CHARACTERIZATION AND EXPRESSION ANALYSIS OF *SPOROCYTELESS (SPL)* GENE IN MONOECIOUS AND GYNOECIOUS LINES OF BITTER GOURD

By

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(2020-11-160)

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KERALA, INDIA

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THESIS

Submitted in partial fulfillment of the
requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University

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COLLEGE OF AGRICULTURE

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KERALA, INDIA

2023

DECLARATION

I, Vijrothi Uma Mahesh Prasad (2020-11-160) hereby declare that the thesis entitled **"Characterization and expression analysis of *SPOROCYTELESS (SPL)* gene in monoecious and gynoecious lines of bitter gourd"** is a bona fide record of research done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"Characterization and expression analysis of *SPOROCYTELESS (SPL)* gene in monoecious and gynoecious lines of bitter gourd"** is a bona fide record of research work done independently by **Mr. Vijrothi Uma Mahesh Prasad (2020-11-160)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of, **Mr. Vijrothi Uma Mahesh Prasad (2020-11-160)** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that this thesis entitled **"Characterization and expression analysis of *SPOROCYTELESS (SPL)* gene in monoecious and gynoecious lines of bitter gourd"** may be submitted by **Mr. Vijrothi Uma Mahesh Prasad**, in partial fulfillment of the requirement for the degree.

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ACKNOWLEDGEMENT

It gives me immense pleasure to greatfully thank and pay my heartiest devotion to ***The Almighty God*** *whose graceful blessings at every step have brought me here upto and without which nothing could have been accomplished.*

With an over whelming sense of legitimate pride and indebtedness to my Major Advisor ***Dr. Deepu Mathew,*** *Professor, CPBMB, College of Agriculture, Vellanikkara, I owe a substantial depth of sincere regard and gratitude for his endless inspiration, ample guidance, optimistic criticism, immense help, untiring interest, constant patience, affectionate advice, and unceasing encouragement which induced self confidence in me throughout this study. It was my pleasure and privilege to be associated with him and work under his close guidance and supervision. This is my turn to express heartfelt thanks to him for having shaped my work culture towards the attainment of perfection.*

I am extremely thankful to ***Dr. Abida P.S.,*** *Professor and Head, Department of Plant Biotechnology, and member of my advisory committee for regular support, unstinted co-operation and valuable suggestions rendered during the study. I owe my deep sense of gratitude to* ***Dr. Pradeepkumar T.****, Professor and Head Department of Vegetable Science, College of Agriculture, Vellanikkara, for his generous guidance, critical examination of manuscript rendered that has helped me a lot for the improvement and preparation of thesis and co-operation throughout the research work.*

My sincere thanks to ***Dr. Rehna Augustine,*** *Assistant professor, Department of Plant Biotechnology, College of Agriculture, Vellanikkara, for her expert advice during the whole venture. I extend my thanks to* ***Dr. Kiran A.G****. during qRT-PCR data analysis. I am greatly indebt to* ***Ravi Shankar*** *sir (DIC) for his sincere help and guidance.*

Whole hearted thanks to my teachers ***Dr.Gibbence****,* ***Dr. Smitha Nair, Dr.Preetha R.,*** *for their expert teaching, moral support and co-operation throughout the period of study.*

My special thanks to the non-teaching staff, especially Shylaja chechi, Simi chechi, Melna chechi, Susmi chechi, Reena chechi, Pushpa chechi, Minnu chechi , Megha Das, for their motherly affection and encouragement throughout my study.

I express my heartfelt thanks to ***Ramnath sir, Sapna Mam*** *from Kerala Veterinary and Animal Sciences University, Mannuthy for their support and guidance provided during real time PCR analysis*

I also use this opportunity to thank my batchmates ***Brindha T.M.,*** *for her moral support during my study. I take this opportunity to extend my gratitude to my seniors* ***Feba chechi, Shivaji sir, Shankar anna, Amala chechi, Nivedhitha, Shri Hari Prasad, Sunny babu, Mullai akka, Sanjay sabu, Anjala chechi, Varsha chechi, Gershom, Venkateswar Reddy sir, Naidu sir, Divya mam, Swathi mam, Manoj sir, Kasi sir, Siva sir, Vishnu sir*** *for their support in different stages of the study.*

I wish to extend my thanks to my friends ***Urati Mahesh, Sushma Reddy, Tejaswi, Tanu C.N., Vamsi Krishna, Abhishek, Sheik Kalesha, Rama Krishna, Sachin, Agnes, Shyamala*** *for their affection which rendered mental strength to me during the research work. Iam grateful to my juniors* ***Ameer, Shijo, Pandya Raj, Abhishek*** *and* ***Aishwarya*** *for their care and affection.*

I express my gratitude to ***DBT*** *for financial support and to* ***Kerala Agricultural University*** *for the financial and technical support for persusasion of my study and research work.*

I can never thank enough to my beloved father ***Satyanarayana****, my mother* ***Padmavathi*** *and my dear brother* ***Siva Prasad*** *for their selfless love, constant encouragement, personal sacrifices, and prayers which helped me to move forward with a smile, in spite of all the hardships in research.*

I wish to thank all others who had helped me in one way or the other in completing the study. Errors and omissions are entirely unintentional.

Vijrothi Uma Mahesh Prasad

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%	: Percent
>	: Greater than
µg	: Microgram
µl	: Microliter
ml	: Milliliter
ß	: Beta
Δ	: Delta
º C	: Degree Celsius
BLAST	: Basic Local Alignment Search Tool
bp	: Base pair
cDNA	: Complementary DNA
CPBMB	: Center for Plant Biotechnology and Molecular Biology
CTAB	: Cetyl Trimethyl Ammonium Bromide
Ct	: Cycle threshold
CV	: Cultivar
DEPC	: Diethyl pyro carbonate
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxyribonucleoside triphosphate
EBI	: European Bioinformatics Institute
EDTA	: Ethylene Diamine Tetra Acetic acid
g	: Gram
KAU	: Kerala Agricultural University
Kb	: Kilo base pairs
L	: Liter
M	: Molar
mg	: milligram
mL	: Milliliter
mM	: Milli molar

mRNA : Messenger RNA

NCBI : National Center for Biotechnology Information

ng : Nanogram

NHB : National Horticulture Board

nm : Nanometer

NTC : No template control

OD : Optical Density

ORF : Open reading frame

PCR : Polymerase Chain Reaction

pH : Hydrogen ion concentration

PVP : polyvinylpyrrolidone

QV : Quality Value

qRT-PCR : Quantitative Reverse Transcription Polymerase Chain Reaction

RNA : Ribonucleic acid

RNase : Ribonuclease

rpm : Revolutions per minute

RT-PCR : Reverse Transcription Polymerase Chain Reaction

TAE : Tris Acetate EDTA

Taq : *Thermus aquaticus*

Tm : Melting temperature

UTR : Untranslated region

V : Volts

Introduction

1. INTRODUCTION

The Cucurbitaceae family includes several important vegetable crops, including bitter gourd (*Momordica charantia* L.; 2n=2x=22). Bitter gourd holds the alternate names bitter melon, balsam pear, bitter cucumber, African cucumber, and *Karela* (Heiser, 1979). India produces roughly 1.2 million metric tonnes of bitter gourd from an area of 99,000 hectares (NHB database, 2018-19). Major states growing bitter gourds are Tamil Nadu, Kerala, Maharashtra, Uttar Pradesh, Andhra Pradesh and Gujarat.

Only 12 of the 47 species in the genus *Momordica*, which originated in tropical Africa, are found in Asia and Australia (Schaefer and Renner, 2010). *Momordica charantia* var. *charantia*, which yields enormous fruits, and *Momordica charantia* var. *muricata*, a wild variety with small, rounded fruits, are the two main botanical varieties of *Momordica charantia* (Chakravarthy, 1990; John and Antony, 2008). It is a trailing vine crop that grows quickly and is extremely valuable in terms of nutrition and medicine. The bitter gourd's immature fruits are often eaten as vegetables and known for its anti-diabetic and other therapeutic effects (Robinson and Decker-Walters, 1997). Given that its fruits are high in vitamin A, vitamin C, iron, phosphorus, and carbohydrates, it is regarded as the most nutrient-dense among the cucurbits (Miniraj *et al*., 1993; Desai and Musmade, 1998; Behera, 2005). Its enormous therapeutic benefits are attributable to the presence of advantageous phytochemicals, which are known to have immune-boosting, antiviral, anti-diabetic, antibiotic, antimutagenic, antihelmenthic and cancer-preventive characteristics. Phytochemicals such as momordin and charantin in fruits are clinically proven for its hypoglycemic activity.

Monoecy, having separate male and female flowers in a plant, is the main sex form of bitter gourd. However, gynoecious sex form with only female flowers, is reported from India and Japan (Ram *et al*., 2002; Iwamoto and Ishida, 2006). Use of gynoecious lines as female parent in the hybridization programme is a cost-effective strategy since the manual labour required for emasculation can be avoided. Though many gynoecious lines are reported in major cucurbits such as cucumber, initially gynoecious bitter gourd lines were reported from KAU (KAU-Mc-Gy101 and KAU-Mc-Gy102; Minnu *et al*., 2022; Sunny *et al*., 2022; Baluchamy *et al*., 2023), IIVR

(Gy-323, Gy-333, Gy263B, Gy-2116, Gy-2135, Gy-318, Gy-144; Ram *et al*., 2002; Moharana *et al*., 2022;), IIHR (IIHRBTGy-491, IIHRBTGy-492; Varalakshmi *et al*., 2014) and IARI (DBGy-201 and DBGy-202; Behera *et al*., 2006; Behera *et al*., 2009) only. A gynoecious inbred, OHB61-5 with high combining ability was also reported from Japan (Iwamoto and Ishida, 2006; Iwamoto *et al*., 2009).

Flowering in plants is a complicated process involving multiple genes and pathways. In the ABC model of flowering, stamen formation is the result of interaction between B and C genes (Bowman *et al*., 1991). The specification of stamen identity in *Arabidopsis thaliana* is controlled by a combination of the B genes *APETALA3* (*AP3*) and *PISTILLATA*, the C gene *AGAMOUS* (*AG*), and the E genes *SEPALLATA* (*SEP1* to *SEP4*). *AG* is understood to activate the transcription of the "floral organ-building" gene *SPOROCYTELESS/NOZZLE* (*SPL*/*NZZ*). *SPL* plays a central role in regulating microsporangium differentiation, because SPL/NZZ mutant anthers fail to form archesporial cells (Schiefthaler *et al*., 1999; Yang *et al*., 1999). Ectopic expression of *SPL*/*NZZ* has resulted in ectopic expression of *AG* and *SEP3* in the outer whorls of flowers, indicating that this gene is engaged in controlling stamen identity via interacting with genes required for stamen identity (Liu *et al*., 2009). In wild-type *Arabidopsis* ovules, megaspore mother cell differentiation required *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*), as demonstrated by the *spl/nzz* mutant failing to develop megaspore mother cell (Zhao *et al*., 2017; Mendes *et al*., 2020).

The chances for the isolation of a gynoecious line from a monoecious population is often rare since gynoecy in bitter gourd is under the control of a single, recessive gene *gy-1* (Ram *et al*., 2006). Thus, with the increasing demand for the development of gynoecious lines in crops such as bitter gourd, genes identified to have definite roles in sex expression assumes importance. The *SPL* genes is shown to have pronounced role in stamen and pistil development in flowers. To proceed with the alteration of the sex forms in any crop by genetic engineering, the candidate gene has to be thoroughly characterized. Also, its expression levels in different sex forms has to be well understood. Thus, the objective of this study was to characterize the *SPL* gene in bitter gourd and to study its expression in the male and female flowers in monoecious and gynoecious sex forms, to formulate the strategies to develop the gynoecious lines through genome engineering.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Background

Bitter gourd (*Momordica charantia* L., Family: Cucurbitaceae, 2n=22) is a vegetable crop widely grown in the tropical and subtropical regions of the world. In cucurbits, especially in cucumber and melon, the impact of gynoecious lines in enhancing crop yield is widely known and has been extensively investigated. Improvement of the trait will be attained by comprehending the genetics and molecular mechanisms behind the determination of gynoecious sex form. Though many gynoecious lines are reported in major cucurbits such as cucumber, gynoecious bitter gourd lines are reported from KAU, IIVR, IIHR and IARI only . The *AGAMOUS* (*AG*) gene, leading C class gene in the ABC model of flowering, acts by activating the transcription of *SPOROCYTELESS* (*SPL*) gene and hence *SPL* is considered very important for the megaspore development in flowers. For the silencing/ over expression of this gene, aimed at altering the sex form of bitter gourd, it is to be sequence characterized.

Enough experimental results are available to connect the role of plant growth regulators to initiate the pathways associated with specific sex expression. The relevant literature on the topics related to this study is reviewed in this chapter.

2.2 Plant sex determination

Majority of angiosperms produce hermaphrodite flowers, which have both male and female reproductive organs. Flowering plants have evolved some sex determination techniques in order to avoid self-fertilisation, which could result in fitness loss owing to inbreeding depression (Darwin, 1876). One such tactic is the establishment of unisexual flowers, in which the male and female gametes of flowers are segregated on several blooms of the same plant (monoecious species) or on distinct individual (dioecious species). As a result, the unisexual flower will either have pistils or stamens.

In order to achieve unisexuality, flowers that are initially flawless go through stage-specific and selective arrest of preformed organ primordia. The distinction lies in the way and stage of floral metamorphosis at which the arrest of reproductive organs occurs, (Dellaporta and Calderon-Urrea, 1994). For instance, the sex organ

primordia entirely terminate in maize, whereas the primordial arrest in cucumber occurs throughout their growth stage (Malepszy and Neimirowcz-Szcytt, 1991; Grant *et al*., 1994).

Sex determination, a prominent phenomenon in cucurbits, is governed during development and results in various sexual flowers on the plants. For the purpose of determining what causes plant sex difference, numerous investigations have been conducted (Ainsworth,1999; Tanurdzic and Bnaks, 2004; Perl- Treves and Rajagopalan, 2006; Boualem *et al*., 2008; 2009; Li *et al*., 2009; Martin *et al*., 2009). However, it is still uncertain what commences the decision-making process for organ development and how it could be carried out.

2.3 Sex expression in bitter gourd

Sex expression is the manifestation of a specific gender in a population of plants (Cruden and Lloyd, 1995; Neal and Anderson, 2005). From ideal to a variety of intermediate forms, sex expression has been modified as a result of evolutionary changes through successive generations and the influence of dominant mutations. Since they have the capacity to harbour different sex forms such as hermaphrodite, monoecious and dioecious, cucurbits are models for researchers looking into how plants determine their sexual orientation (Robinson and Decker-Walters, 1999).

Monoecy and gynoecy are the predominant sex forms in bitter gourd but androecy and andromonoecy are also sporadically observed in nature. Furthermore, hermaphrodite plants are also reported (El-Shawaf and Baker, 1981).

Monoecious bitter gourd plants begin to produce male flowers, followed by female and male flowers alternatively, to terminate with a female flowering phase (Shifriss, 1961). Gynoecism occurs when all flower nodes yield female flowers. In various parts of the world, gynoecious plants with exclusively pistillate flowers have been discovered.

Phytohormones, chemical inhibitors and environmental conditions such as day length, humidity, and temperature, influence how sex determining genes are regulated (Meagher,2007). Under short photoperiod and low temperature (25 °C day/ 15 °C night), gynoecy progresses. By adopting the short-day treatment from seedling

emergence until sixth leaf stage, a high frequency of pistillate flowers can be induced (Behera et al., 2011).

The plant hormone ethylene exerts a feminising impact on bitter gourd, cucumber, and melons. Thus, to create hermaphrodite flowers, to maintain the gynoecious population, ethylene inhibitors such as GA_3, silver nitrate, and silver thiosulfate are applied after the first female flower (Matsumura et al., 2014).

2.4 Sources of gynoecious bitter gourds

Monoecious sexuality dominates in bitter gourds, but gynoecious forms are documented from India, China, and Japan (Behera et al., 2006). Though gynoecy is uncommon, Zhou et al. (1998) have developed a population of bitter gourd with a greater amount of pistillate flowers and used it for hybrid development.

Ram (2002) identified three gynoecious bitter gourd plants, Gy23, Gy63, and Gy263B, with complete expression of gynoecy, and this is the first report on its characterization. Each plant is crossed with its respective monoecious population (Ram *et al.,* 2002). Subsequently, through selfing and sib-mating, they created populations with a greater amount of pistillate flowers.

The two gynoecious lines, DBGy-201 and DBGY-202, were isolated from a native source of the related wild species, *M. charantia* var. *muricata* (Behera *et al.,* 2006). By selfing or crossing to the same lineage, Iwamoto, and Ishida (2006) created a gynoecious inbred from the cv. Aochu-naga that included a high proportion of female flowers.

The gynoecious bitter gourd line OHB61-5 from Okinawa, Japan, with only female flowers, is thought to be a spontaneous mutant (Matsummra *et al*. (2014). Varalakshmi et al. (2014) reported two gynoecious bitter gourd lines, IIHRBTGy-491 and IIHRBTGY-492.

The consistency of the gynoecious sex expression was assessed in the gynoecious line KAU-MCGy-101 identified at Kerala Agricultural University. Since only female flowers were generated throughout the growth phase in all the plants arising from the sib-mated population, the gynoecious sex expression was found consistent in the inbred (Minnu, 2019). The inbred lines had superior average fruit weight, fruit length, and fruit girth when compared to the first recorded gynoecious

lines, indicating the potential for imparting earliness and higher productivity in the breeding programmes.

From the gynoecious bitter gourd accession NBGH-167, Jadhav *et al*. (2018) selected four gynoecious lines, Gy-1, Gy-14, Gy-15, and Gy 34, with 100% gynoecy nature, using plant by plant selection of its transgressive segregants in progeny row method.

2.5 Inheritance of gynoecy in bitter gourd

Using the gynoecious line Gy263B and monoecious inbred line Pusa Do Mosami, Ram et al. (2006) have studied the inheritance of gynoecy in bitter gourd. F_1 was backcrossed with Gy263B to generate test cross progeny. The F_2 progeny segregated for gynoecy in the ratios of 3:1 (3 monoecious: 1 gynoecious plants) whereas the test cross progeny segregated in the ratio of 1:1. This suggested that gynoecy is governed by a single recessive gene. According to cucurbits gene nomenclature, a gene symbol *gu-1* is suggested for the expression of gynoecism in bitter gourd (Robinson et al., 1976). In contrast to their discovery, Iwamoto and Ishida (2006) proposed a partial dominant nature of gynoecism in bitter gourd.

After crossing two gynoecious lines, DBGY-201 and DBGY-202 with two monoecious cultivars, Pusa Do Mausami, and Pusa Vishesh, Behera *et al*. (2009) detected the segregation of gynoecy in F_2 populations in the ratio of 3:1. Their findings supported the reports by Ram et al. (2006) that the bitter gourd has a single recessive (*g-1*) regulation of the gynoecious sex form. Poole and Grimball (1939) first noted a similar pattern of inheritance for gynoecy in melon.

The gynoecious line OHB61-5 was crossed with the monoecious line OHB95-1A, leading to about 5% more female flowers per plant (Matsummra et al., 2014). Segregation ratio for gynoecy in F_2 population was 3:1, suggesting that their findings are consistent with earlier reports showing the monogenic recessive nature of the gynoecy trait. Mishra et al. (2015) provided more support for using the bitter gourd cross DBGy201 x S-2 and DBGy201 × Pusa Do Mausami.

To ascertain how gynoecy is inherited, two cultivated inbred lines, the gynoecious K44 and monoecious Dali-11, were crossed and resulting F_2 population was examined Cui *et al.* (2018). The population was split into two groups and raised under two distinct conditions. Even though *gy* was a qualitative feature in their data, a

shift in the pattern of segregation between these two groups was observed. One of the populations revealed a 3:1 ratio of gynoecy segregation. The other population displayed two-genic segregation (15:1), indicating that at least two genes may interact to control gynoecy.

In order to study the inheritance pattern of gynoecy, Rao *et al*. (2018) crossed the gynoecious bitter gourd line DBGy-201 with Pusa Do Mousami having 6% pistillate flower frequency. The F_1 population was monoecious with a dominating pistillate frequency of 33%. The back cross population similarly segregated in a 1:1 ratio, explaining the monogenic recessive inheritance nature of gynoecy in bitter gourd. F_2 population fit well in the predicted ratio of 3 monoecious: 1 gynoecious.

In general, the scholars' perspectives on the heredity of gynoecy in bitter gourd is diverge. Gynoecy is largely believed to be regulated by a monogenic recessive gene (*gy-1*) (Ram et al., 2006; Behera et al., 2009; Matsummura et al., 2014), while the remaining studies have shown that the condition is either partially dominant (Iwamoto and Ishida, 2006) or semi-dominant. Cui et al. (2018) also reported two pairs of gynoecy-related genes.

2.6 Candidate genes for gynoecy

Plant science's trendiest area of study is the determination of plant sex, which is connected to yield and quality. The sex-determining genes in cucurbits are orthologous and remarkably conserved. Because of its broad range of floral sex types, cucumber is regarded as a model organism for sex determination (Tanurdzic and Banks, 2004). Since the cloning of many key sex-determining genes in cucurbit species, there have been notable advances in understanding the mechanisms behind this crucial overall development in flowering plants (Boualem *et al*., 2008; Li *et al*., 2009; Martin *et al*., 2009). A highly regulated network of interconnected genes controls the process at various points throughout floral morphogenesis on many omics systems (Pawekowicz *et al*., 2019).

In 2010, Guo et al. adopted digital expression profiling to analyse the transcriptome fluctuations between two nearly isogenic cucumber lines: the WI1983G gynoecious line and the WI1983H hermaphrodite line. An aggregate of 214 genes with differential expression were found, of which 90 and 124 genes, respectively, were elevated in gynoecious and bisexual flowers. Additionally, they listed a few

transcription factors (TFs), such as auxin response and zinc finger TFs, whose expression is only increased in hermaphrodite flowers.

In order to find probable candidate genes controlling gynoecy in bitter gourd, Gunniah *et al*. (2014) conducted an *in silico* differential gene expression analysis between gynoecious line Gy323 and monoecious line DRAR-1. In Gy-323, 4131 genes were up-regulated and 3734 genes were down-regulated, for a total of 7865 genes that were differentially regulated between the two lines. Potential candidate genes for gynoecy may be found among the up-regulated genes that play a substantial role in the manufacture of ethylene or the development of flower organs. This has led to the identification of a number of possible candidate genes for gynoecy, including *ACS*, *ACO*, *ETR*, and *ERFS*. An *Arabidopsis* homologue BTB/POZ domain-containing protein that controls the development of floral organs showed the greatest upregulation for gynoecious lines, with a seven-fold increase than the monoecious line.

Similar to this, in bitter gourd, RNA-seq technology was used to compare the transcriptome dynamics of female flowers of a gynoecious line DBGY-201H with hermaphrodite flowers of the same line (Behera *et al*., 2016). The 477 genes identified have shown a significant variation in gene expression, of which 237 were down-regulated and 59 were up-regulated in DBGY-20H sample. Additionally, they found differently expressed ESTs between two flower types, enabling gene discovery and details about the molecular basis of sex determination.

Cui *et al*. (2018) created a RAD-based genetic map for bitter gourd using an F_2 population derived from the gynoecious line K44 and monoecious Dali-11. The map had 11 linkages groups, and the QTL/genes *gy*/*fffn*/*fin* regulating the sex expression involved in gynoecy, the first female flower node and the female flower number, respectively were placed in the first linkage group. By mapping the flanking RAD tags to the OHB3-1 genome, they have also provided the list of potential genes influencing the expression in bitter gourd. Additionally, they validated that the *gy* locus, which Matsumura *et al*. (2014) discovered, is located at the distal end of linkage group 1.

In order to determine gynoecy in bitter gourd, Matsumura *et al*. (2020) discovered the gene *NUA* (*Nuclear Pore Anchor*) connected with the QTL. The 60 kb

gene was primarily made of introns and had only 6,240 bp coding region. Through BLAST search, an *Arabidopsis thaliana* homolog with 6,345 bp coding sequence that is known to have a significant impact on stamen length and anther size was found, indicating that *NUA* is a probable candidate gene for gynoecy in bitter gourd.

It is widely acknowledged that plant hormones like auxin, ethylene, and gibberellin have a significant impact on how plants behave in sex. Studies have revealed that the majority of the genes that are differentially expressed between monoecious and gynoecious bitter gourds are connected to the signal transduction of plant hormones, such as auxin and ethylene production, which are covered separately below.

2.6.1 Ethylene mediated sex determination

The phytohormone ethylene participates in a number of processes throughout the plant life cycle, including seed germination, root hair formation, root nodulation, floral withering, and fruit ripening (Abeles, 1973; Lieberman, 1979; Yang, 1985, Yang and Hoffman, 1984; Mattoo and Suttle, 1991). It is acknowledged as a powerful regulator of plant growth and development (Ecker, 1995).

ACC (1-aminocyclopropane-1-carboxylic acid) and S-adenosyl methionine (S-AdoMet) are ethylene precursors. The enzyme ACC synthase transforms S-AdoMet into ACC as the first step in the biosynthesis of ethylene. The enzyme ACC oxidase eventually converts ACC to ethylene (Yang and Hoffman, 1984; Kende, 1993). In addition, genes in the ethylene signalling pathway such *ETR*, *ERS*, *EIN*, and *ERF* are crucial for controlling ethylene in plants. The main determinant in determining sex is ethylene, which has a strong correlation with femaleness (Rudich *et al*., 1972a, 1976; Yin and Quinn, 1995). Compared to monoecious cucumber plants, gynoecious plants produce higher amounts of it (Rudich *et al.,* 1972b; Trebitsh *et al*., 1997). Furthermore, it was shown that the development of female flowers was decreased when ethylene biosynthesis and action were blocked (Beyer, 1976; Atsmon and Tabbak, 1979; Takahashi and Suge, 1980; Takahashi and Jaffe, 1984).

2.6.1.1 Ethylene related genes

Two ACS genes, *CsACSIG* and *CsACS2*, two ACC oxidase genes, *CsACO2* and *CsACO3*, three ethylene receptor genes, *CsETRI*, *CsETR2*, and *CsERS*, and a MADS-

box gene, *ERAF17* (Ando *et al*., 2001), were identified to act as ethylene receptors, leading to the formation of female flowers in cucumber.

Ethylene response factor (ERF) transcription factors are distinguished by the presence of a DNA-binding domain of the AP2/ERF type that is unique to plants (Lin *et al.,* 2009; Giovannoni and Klee, 2011). ERF proteins participate significantly in ethylene biosynthesis by regulating the transcription of *ACS* and *ACO* genes (Zhang *et al*., 2009; Klee and Giovannoni, 2011; Liu *et al*., 2016). These genes accomplish the responses by promoting the initiation of the expression of downstream ethylene-responsive genes (Xiao *et al*., 2013 and Li et al., 2016; Tao *et al*., 2018).

2.6.1.1.1 ACS genes

The *ACC synthase* gene controls most aspects of ethylene production (Yang and Hoffman, 1984). This enzyme is encoded by a multigene family, and each of its members exhibits differential expression in response to various stimuli (Huang *et al*., 1991, Nakagawa *et al.,* 1991, Olson *et al*., 1991, Rottmann *et al*., 1991, Liang *et al*., 1992, Zarembinski and Theologis, 1993, Destefano-Beltran *et al*., 1995). By locating specific *ACC synthase* (*ACS*) genes involved in the growth of pistillate flowers, it is possible to comprehend how ethylene regulates the molecular manifestation of sex.

The apex of gynoecious plants showed higher levels of *ACC synthase* activity compared to the monoecious plants (Trebitsh *et al*., 1987). When genome-wide transcriptome profiling was done on cucumbers, it was discovered that gynoecious and monoecious cucumbers differed in the expression of the gene *ACS*. The mutation in ACS and auxin-related genes is mostly responsible for the differential expression (Wu *et al*., 2010).

Gynoecy was associated with high-level expression of *CsACS1*, *CsACS2*, and *CsACS11* and enhanced endogenous ethylene levels in cucumber (Knopf and Trebitsh, 2006; Saito *et al*., 2007; Boualem et al, 2016; Chen *et al*., 2016).

Monoecious (cv. Marketmore76; *MMff*) cucumbers were found to have only one copy of the *Cs-ACS1* gene, whereas gynoecious line had at least one additional copy of this gene (cv. Marketmore 76F; *MMFF*) (Trebitsh *et al*., 1997). There may be more *Cs-ACS1G* copies in the gynoecious line as a result of the increased pace of sex change. The auxin-inducible *Cs-ACS1G* is present in the monoecious lines' genomes.

Therefore, it was concluded that the monoecious cucumber plant still has the ability to produce female flowers, and that ethylene and auxin manipulation can encourage female flowering. They proposed that the *Cs-ACS1G* is tightly connected to the *F* locus and may be essential for cucumber sex differentiation.

Wu *et al.* (2010) further verified that gynoecious cucumbers contain a second copy of *CsACS1G*. Li *et al*. (2020) validated the finely tuned structure of the *F* locus in the gynoecious cucumber and elucidated that only *CsACS1G*, a duplicate copy of *CsACS1* with recombinant distal promoter, is responsible for gynoecy out of the three genes (*CsACS1*, *CsACS1G*, and *CsMYB*) in the *F* locus. Additionally, cucumber's gynoecy became unstable as a result of the loss of this extra copy by uneven crossing over during meiosis.

Among the genes encoding ACC synthase, only the mRNA of *Cs-ACS2* was found near the apical end of gynoecious cucumbers. It was discovered to co-segregate with the *M* locus. Therefore, it was suggested that the *Cs-ACS2* gene may play a role in regulating the growth of female flowers at the tip of gynoecious cucumber plants (Kamachi *et al*., 1997). Yamasaki et al. (2003b) proposed that the *CsACS4* gene is involved in the diurnal regularity of ethylene evolution.

According to a modified model for sex determination in cucumbers (Li *et al*., 2012), by cooperating to create male, female, or perfect flowers, *CsACS2* and *CsACS1G* encode ACC synthase enzymes. The *F* gene is initially activated in female flower bud primordia, and ethylene will support the growth of female flowers. Additionally, *M* is activated, and *M* then begins to express positive feedback. Female flowers are produced as a result of the constant arresting of stamen primordia development by the translation of the *M* gene mRNA beneath the region of the pistil primordia.

In carpel primordia of buds that are expected to grow into melon carpels, *Cm4CS-7* expresses itself specifically (Boualem *et al*., 2009). After *ACS* expression, staminal growth is probably inhibited due to inter-organ communication. Ethylene effects the development of female flowers in cucumbers by inducing DNA damage in the primordial anther of female flowers that is specific to an organ (Wang et al., 2010). The component gene of the ethylene signalling pathway, *CsETRI*, was

downregulated both temporally and geographically in the stamens of stage-6 female flowers.

In contrary, in watermelon, ethylene promotes the growth of male flowers. Both the watermelon *ACS* genes, *CitACS1* and *CitACS3*, were expressed in the floral tissues, but *CitACS1* was also found in the vegetative tissues, indicating that it is important for plant development and growth. The *CitACS3* was expressed in open flowers and in staminate or hermaphrodite floral buds, but not in female flowers. Minkov *et al*. (2008) provided an explanation for the upregulation of *CitACS3* and its function in ethylene-regulated anther formation.

An SNP in the highly conserved region of *CmACS-7* gene impairs the protein function and reduces the enzyme activity of the ACC synthase in melon (Bouleam *et al*., 2008). In female flowers, this enzyme activity inhibits the growth of the stamen rather than enhancing the carpel development. As a result of the enzyme's inaction, hermaphrodite flowers are produced and plants become andromonoecious. This *CmACS7* gene, which is the homolog of *CsACS2* in cucumber, is encoded by the stamen-inhibiting '*A*' locus of the melon (Bouleam *et al.,* 2009). Similarly, in watermelon, monoecious lines with active ClACS7 enzyme yield female flowers, but those with inactive enzyme produce hermaphrodite blooms (Bouleam *et al*., 2016).

CitACSA, a watermelon gene that Manzano *et al.* (2016) identified and characterised, was strikingly similar to previously described genes for ethylene biosynthesis known to be involved in andromonoecy, CmACS7, CsACS2, and CpACS274. The pistillate flowers are where this gene's encoding ACS type III enzyme is most prominently expressed. In andromonoecious lines, a mis-sense mutation in the conserved region (C_{364W}) of this gene was found, and the amounts of ethylene decreased concurrently. As a result, scientists were able to explain how the mutation prevented the ethylene production necessary for the detainment of stamens in flower buds intended to become female, leading to hermaphrodite flowers for andromonoecy.

By suppressing the male flower-promoting gene *CmWIP1*, Bouleam *et al.* (2015) demonstrated how the melon gene *CmACSII* determines the female flower. Therefore, the lack of *ACSII* function results in andromonoecy by causing stamen formation. Similar to cucumber, it was proposed that*sACSI* be associated with the '*A*'

locus. In response to ethylene signalling, the ERF protein genes *CsERII* and *CmERFII* in cucumber and melon, respectively, facilitate ethylene-regulated transcription of the *CsACSII* or *CmACSII* genes (Tao *et al*., 2018).

Silver thiosulphate (STS) is an ethylene regulator that alters the sex of plants by preventing them from detecting ethylene. Spraying STS at 6.0 mM on bitter gourd transforms female flower buds into hermaphrodite ones. Kumari (2015) studied how *McACS* genes are expressed during four stages of transformation of female buds into ideal buds. After STS spray, *McACS2* expression was lowered, and during the development of hermaphrodite flowers, expression got enhanced. Therefore, it is proposed that silver ions will diminish ethylene perception, which will then result in a decrease in the concentration of *McACS* transcripts in the flower buds of gynoecious line (DBGy-201). This provides compelling evidence that ethylene production, which is mediated by *McACS2*, is connected to sex determination in bitter gourd. Gunniah *et al.* (2014) used *in silico* differential gene expression analysis among monoecious and gynoecious plants to identify a list of probable candidate genes involved for gynoecy in bitter gourd. The list was dominated by genes related to ethylene, including *ACS*, *ACO*, *ETR*, and *ERS*.

A synteny mapping and phylogenetic analysis of conserved genes has shown that bitter gourd is more close to watermelon rather than cucumber or melon (Urasaki *et al.*, 2017). Since the sex determining genes are already discovered in cucumber and watermelon, the bitter gourd genome was compared to those genes to locate orthologous genes. Three proteins MOMC3 649, MOMC46 189, and MOMC518 1 were thought to be the respective bitter gourd orthologs of *CmAcsII* and *CmAcs7*. Following confirmation, it was shown that pistillate flowers expressed MOMC3 649g, which encodes a protein similar to *CmAcsII,* and MOMC518 1g encodes a protein similar to CmAcs-7. The findings supported earlier research that showed significant expression of *ACSII*, *ACS7*, and *ACS2* in female flowers of cucumber and bitter melon.

2.6.1.1.2 *ACO* genes

In pea and sunflower seedlings, high *ACC oxidase* activity and transcript levels were related to actively dividing tissues, respectively (Liu *et al*., 1997). Developing tomato flowers showed patterns of organ-specific *ACO* expression (Barry *et al*., 1996). Two

ACO genes were selectively expressed in the growing pistil tissue of *Petunia* (Tang *et al*., 1993).

Three complete cDNAs for the *CsACOI* and *ACO* genes were generated by Kahana *et al*. (1999). In cucumber, different sex types, developmental stages, floral kinds and organs responded differently to etherel treatment in terms of *CsACO2* and *CsACO3*. Additionally, they showed that the expression of *CsACO* in the shoot apex has no bearing on sex determination and the mechanism regulating ethylene production during flower development is a complex.

An SNP makes CsACO2 enzyme , reduce ethylene release from shoot tips during the crucial stage of sex determination, carpel development gets prevented and convert cucumber plants to andromonoecious Chen *et al*. (2016). The melon gene *CmACO3*, an ortholog of *CSACO2*, displayed a similar expression pattern in the area of carpel development. This result, contradictory to the report of Kahana *et al.* (1999), was not reached from the bud of the key stage of sex determination.

2.6.2 Transcription factors (TFs) in sex determination

TFs, the sequence-specific DNA-binding factors, control the transcription of genes (Singh *et al.,* 2002). Neo-functionalization and sub-functionalization of TFs are important for the differentiation of plant morphology Sharma *et al*. (2013).

2.6.2.1 MADS box genes

The process of determining sex in flowering plants is intricate and involves a number of genes that are variably expressed at distinct tissues and developmental stages (Charlesworth & Mank, 2010). One of these is the gene for the MADS box family of transcription factors, which is important for plants during their reproductive stage. One of the first isolated MADS box genes is the *Arabidopsis AGAMOUS* (*AG*) gene, which encodes the floral homeotic genes (Yanofsky *et al*., 1990). As shown by overexpression and mutant phenotypes, *AG*, a class C homeotic gene that plays a crucial regulatory role in defining the identity of stamens and carpels. Its interaction with other MADS-box proteins is crucial for the induction of floral organs in *Arabidopsis* (Honma and Goto, 2001). In monoecious cucumber, class C homeotic genes determines position-dependent arrest of specific reproductive organ

development, a fundamental mechanism in unisexual flower development (Kater *et al*., 2001).

MADS box TFs contain the highly conserved MADS box and the moderately conserved K-box, a domain that creates coiled-coil structures that enable their dimerization (Ma *et al*., 1991). The MADS boxes attach to a particular CArG box in the promoter region of target gene and control the expression.

Purugganan *et al*. (1995 have analysed the amino acid sequences of MADS box genes from various species to classify them into groups B, C and other groups such as *AGAMOUS* (*AG*), *APETALA2* (*AP2*), *APETALA3* (*AP3*)/*PISTILLATA*, and *APETALA1*/*AGL9*.

The *AG* homologs are present in all the plants and functional studies using ectopic expression have shown that they play a crucial role in determining the identity of stamen and carpel (Mandel *et al*., 1992; Kempin *et al*., 1993; Tsuchimoto *et al*., 1993; Pnueli *et al*., 1994; Saedler and Huijser, 1994; Kang *et al*., 1995).

Previously reported MADS-box genes known as *AGAMOUS-LIKE6* (*AGL6*) are found in many eukaryotic genomes, including those of gymnosperms and angiosperms (Becker and Theissen, 2003). The *AGL6-like* genes play important roles in the formation of angiosperm flowers and overexpression of *AGL6* orthologs from orchid (Hsu, 2003) and hyacinth (Fan, 2007) in *Arabidopsis* causes flower homeotic transition and promotes flowering.

MAD gene expression is not required for the pathway that results in the arrest of the reproductive organ in cucumber unisexual buds (Perl-Treves *et al*., 1998). In contrast, Ando *et al*. (2001) reported that expression of the MAD box gene *ERAF17* in cucumber apical floral buds would control the induction of female flowers by ethylene.

Table 2.1. List of genes from MADS box family that participate in floral induction

Sl. No.	**Gene**	**Crop**	**Reference**
1	Cucumber MADS box gene 1 *CUM1* and *CUM10*	*Cucumis sativus*	Kater *et al.* (1998)
2	*TaAP3, TaAGAMOUS* and *TaMADS1 3*	*Triticum aestivum*	Su *et al.* (2019)
3	*OsMADS6*	*Oryza sativa*	Li *et al.* (2010)

2.6.2.1.1 MADS-box genes in *Momordica*

In bitter gourd, a conserved MADS box protein family area was used to isolate the homologue of the *AG-like* gene known as *BAG*. In *Arabidopsis thaliana*, CUM10, cucumber MADS-box protein (95%) and AGL11 were discovered to have a high degree of amino acid sequence similarity.

On the basis of the conserved MADS box sequences, Peng *et al*. (2005) identified two MADS box genes, *McAG2* and *McAG6*, from female flower buds of bitter gourd. These genes had been fully cloned and sequenced in preparation for phylogenetic analysis and sequence comparison. The amino acid sequences of the genes *McAG2* and *McAG6* were remarkably similar to those of the *AGL6* and *AG-like* genes, respectively. Additionally, a high degree of similarity was found between the *McAG2* and *CUM1* (Cucumber MADS box gene 1) proteins. *McAG2* and *McAG6* showed their highest levels of expression in the carpel and shoot apex, respectively. The bitter gourd *McAG6* gene was the first *AGL6-like* gene in the Cucurbitaceae family, and its high level of expression in vegetative tissues was intriguing because it was not common in the previously studied plants.

Two genes in bitter gourd were found involved in controlling the development of floral organs according to the pattern of gene expression in various tissues. In order to characterise these genes and identify the various MADS-box members of this family, genome-wide analyses of bitter gourd MADS-box genes was done (Hseih and Do, 2019). The phylogenetic analysis had shown that MADS box proteins from bitter gourd is closely related to watermelon. As per gene expression clustering of the transcripts, three potential genes associated with the sex determination mechanism

McMADS1, *McMADS10* and *McMADS22* were discovered. Additionally, they claimed that hormones such as GA and various environmental factors might affect these genes, which seems to be at conflict with the earlier results of Perl-Treves *et al*. (1998) in cucumber.

Mohanty and Joshi (2019) cloned 17 *MdMADS* genes from the flower buds of *Momordica dioica*. Based on the motifs, they divided the genes into MIKC (11 genes), MIKC (4 genes), and M-type (2 genes) subgroups. Through transcriptome analysis, 11 MIKC genes were found related to floral homeotic functions whereas four MIKC genes were related to male gametophyte development and two M-type genes to female gametophytic development. These genes cause sexual dimorphism in *M. dioica*.

2.6.2.2 Auxin mediated sex determination

Auxin causes the creation of female flowers and boosts the synthesis of ethylene (Takahashi and Jaffe, 1984; Galun *et al*. 1962; Atsmon and Tabbak, 1979). When auxin was present, certain *ACS* genes expressed more (Trebitsh *et al*., 1987).

Auxin can induce the genes that directly govern the biogenesis of sexual organs. Auxins act through the transcriptional control of gene families such as (*Aux/LAA*), Gretchen Hagen3 (*GH3*), slight auxin up RNA (*SAUR*), and auxin factor (*ARF*) (Shen *et al.,* 2014; Zouin *et al.,* 2014).

2.6.2.2.1 *ARF* genes

The family of functionally unique DNA-binding proteins known as auxin response factors (ARFs) provides specificity to auxin response by choosing target genes as factors. They are key players in the auxin signalling system, and by interacting with the conserved auxin response element (AuxRE) in the promoter region of numerous auxin response genes, they have the ability to either activate or inhibit transcription of those genes (Tiwari et al., 2003)

Liu *et al.* (2015) discovered many ARF genes in papaya and examined their pattern of expression during distinct developmental stages. The expression of the genes *CPARF1*, *CPARF2*, *CPARF4*, *CPARFS*, and *CPARF10* peaked early in the development of the flower and then gradually decreased during the remaining phases.

One of the auxin-regulated genes, the *aux responsive factor* gene (*arf*), regulates the growth of pistillate flowers in tomato, arabidopsis, and papaya (Li *et al*., 2016).

The auxin signal transduction pathway is significantly impacted by the association between aux/IAAs and ARF. Auxin suppresses the function of the ARF protein by dimerizing with Aux/IAAs in low concentrations, while it activates it in high concentrations (Ulmasov *et al.*, 1997; Hagen and Guilfoyle, 2002).

Through *de novo* transcriptome sequencing, Shukla *et al*. (2015) generated 65,540 transcripts for monoecious (DRARI) and gynoecious (Gy323) lines of bitter gourd. The digital gene expression analysis to compare the transcript count and related differential expression pattern has shown that genes showing high-level differential expression are associated with hormone signalling and response. From among them, 56 ARF genes, which are controlled by auxin and crucial for gene expression and development were discovered.

2.6.3 WRKY transcription factors

In plants, WRKY proteins make up a huge family of TFs. Members of this family have one or two conserved WRKY domains, with the C terminus containing the C2HC or C2H2 zinc-finger protein motif and the N terminus containing the conserved WRKYGQK heptapeptide sequence (Ishiguro *et al*., 1994). In accordance with the shape of the C-terminal zinc-finger motif and the number of WRKY domains, the WRKY family will be split into I, II and III groups (Rushton *et al*., 2010). The W-box [(T)TGAC(C/T)] present in the promoter regions of numerous target genes is recognised by members of this family.

Transcriptome analysis of monoecious and gynoecious cucumbers revealed that WRKY DNA-binding protein is important for sex determination (Guo *et al*., 2010). This is the first publication to link WRKY variables with plant sex determination, despite the fact that its involvement in stress response and different phases of plant growth were well established. WRKY TFS is essential for controlling plant signals, especially in the ABA response pathway (Rushton *et al*., 2012). According to Rudich *et al*. (1974), ABA encourages female flowering tendency while inhibiting GA, which increases male flowers. In *Arabidopsis*, WRKY75 negatively regulates the GA signalling pathway (Zhang *et al*., 2018) and favourably regulates

floral initiation by interacting with DELLA proteins (Peng *et al*., 1997; Silverstone *et al.,* 1998 and Pysh *et al*., 1999).

The *de novo* transcriptome sequencing and comparison of TFs of monoecious (DRARI) and gynoecious (Gy323) bitter gourd lines have revealed the potential sex-determination candidate genes (Shukla *et al.*, 2015). Using the UniProt non-redundant protein and TAIR (The Arabidopsis Information Resource) data sets, *Momordica* transcripts were functionally annotated to *Arabidopsis thaliana* TF sequences and classes of TFs primarily involved in the differential pattern formation were identified. This study reported 80 genes related with WRKY transcription factors.

2.7 Role of *SPL* gene in plant sex determination

Flowering in plants is a complicated process involving multiple pathways and genes. According to ABC model of flowering as proposed by Bowman *et al*. (1991), stamen formation is the result of interaction between B and C genes. The specification of stamen identity in *Arabidopsis thaliana* is controlled by a combination of the B genes *APETALA3* (*AP3*) and *PISTILLATA*, the C gene *AGAMOUS* (*AG*), and the E genes *SEPALLATA* (*SEP1* to *SEP4*). *AG* is understood to activate the transcription of the "floral organ-building" gene *SPOROCYTELESS/NOZZLE* (*SPL*/*NZZ*). *SPL* plays a central role in regulating microsporangium differentiation, because *SPL/NZZ* mutant anthers fail to form archesporial cells (Schiefthaler *et al*., 1999; Yang *et al*., 1999). Liu *et al*. (2009) reported that ectopic expression of *SPL*/*NZZ* results in ectopic expression of *AG* and *SEP3* in the outer whorls of flowers, indicating that this gene is engaged in controlling stamen identity by interacting with the genes required for stamen identity.

In wild-type *Arabidopsis* ovules, megaspore mother cell differentiation required *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*), since the *spl/nzz* mutant failed to develop an MMC (Zhao *et al*., 2017; Mendes *et al*., 2020). In cytoplasmic male sterile mustard plants, expression of *SPL-like* gene was lost in the flower buds. Mitochondrial-specific inhibitors have repressed the expression of *SPL-like* gene and the shape and vigour of pollen grains were severely affected but pistil fertility remained unaltered (Yang *et al*., 2008). The anther primordium, PMCs, and tapetum all express the *SPL* (Schiefthaler *et al*., 1999). The pollen sac cells in *spl* mutant

flowers do not produce microsporocytes, and the tapetum does not develop. The *SPL* thus has a role in the differentiation of microsporocytes and the development of an anther wall.

The C-class MADS box protein AGAMOUS (AG), which binds to the CArG-box-like sequence in the 3' region of *SPL*, directly controls the transcription of *SPL* (Ito *et al*., 2004). The *SPL* is important in defining stamen identity because it causes the ectopic expression of stamen identification genes such *AG*, *SEPALLATA* 3 (*SEP3*), and *APETALA*2 (*AP2*) in the outer whorls of flowers (Liu et al., 2009). On the other hand, in *Arabidopsis*, *SPL* is expressed in the ovule primordium, nucellus, MMC, and growing integuments. A halted nucleus and the lack of the megasporocyte and embryo sac were the results of the *SPL* mutation.

The molecular mechanism and characterisation of *SPOROCYTELESS* in controlling the ovule development of *Arabidopsis* showed that *SPL* performs as a transcriptional repressor similar to an adapter (Baoye Wei *et al.,* 2014). It enlists *TOPLESS/TOPLESS-RELATED* (*TPL*/*TPR*) co-repressors to suppress the transcription factors that resemble *CINCINNATA* (*CIN*) such as *TEOSINTE BRANCHED1*/*CYCLOIDEA/PCF* (*TCP*). It also recruits *TPL/TPR*s using its EAR motif at the C-terminal end and inhibits *TCP*s using its N-terminal portion. They showed that the abnormalities of megasporogenesis in *SPL* mutants can be partially mimicked by either disruption of *TPL/TPR*s or overexpression of *TCP*s. Additionally, *TCP* disruption results in abnormalities that are similar to *spl-D* gain-of-function mutants. These findings outline the method through which *SPL*, along with *TPL/TPR*s, regulates ovule development by inhibiting the actions of essential transcription factors.

Studies of the *YUCCA (YUC)* genes (*YUC1*, *YUC2*, *YUC4* and *YUC6*) that encode the key enzymes in auxin biosynthesis provided direct evidence that auxin homeostasis determines the morphology of lateral organs (Zhao *et al.,*2001; Chang *et al.,*2006). The molecular characterization studies revealed that these *YUCCA (YUC)* genes are modulated by SPL. It's shown that in *spl-D* mutant plants, the expression of *YUC2* and *YUC6*, two essential genes in auxin production, was markedly suppressed. Additionally, the *spl-D* plants exhibited pleiotropic developmental flaws such as a decrease in shoot apical dominance, a reduction in the

number of lateral roots, and a simpler venation pattern suggesting that *SPL* may regulate auxin biosynthesis. According to the genetic and phenotypic studies of the *spl-D*/*yuc6-D* double mutant, *SPL* may control auxin homeostasis by inhibiting the transcription of *YUC2* and *YUC6* and taking role in lateral organ development (Li *et al.,* 2008).

The *HYDRA* gene is responsible for the production of spores in tomato. Through cloning and expression analysis it was identified that *HYDRA* encodes the tomato orthologue of *SPL/NZZ* of *Arabidopsis* and involves in germ line formation and fruit set in tomato and it was reported that SPL-like genes work as down regulation of fruit set in fleshy fruit plants and have an evolutionary conserved role in controlling sporogenesis in plants (Pilar-Rojas *et al.,* 2017).

Phosphorylation of the *SPL* gene by the protein kinase MPK3 and MPK6 are required for the development of anthers in *Arabidopsis*. Phosphorylation by MAPK6 maintains the *SPL* protein stability and improves the quantitative and spatial stability of anther cell differentiation.

The *SPL* along with *BAM1/2* involves in early anther development. Expression of *BAM1* in *spl* mutant and expression of *SPL* in bam1-bam2 double mutant were analysed. While *BAM1* expression was decreased and distributed unevenly in the *spl* mutant anther, *SPL* expression increased in the *bam1-bam2* mutant. This showed that *SPL* positively regulates *BAM1* expression, but that *BAM1*/*BAM2* play a role in limiting *SPL* expression. In order to regulate the equilibrium across sporogenous and somatic cell fates, it was suggested that *SPL*(*NZZ*) and *BAM1*/2 also create a positive-negative feedback loop (Hord *et al*., 2006).

The *SPL* and polarity genes such as the *YABBY* family, the class-III *Homeodomain Leucine Zipper* (*HD-ZIP III*) family, the *KANAD* (*KAN*) family, *ETTIN/AUXIN RESPONSE TRANSCRIPTION FACTOR3* (*ARF3*) and *ARF4* participate in the creation of the adaxial-abaxial pattern in ovules. YABBY family proteins, including FILAMENTOUS FLOWER (FIL), INNER NO OUTER (INO), and YABBY 3 (YAB3), determine the abaxial cell fate of ovules. By physically interacting with *FIL*, *INO* and *YAB3*, the *SPL* represses *INO* transcription in both time and space.

In a cucumber mutant with highly impaired anther and ovule development, *SPOROCYTELESS* (*SPL*)/*NOZZLE* (*NZZ*) expression was almost eliminated. Particularly in the growing anthers and ovules, *CsSPL* was expressed. Reduced male and female fertility with abnormal pollen and impaired ovule development were the results of *CsSPL* knockdown. Importantly, *CsSPL* positively regulated *CsWUS* expression through direct interactions with *CsWUS* (*WUSCHEL*) in the nucellus and *YABBY* family genes in integuments. In contrast, *CsPHB* (*PHABULOSA*), an *HD-ZIP III* gene that is only expressed in the nucellus, promoted *CsSPL* expression by binding to the CsSPL promoter. As a result, *CsSPL* functions as an adaptor to connect *CsPHB* and *CsWUS* activity, establishing a hitherto unrecognised regulatory mechanism that directs the development of the sex organ in plants (Xiaofeng Liu *et al.,* 2018).

Better knowledge of the molecular and genetic mechanisms governing the pattern development and growth control during floral organogenesis was made possible by the characterization of the *NOZZLE* gene. Additionally, it was stated that *NOZZLE* connects these developments as the ovule is developing. *NOZZLE*, an essential component of the ovule primordium, inhibits the actions of *BELL*, *AINTEGUMENTA*, and *INNER NO OUTER*, all of which encode putative transcription factors, in the region that will eventually become the nucellus. It has been established that the chalaza identification genes, *NOZZLE* and *BELL* have complementary roles in determining the ovule's potential chalaza.

Moreover, *NOZZLE* controls the number of cells and, thus, the length of the funiculus by competing with *AINTEGUMENTA* and *INNER NO OUTER* function. The growth of the integuments also requires *NOZZLE*. It has been demonstrated that *AINTEGUMENTA* and *INNER NO OUTER* are transcriptionally controlling *NOZZLE* during the earliest stage of this process. Therefore, *NOZZLE* indicates a downstream target of these two genes in the development of integument.

Materials and Methods

3. MATERIALS AND METHODS

The research work entitled "Characterization and expression analysis of *SPOROCYTELESS* gene in monoecious and gynoecious lines of bitter gourd" was carried out at Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Thrissur during the period 2020-2023. This chapter includes the details of materials used and the methodologies employed in the research.

3.1 Materials

3.1.1 Plant material

Seeds of monoecious cultivar Priyanka and gynoecious line KAU-Mc-Gy-102 were collected from the Department of Vegetable Science, College of Agriculture, Thrissur. These genotypes were used to study the sequence variation of the *SPOROCYTELESS* gene as well as the level of expression of this gene in developing floral buds.

The seeds were sown separately in pots and plants maintained till they reached flower bud stage.

3.1.2 Laboratory chemicals and equipment

LR and MB grade chemicals were purchased from Sigma Aldrich Pvt. Ltd., SRL Chemicals Pvt. Ltd., and HiMedia. Sigma Aldrich Pvt. Ltd., including DEPC (diethyl pyrocarbonate), RNA Later solution, RNase ZAP, and TRI reagent. Thermofisher Scientific provided the 2x RNA loading dye, Riboruler RNA low range ladder and Verso first strand cDNA synthesis kit. Bio-Rad Laboratories provided the SsoAdvanced Universal SYBR Green Supermix used for qRT-PCR analysis. GeNei Labs Pvt. Ltd. provided the dNTP mix, Taq DNA polymerase, and Taq buffer used in the PCR. HiMedia provided the PCR grade water that is suitable for PCR. GeNei Labs Pvt. Ltd.'s StepUpTM 100 bp DNA ladder and Biolit ProxiO100bp DNA ladder from SRL Chemicals Pvt. Ltd. were also used in this research

The laboratory equipment included the electronic balance (Shimadzu), icematic (F100 compact), fume hood (CAFH 1500), water bath (Rotek), high speed refrigerated centrifuge (KUBOTA 3500 and Eppendorf), microcentrifuge (SPINWIN^{TM} MC-100, SPINWIN TM MC-01), micropipettes (Eppendorf), pH metre

(EU Tech instrument pH tutor) and thermal cycler (BioRad). The glassware was supplied by Borosilicate, while the plasticware were purchased from Tarsons India Ltd. For the quantification and quality control of nucleic acids, Nanodrop ND-1000 spectrophotometer and gel electrophoresis unit (BioRad Gel Doc^{TM} XR+) were utilised. QuantityOne software was used for the analysis of gel images. The StepOnePlus Real-Time PCR machine (Applied Biosystems) at Kerala Veterinary and Animal Sciences University was used for the RT-qPCR analysis.(Annexure-I)

3.2 Methods

3.2.1 Isolation and characterization of *SPOROCYTELESS* gene

3.2.2 Primer designing

Three pairs of primers were designed for the PCR amplification of full length of *SPOROCYTELESS* gene in the leaves of both monoecious and gynoecious bitter gourd lines. Reference sequence was downloaded from the NCBI Genome database and Primer3 software was used. Primer sets were synthesised by Sigma-Aldrich Pvt. Ltd.

3.2.3 Isolation of total genomic DNA from bitter gourd

First to third leaves from the shoot tips of each genotype were collected in ice box. Long term storage, when required, was done at -80°C. The leaves were washed with sterile water and then cleaned with 70% ethanol.

The CTAB method (Rogers and Bendich, 1994) was used to extract the genomic DNA. Material required for DNA isolation are listed in Annexure II.

Procedure

- 0.1 g of clean leaf tissue was ground using liquid nitrogen in a pre-chilled mortar and pestle
- One mL of preheated CTAB extraction buffer, a tiny amount of PVP (Polyvinyl Pyrolidone), and fifty micro litres of ß-mercaptoethanol were added to the mortar.
- The homogenate was transferred to a two-mL autoclaved centrifuge tube, 500 µL more CTAB buffer added in each tube, mixed gently, and incubated at 65 °C for 20-35 minutes with periodic mixing and moderate inversion

- To create an emulsion, an equal volume of chloroform: isoamyl alcohol (24:1) was added and gently mixed by inversion. The tubes were centrifuged for 15 minutes at 4 °C at 10,000 rpm.
- After centrifugation, DNA and RNA were in the top aqueous layer, proteins and tiny particles in the middle layer and chloroform, pigments and cell debris in the lowest layer.
- The top aqueous layer was cautiously pipetted into a clean centrifuge tube and mixed with an equal amount of chloroform:isoamyl alcohol (24:1) and one tenth volume of 10% CTAB solution. Mixed the ingredients gently and centrifuged at 10,000 rpm for 15 minutes at 4 °C.
- The aqueous phase was transferred to a two-mL sterile centrifuge tube, to which 2-3 µ L of RNase was added, mixed thoroughly, and incubated for 1.5 hours at 37 °C. Equal volume of Chloroform to isoamyl alcohol (24:1) added, and the mixture was centrifuged at 10,000 rpm for 10 minutes at 4 °C.
- Supernatant was carefully pipetted out to a clean 1.5-µL centrifuge tube and 0.6 volume of chilled isopropanol was added. To witness the development of threads, the tubes were gently inverted in a horizontal position, and the contents were allowed to precipitate. The tubes were maintained at -20 °C for 30 to 60 min. to allow complete precipitation.
- Tubes were centrifuged for 15 min. at 4 °C and 10,000 rpm.
- Pellet was washed with 70 % ethanol (200 µL) for 5 min. at 10,000 rpm. After washing, ethanol was decanted.
- Then the pellet was washed with 100 µL of 100 % ethanol for three min. at 10,000 rpm
- The pellet was then dissolved in 50 µL of autoclaved distilled water and stored at -20 °C

3.2.4 Quality assessment by agarose gel electrophoresis

Quality of the extracted DNA was evaluated by electrophoresis on 0.8 % agarose gel (Sambrook et al., 1989). Reagents and apparatus used in agarose gel electrophoresis are listed in Annexure III.

Procedure for gel casting and electrophoresis

- A 60 mL gel casting tray was placed in a gel casting device, and a comb was put vertically about an inch from one end of the tray such that there would be a space of one to two millimetres between the comb's teeth and the tray's surface
- Agarose gel (0.8 %) was made by mixing 0.48 g of agarose with 60 mL of 1X TAE in a conical flask, then heating the mixture in a microwave for 45–60 sec. or until the agarose was completely dissolved and the solution was clear
- The solution was allowed to cool to around 42-45 °C at ambient temperature and then 5µL of ethidium bromide dye with a working concentration of 0.5 g/mL was added and mixed to ensure even dispersion. The delicate mixing process prevented the formation of bubbles.
- The warm semi-solid solution was gently poured into the casting tray, filling it to a height of approximately 5-6 mm, and then left to cool and harden for approximately 30–40 minutes. After the gel got solidified, the casting tray and comb were carefully removed.
- The gel tray was positioned within the horizontal electrophoresis unit's gel tank with the wells facing the cathode. IX TAE buffer solution was poured into the gel tank until all the wells were immersed.
- The first well was added with 4 µL of 100 bp DNA ladder
- Five µL each of the DNA samples were prepared by mixing with 1 µL each of 6X gel loading dye. The mixture was then loaded into each well.
- The tank was sealed, the anode and cathode were connected to the power pack, and electrophoresis was performed at a constant voltage of 70 V.
- When the tracking dye reached two-thirds of the length of the gel, electrophoresis was stopped, gel removed from the apparatus and bands docked under a UV trans-illuminator.
- Gel disposed properly, buffers disposed or transferred back, if reusable, and gel tank cleaned up.

3.2.5 Gel documentation

A UV trans-illuminator was used to check for bands on the electrophoresed gel. Due to the ethiduim bromide dye, which binds to the hydrogen bonds between the

double helix, the DNA fluoresces when exposed to UV light. This dye is activated at 260 nm and emits yellowish orange light at a wavelength of 590 nm.

Using Quantity One software, bands were recorded in the gel documentation system (BioRad Gel DOC-It^{TM} imaging system). The gel was examined for presence of RNA and protein as well as the structural integrity and clarity of the DNA band. A thick band lighter than 100 bp showed the presence of RNA whereas proteins clogged the wells and made to fluoresce under UV. Degraded DNA appeared as smear whereas intact good DNA resulted a single heavy band just emerged from the well.

3.2.6 Assessment of quality and quantity of DNA

A spectrophotometer (NanoDrop ND-1000) was used to evaluate the quality and quantity of the extracted DNA. While proteins have the highest absorbance at 280 nm, nucleic acid exhibits its maximum absorbance at 260 nm. At both wavelengths, the absorbance was measured, and the purity was calculated using the A_{260}/A_{280} ratio. DNA of acceptable quality and reasonably free of proteins and RNA will have a ratio between 1.8 and 2.0. A result of more than 2.0 suggests RNA contamination, whereas a ratio of less than 1.8 indicates contamination with proteins. The amount of DNA in a pure sample was determined using the relation shown below.

$$1 \text{ OD at } 260 \text{ nm} = 50 \text{ } \mu\text{g DNA mL}^{-1}$$

Hence, the OD_{260} reading obtained from the sample multiplied with 50 gives the total quantity of DNA (µg/mL).

Procedure

- NanoDrop spectrophotometer was connected to the system, which was installed with the software ND-1000
- The option 'nucleic acid' was selected in the software
- The upper and lower measurement pedestals were carefully cleaned with the sampling arm raised, and one µL of distilled water was pipetted onto the lower measurement pedestal.
- Sampling arm was closed, and the operating programme started the spectrum measurement.
- Reading for the blank sample was set to zero

- Similarly, the all samples were measured and after the last sample, the sampling arm, as well as the upper and lower pedestals, were carefully cleaned using a soft laboratory wipe

3.2.7 Thermal amplification in PCR

Three primer pairs were used to PCR amplify the *SPOROCYTELESS* gene from bitter gourd DNA. Gradient PCR at 55 to 60 °C range were performed to determine the ideal annealing temperatures for each primer pair. Composition of the PCR reaction mixture is presented in Table 3.1. The DNA template was added separately after the aliquots of the PCR reaction mixture had been added to the 0.2 mL PCR tubes. Thermal profile for gradient PCR is presented in Table 3.2.

Table 3.1. Components of PCR reaction mixture

Sl. No.	PCR components	Volume (µL)
1	Genomic DNA (50 ng/µL)	2.0
2	10X *Taq* assay buffer A with $Mgcl_2$	2.0
3	dNTP mix (2.5mM each)	1.8
4	Forward primer (100pM)	1.0
5	Reverse primer (100pM)	1.0
6	*Taq* DNA polymerase(3U/µl)	0.4
7	PCR grade water	11.8
	Total volume	20.0

Table 3.2. Thermal profile for gradient PCR

Sl. No.	Steps	Temperature (°C)	Time	Cycles
1	Initial denaturation	95	5 min.	-
2	Denaturation	95	30 sec.	30
3	Primer annealing	60	30 sec.	
4	Primer extension	72	1 min.	
5	Final extension	72	8 min.	
6	Hold	4		-

3.2.7.1 Analysis of PCR amplicons by electrophoresis

PCR products were electrophoresed on 2 % agarose gel stained with ethidium bromide. A 100 bp DNA ladder (SRL Biolit) was run to compare and estimate the amplicon sizes.

3.2.8 Gel purification

DNA was extracted from the amplicons in the agarose gel for subsequent sequencing on Sanger platform.

Using the gel and PCR clean-up kit (Macherey-Nagel™ Nucleospin™), the PCR amplicons of the expected size were eluted from the agarose gel.

Materials required

- Buffer NT1 (provided with the kit)
- Buffer NT3 (provided with the kit)
- Nuclease free water

Procedure

- From the agarose gel, the amplicon of the expected size was cut with a sharp blade.
- The sliced gel was placed in a microcentrifuge tube and 200 µL of Buffer NT1 was added to each 100 mg of the gel.
- Incubated the tube at 50 °C for 5–10 min. and got the gel slice dissolved.
- A quick vortexing was performed for 1-2 minutes
- The sample was put into the gel clean up column using a 2.0 mL collection tube, which was included with the kit.
- Centrifuged at 11000 g for 30 sec. and flow-through discarded
- The column was placed back to the collection tube
- Buffer NT3 (700 µL) was added to the column and centrifuged for 30 sec. at 11000 g
- The flow-through was discarded
- This action was repeated once more
- The tube was centrifuged again for one minute at 11000 g to remove even traces of Buffer NT3

- The column was then incubated for 2–5 minutes at 70 °C
- The column was inserted into a 1.5 mL tube, 10 µL of nuclease-free water was added, and centrifuged at 11000 g for one min.
- This step was repeated once again.
- Eluted DNA was maintained at -20 °C and 4.0 µL of eluted DNA was used to check the quality on 0.8 % agarose gel

3.2.9 Sequencing and sequence analysis

3.2.9.1 Sequencing of PCR product

PCR products, except those eluted from the gel, were subjected to column purification. PCR amplicons generated using three primer combinations, in both monoecious and gynoecious lines, were paired-end sequenced on Sanger platform using the specific primers (Sanger et al., 1977).

3.2.9.2. Sequence data analysis

The forward and reverse sequences generated from an amplicon using the respective primers were aligned to a single, continuous sequence. Online sequence assembly programme CAP3 available at PRABI-Doua was used to create the continuous sequences from the overlapping forward and reverse complementary sequences (Huang and Madan, 1999).

Contiguous sequences generated using three primer pair were combined to develop the sequence of *SPOROCYTELESS* gene in monoecious and gynoecious lines. Additionally, the sequence from these lines was aligned with reference sequence in GenBank using multiple sequence alignment tool Clustal Omega available at European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Goujon *et al.* 2010). Identical bases at any position were indicated by an asterisk (*), whereas non-identical bases missed it. In/Dels were indicated by a gap within the sequence.

3.2.10 Expression analysis for *SPOROCTLESS* gene

To confirm the role of *SPOROCYTELESS* gene in flowering in bitter gourd, its expression in the developing buds of monoecious and gynoecious lines was assessed using real time quantitative PCR (RT-qPCR) analysis.

3.2.10.1 Collection of flower bud samples

Floral buds were collected from pot raised monoecious and gynoecious lines of bitter gourd. The collected samples were wiped with RNase ZAP (Sigma) and stored in RNA Later (Sigma) solution till RNA extraction. When necessary, the buds were stored at -80 ºC for long term.

3.2.10.2. RNA isolation

The following general precautions were followed before the isolation of RNA.

- Microcentrifuge tubes, microtips, mortars, and pestles, and other materials needed for RNA isolation, were treated with 0.1 % DEPC overnight and doubly autoclaved before use
- To prepare the TAE buffer and 75% ethanol, double autoclaved DEPC treated water was used
- Before usage, the work bench, all apparatus including the gel electrophoresis machine, were cleaned with 75% ethanol and RNase ZAP (Sigma).

The procedure followed for isolation of good quality RNA from the floral buds is given below.

Reagents used

- TRI reagent (Sigma)
- Chloroform
- Isopropanol
- 75 % ethanol
- Nuclease free water

Procedure

- Ground 100 mg of buds to fine powder, in liquid nitrogen, using pre-chilled DEPC-treated mortar and pestle
- When the powdered tissue was fully thawed, it was transferred to a 1.5 mL microcentrifuge tube
- One mL of TRI reagent was added to the powdered tissue
- Vortexed the tube for 15 sec. and incubated for 10 min. at room temperature
- To this, 200 µL of chloroform was added

- Homogenized the contents for 15 sec. and incubated for 5 min. at room temperature
- Centrifuged the contents at 12000 g, at 4 °C, for 15 min.
- Supernatant was pipetted out to a new 1.5 mL microcentrifuge tube
- To this, 500 µL of ice-cold isopropanol was added and incubated for 10 min. at room temperature, for precipitation of RNA
- Contents were centrifuged at 12000 g for 10 min. at 4 °C and supernatant discarded
- The pellet was washed with 1.0 mL of 75 % ethanol and centrifuged at 7500 g for 10 min.
- The supernatant was removed and the RNA pellets was dried under ambient conditions
- Pellet was dissolved in 20 µL of nuclease free water

3.2.10.3 Analysis of quality of the isolated total RNA

Agarose gel electrophoresis was used to evaluate the quality of the total RNA.

Reagents used

- Agarose (SRL Chemicals Pvt. Ltd.)
- Stock solution of TAE buffer (50X, pH 8.0) prepared in DEPC treated water [T buffer (1 M) (242.2 g/L), EDTA 0.5 M (pH 8.0, 100 mL), Glacial acetic acid (57.1 mL), total volume made up to 1 L]
- Double autoclaved DEPC treated water
- RiboRuler^{TM} low range RNA ladder (Thermofisher Scientific)
- Loading dye (6x) (GeNei)
- Ethidium bromide (10 mg/mL stock)

Procedure

- 1X TAE buffer was prepared using double autoclaved and DEPC-treated water from a 50X stock solution. To prepare 1 % agarose gel, 60 mL of 1X TAE buffer and 0.6 g of agarose were taken in a conical flask and heated in a microwave oven.
- The clear solution obtained after complete melting of the agarose was allowed to cool under room temperature

- When the temperature of the solution reached nearly 45-50 ºC, 3.0 µL. of ethidium bromide was added and thoroughly mixed
- Gel casting, electrophoresis and documentation were performed as detailed under Section 3.2.4

For electrophoresis, 4.0 µL of RNA sample was mixed with 2.0 µL of loading dye and loaded in the well. RNA ladder (3.0 µL) was loaded in the first well.

Quantity and quality of the isolated RNA was assessed using NanoDrop spectrophotometer, following procedure given in Section 3.2.6.

3.2.10.4 Real Time-Quantitative Polymerase Chain Reaction

3.2.10.4.1. First strand cDNA synthesis

The RevertAid First strand cDNA synthesis kit (ThermoFisher Scientific) was used to prepare the first strand of cDNA from the RNA, following manufacturer's protocol.

Reagents used

- 5X reaction buffer
- Oligo(dT) primer
- RevertAid M-MuLV RT (200 U/µL)
- Ribolock RNase inhibitor (20 U/µL)
- Nuclease free water

Procedure

After thawing, the components of the kit were briefly spun and stored on ice.

- Following components were added into a sterile nuclease free 0.2 mL microcentrifuge tube on ice

  Template RNA - 2.0 µg

  Oligo(dT) primer - 1.0 µL

  Nuclease free water - To make up total volume to 12 µL

- The components were carefully homogenized and vortexed for 1 minute

  The tube was incubated at 65 °C for 5 minutes (preferably in a thermal cycler) and then moved to ice to add the following components in the given order

Table 3.3 Components of cDNA reaction mixture

Components	**Volume** (µL)
5X reaction buffer	4.0
RevertAid M-MuLV RT	1.0
Ribolock RNAse inhibitor	1.0
10mM dNTP mix	2.0
Total	20.0

- Contents were mixed gently through vortexting.
- The tube was incubated at 42 °C for 60 min and then warmed for 5 min. at 70 °C to end the reaction
- The cDNA was maintained at -20 °C

3.2.10.4.2 Designing of primers for RT-qPCR analysis

Using the sequences of coding regions in *SPOROCYTELESS* gene, a pair of primers was designed using Primer3 software. Primers were also designed for the endogenous control gene *actin*.

3.2.10.4.3 Confirmation of first strand cDNA synthesis

Using *actin* gene-specific primers, the first strand cDNA was PCR amplified at 60 °C annealing temperature.

Table 3.4 Components of PCR reaction mixture used to confirm the synthesis of first strand cDNA

Component	**Volume (µL)**
cDNA	2.0
10X *Taq* buffer A (with 1.5 Mm $MgCl_2$)	1.5
dNTP mix (2.5 mM each)	1.5
Forward primer (SPL-RT -F)	1.0
Reverse primer (SPL-RT -R)	1.0
Taq DNA Polymerase	0.5
Molecular Biology grade water	7.5
Total	15.0

3.2.10.4.4 RT-qPCR analysis

Through RT-qPCR analysis using SYBR Green chemistry, expression of the *SPOROCYTELESS* gene in floral buds of monoecious and gynoecious bitter gourd lines was studied. The *actin* gene was used as endogenous control and two biological replicates and three technical replicates for the test gene and endogenous control gene were done. Sso Advanced Universal SYBR Green Supermix (Bio-Rad) was used in the experiments, which were carried out on the StepOne Plus Real-Time PCR machine. The Table 3.5 shows the components of the RT-qPCR reaction mixture. The reaction was configured in accordance with the temperature profile shown in Table 3.6. The melt curve analysis was performed to verify the accuracy of the amplicons. Using the formula $2^{-\Delta\Delta Ct}$, the relative level of expression of *SPOROCYTELESS* gene was normalised with the expression of the endogenous control *actin* gene (Livak and Schmittgen, 2001).

Table 3.5 Composition of the reaction mixture used for RT-qPCR

Components	**Volume (µL)**
Sso Advanced™ Universal SYBR Green Supermix	10
Forward primer	1.0
Reverse primer	1.0
DNA template	5.0
Molecular biology grade water	3.0
Total	20.0

Table 3.6 Thermal profile followed for RT-qPCR

Steps	**Temperature (°C)**	**Time (sec.)**
1.Initial denaturation	95	30
2.Denaturation	95	15
3.Annealing	60	30
4.Extension	72	30
Melt curve analysis	95	15
	60	60
	95	15

Step 3 and 4 were carried out for 45 cycles

3.2.10.4.5 Calculation of fold change

Ct values were obtained from the RT-qPCR assay, which were then used to calculate the ΔCt and ΔΔCt of the target gene, in comparison to the house-keeping gene.

ΔCt = Ct of gene of interest - Ct of internal control, where the reference gene is taken as internal control.

ΔΔCt = ΔCt of Sample A - ΔCt of Sample B = [(Ct of gene of interest - Ct of internal control)

for Sample A - (Ct of gene of interest - Ct of internal control) for Sample B] where, Sample B is the untreated sample, which is monoecious flowers, and Sample A is treated sample, which is gynoecious flowers. Fold change was calculated as $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008).

Results

4. RESULTS

The research work entitled "Characterization and expression analysis of *SPOROCYTELESS* gene in monoecious and gynoecious lines of bitter gourd was carried out with the aid of facilities available at Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur during the period 2020-2023. This chapter details the results of the research work.

4.1 Planting material

Monoecious bitter gourd cultivar Priyanka and gynoecious line KAU-Mc-Gy-102 were used to sequence characterize the *SPOROCYTELESS* gene as well as to study its expression level in developing floral buds.

The seeds were sown in separate pots and plants maintained till they reached the bud stage (Plate 4.1).

4.2 Retrieval of gene sequences and primer designing

The *SPOROCYTELESS/SPL* or *NOZZLE/NZL* gene's nucleotide sequence (2132 bp) was retrieved in FASTA format from the NCBI GenBank (XM_0222839995.1)(Fig. 4.0). Three pairs of primers were designed with the desired product size, GC content, melting temperature, and least primer dimerization (Tables 4.1 and 4.2).

Table 4.1 Primer pairs designed for *SPOROCYTELESS* gene

Primer	Sequence (5'-3')
SPL-BG-1-FP	TCCCCATTTGAAAGAAAACCCC
SPL-BG-1-RP	TTTGCTCTTCTTCTGCCATTCC
SPL-BG-2-FP	TCCCTGATTGAGCTTTGTGAAC
SPL-BG-2-RP	GGGGCTCTATCTGGACATTAAGA
SPL-BG-3-FP	ACCCGGGACAGGCTTTTC
SPL-BG-3-RP	TGAACCCAAAAGACAGAACATTT

Table 4.2 Features of the primer pairs designed for the study

Sl. No.	Name of Primer	Length (bp)	Tm (ºC)	Product size (bp)
1	SPL-BG-1-FP	22	63.2	
2	SPL-BG-1-RP	22	63.4	718
3	SPL-BG-2-FP	22	69.2	
4	SPL-BG-2-RP	23	74.1	912
5	SPL-BG-3-FP	18	64.7	
6	SPL-BG-3-RP	23	65.5	1000

4.3 Molecular characterization and analysis of *SPL* gene

DNA was isolated from the emerging leaves and electrophoresed to check the quality. High molecular weight, intact, clear, and bright bands have demonstrated their high quality (Plate 4.2). All DNA samples examined in spectrophotometers had $A_{260}/_{280}$ absorbance ratios between 1.8 and 2.0, indicating their acceptable quality. DNA from KAUMCGy-101 and Priyanka had 665.81 and 585.86 ng/µL concentration with A_{260}/A_{280} absorbance ratio of 1.84 and 1.90, respectively.

Thermal cycling at annealing temperature of 60 °C has generated the bands of the predicted sizes, 718, 900 and 1000 bp (Plates 4.3a and 4.3b).

PCR products were paired-end sequenced on Sanger platform. Forward and reverse sequences having a Quality Value (QV) score of 20 or higher were used for subsequent analysis. . For monoecious and gynoecious lines, contigs with 2381 and 2391 bp were generated (Figs. 4.1 and 4.2). Contiguous sequences generated were used for multiple sequence alignment with monoecious, gynoecious, and reference sequences (Figs. 4.3, 4.4, 4.5, 4.6 and 4.7). Two base-variations found in the sequence of gynoecious sample (G and A) compared to monoecious (T and C, respectively) were subsequently found to be misrepresentations, on cross verification with chromatogram peaks (Figs. 4.8 and 4.9). No valid variations were found between the sequences of gynoecious and monoecious lines.

Homology search for the sequences using BLASTn showed 100 % identity and 79 % query coverage with the predicted sequence of *Momordica charantia* transcript variant (LOC111010534) (Accession no XM_02283995.1) (Figs. 4.10 and

4.11). Analysis of sequences using BLASTx showed 99.09 % identity with the same transcript variant (Accession no XP_O22139687.1) (Figs. 4.12 and 4.13). BLASTp analysis of the sequences showed 100 % identity with protein sequence of *Momordica charantia* (Accession no. XP_022139688.1) (Figs. 4.16 and 4.17).

The amino acid sequence of the *SPL* gene from monoecious and gynoecious lines were deduced using the ExPASy translate tool (Figs. 4.14 and 4.15).

Analysis using ORF finder showed that the nucleotide sequence had 19 ORFs in both lines with ORF4 as the longest with 768 bp and coding for 255 amino acids (Figs. 4.18 and 4.19).

Plate 4.1 Monoecious and gynoecious lines raised in pots

FASTA

PREDICTED: Momordica charantia uncharacterized LOC111010534 (LOC111010534), transcript variant X2, mRNA

NCBI Reference Sequence: XM_022283995.1

GenBank Graphics

>XM_022283995.1 PREDICTED: Momordica charantia uncharacterized LOC111010534 (LOC111010534), transcript variant X2, mRNA

TTGGATTGCAAAGGATCATCATATCCCCATTTGAAAGAAAACCCCAAAAACAAACCAACCCTAAAAGTGA
AGAATTGGGTTGTAAAGTTGGGTTGGCCTCGAGCTCTTTTTGGAACTAAAATTCCATAATTTTAGAGAGA
GAAAGAGGAATTCTGAGAGATGGGTTTTGGTTTTAGATTACATGGAAACAAAACCCACATTGTAGTAGTG
GATGAGAGAGGGAGGAGAGTGAGTGATTTTCTCCACAGTCAAAAAGAAAACGAGGAGTGGAGCGACGATT
TTGGCGACACTATCGATTCTCAAAATCCTCACAGTATGTAAAATTTGTAATGCATTTTTTCTTCTTCCAA
CTGCTTTGATTTTTTCTTCCATTCTCAGACATGCACTGGATTTGAGAATAATCTCACCCTTATCAGTCTC
TTTTGTTGGTTCTTGGGGTTGTTCGTCCTCCGCGAGATTTCCGGTAACTTCATTTTGCTTCGATAGTTCC
GCTCCCCTTCTATATCTCGTCCTCAGATTTCTCAGTTTTACAGGTTCTCTCTGAAACTTCGTATAGATTT
TTGTTTTTGTCCGTTGCTTGTTTTTTTCCCTGATTGAGCTTTGTGAACTGGAATAGAGGTTTTAGTACTG
CAACAGACTTGTTTAGAAATTAGGGTATTGGAATCTTATCTGGGTTGTTGCTTGCTTGAGGAATCTTAGT
TTTTGTTTTGAAGTATAGATGGAAGATAATAGGAATGGCAGAAGAAGAGCAAAAACAGAGGTGTTATAGT
AATAGATTTGGGAGTTTTGGGACAATTGGTGGTGTTGGTGGTAGATCTTCTTCTAAAAAACCAAAGCCAA
AGACTAAGAAAGTTCCACAGAGAGGGCTTGGTGTTGCGCAGCTTGAGAAGATCAGATTAGAGGAACAGCA
GAAGAATGATGCAGCTGCTGCAATTTTCTCATCCCCTTCTCCATTGTCACCTACCAAATCCTCCTCTTAT
CTGTCGTTGCCCGTTCCCAGTTTTCGCCAGTCGAATCAGTCATCGTCCTCGTCTTCATTCCCTTCGCCTC
CTCTGGTGAACCTCTCCTCTTCCAGCTCAATGTTTGGGCCACCTCTGCCTGTTCTGAACATGGACGTTAG
AGATTCTTTCACTGTTCCATTGGTGGATCAGGCAAAGAGTGGTGGATCTGAAACTGGGTTGTCTGCAGTT
ACAATCATGGAGCAAGGAAATGCTCTCAAATGGCAGAGCTCTTGTGAATACTATCTCGAGAAGGAGAATT
ACGGGGTCGACCCGGGACAGGCTTTTCGCTCAAATTTCAATCTCCCATATGAAGTTAACCCTGCCTGGCC
TTCCCCAGAACTGCTCCAAAGAGCACAGCAATACCAATCACCTTCTCCAATGGTGAATAATGTCTCATCA
ACAACTTCATTGTCTTCTGGTCTTAATGTCCAGATAGAGCCCCCTTCAAACCAAAGCTATTATTGTGGCA
ACTACTCAACCATCTGGGCAGACAAGGAAGAGAAGATGGTTGGCATGAAGAGATTGTCTCTCTTAACTCC
AAACAACCCAAGTGGACCGACTTTCGATTACGACCGCTCTTCCTTCACTGTTCCAAATAGATCTGATGAT
TCACCTTCACATGGCAATGGAAGCCTGCCAAGTTTTCCAGAGGATCATACGCCGAGCTTAACACCGAGCT
CCGAGGACGACTTTGGTGGAAACTTCCTCACCCTGGCTTCTCTTGCTACTCGTTGGACATCATGTCCAAG
CTCTAACAATGTTAAGTCCCCTCCAACTTATCCTCTTCTGCAGAACCTTGTGCATCACCCCGATTCAGAA
CCGTTTCATTTTCAGGGAGGAACGAAACCTGTTGGATTGGCCGAAAAGCAGCCATTCTACAGCTTCCTTC
CAGCTGCAGAGGCAAAAACTGGACAAGAAACTGCCACTTCTGTGAACAAATTCAAGGGTGGCGAAGTAGG
AGAAAGAGTTGATTTAGATTTGAAGTTGTAGAAGTTGTAGAAACCTGTATGTTACTCTTTTAAATGTTCT
GTCTTTTGGGTTCAAATATATGTAGGTTTTAAATTATGAAATTTTGTGTAAGAAACTCAAAATCTTACCT
TCAAAGTTGTTTTGTTAGGTTCTGTTGGATAA

Fig 4.0 FASTA sequence of the *SPOROCYTELESS/NOZZLE* gene obtained from the NCBI

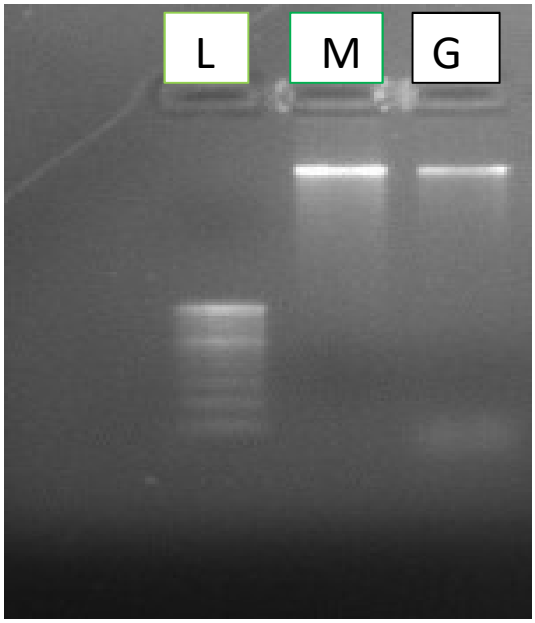


Plate 4.2 Agarose gel profile of total DNA isolated from bitter gourd leaves. L: Ladder, M: Monoecious, G- Gynoecious

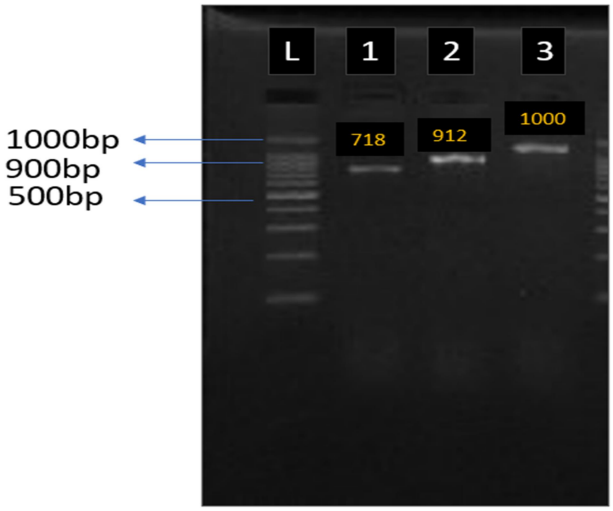


Plate 4.3a. PCR amplification of SPL gene target in monoecious bitter gourd cultivar. L- Ladder, 1- SPL-BG-1, 2-SPL-BG-2, 3-SPL-BG-3

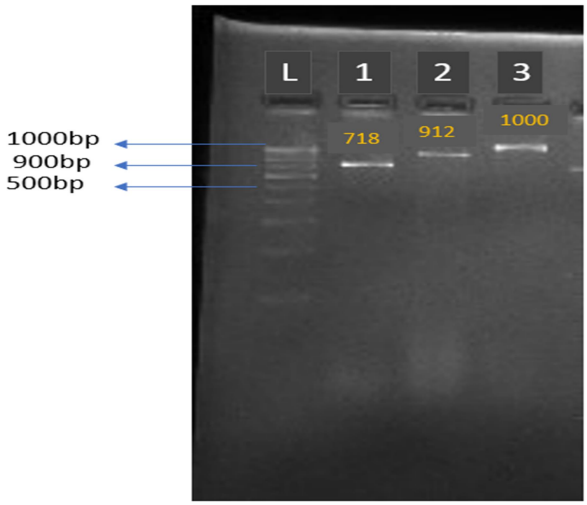


Plate 4.3b. PCR amplification of SPL gene target in monoecious bitter gourd cultivar. L- Ladder, 1- SPL-BG-1, 2-SPL-BG-2, 3-SPL-BG-3

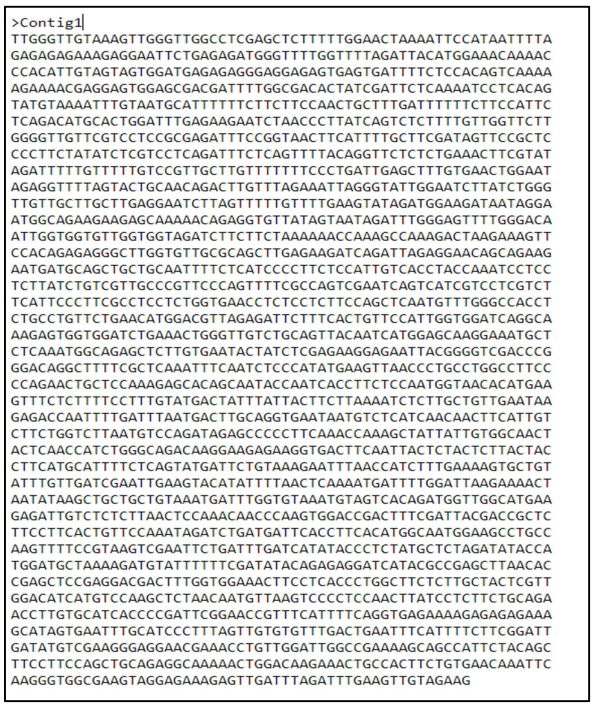


Fig. 4.1. The contig obtained after assembling the forward and reverse sequences of all the three sets of primers was 2381 bp long for monoecious

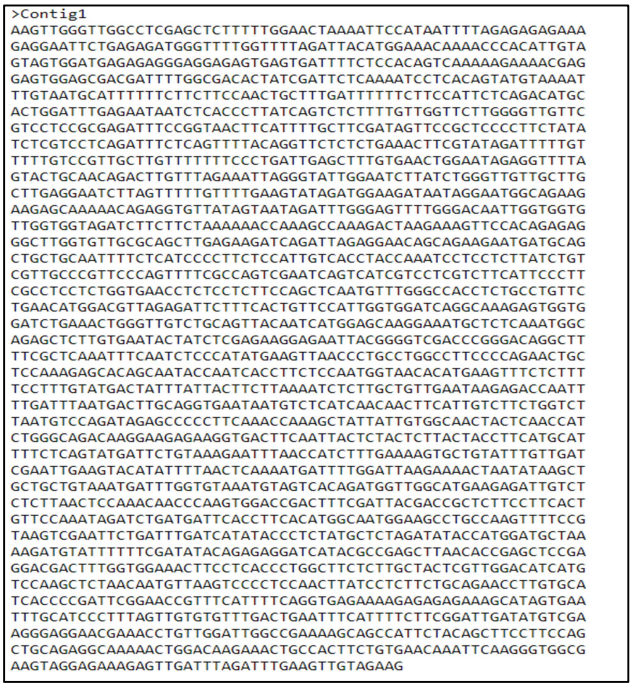


Fig 4.2. The contig obtained after assembling the forward and reverse sequences of all the three sets of primers was 2391 bp long for gynoecious

Fig. 4.3. Multiple sequence alignment of *SPL* gene

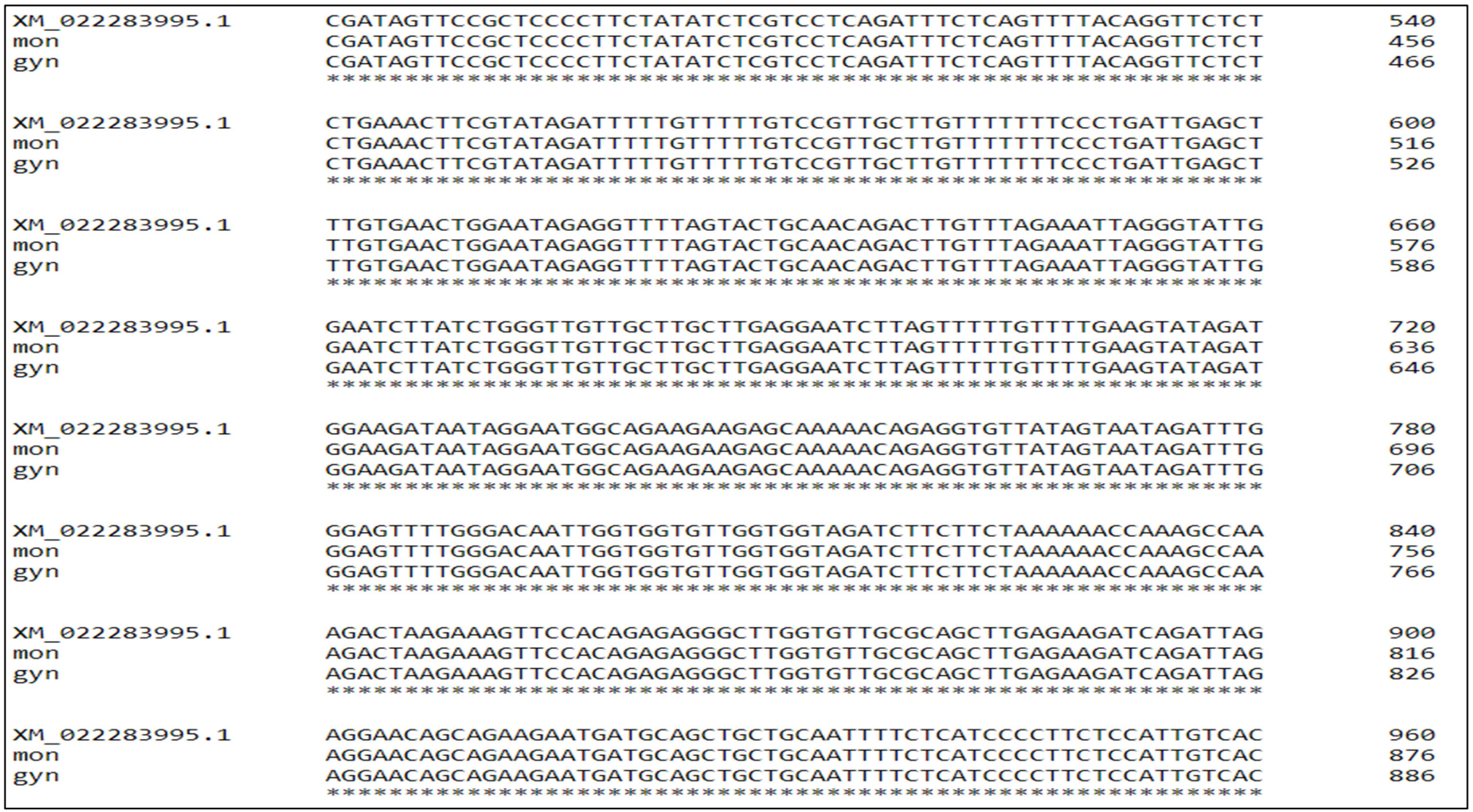


Fig .4.4. Multiple sequence alignment of *SPL* gene

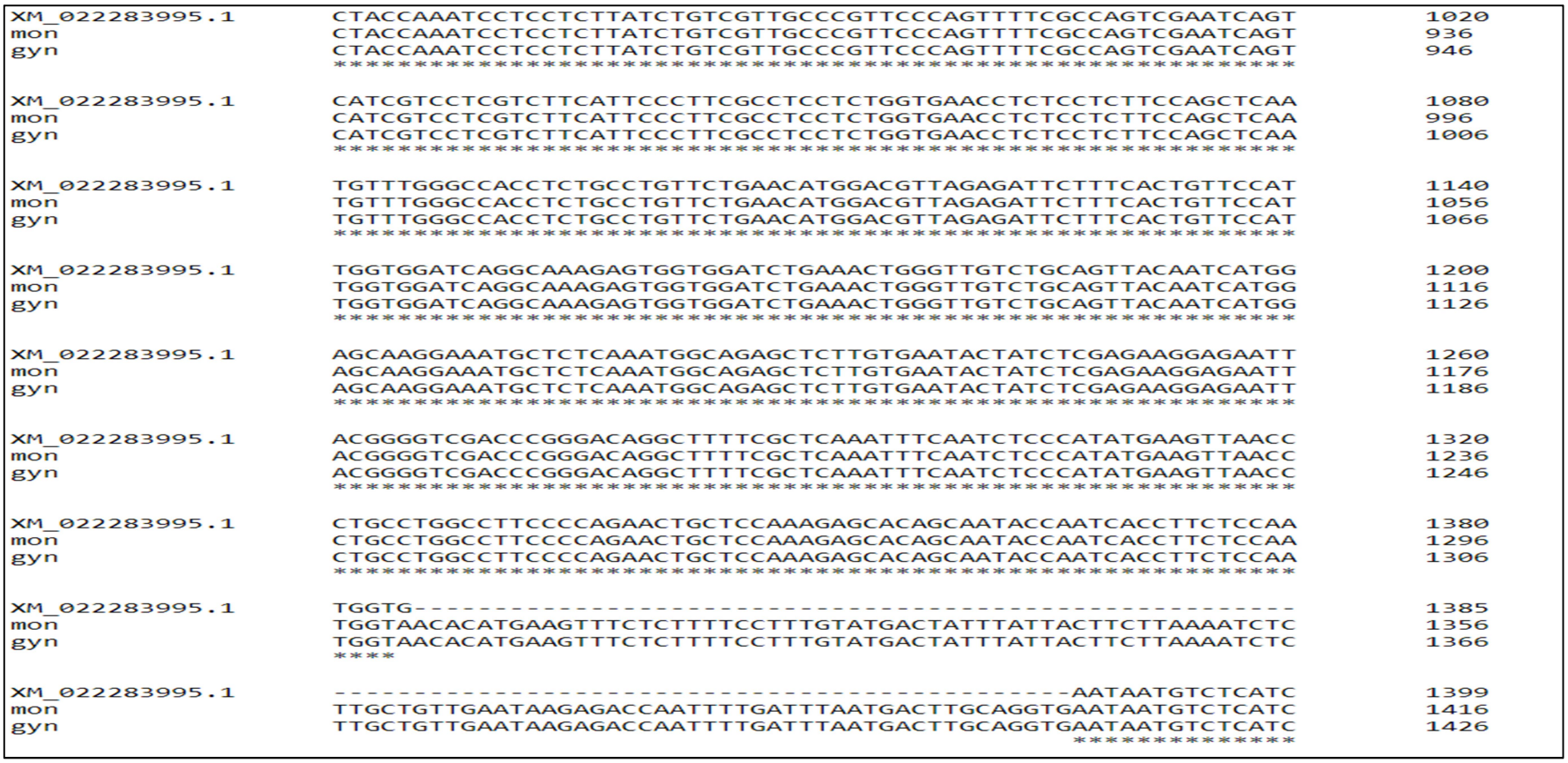


Fig. 4.5. Multiple sequence alignment of *SPL* gene

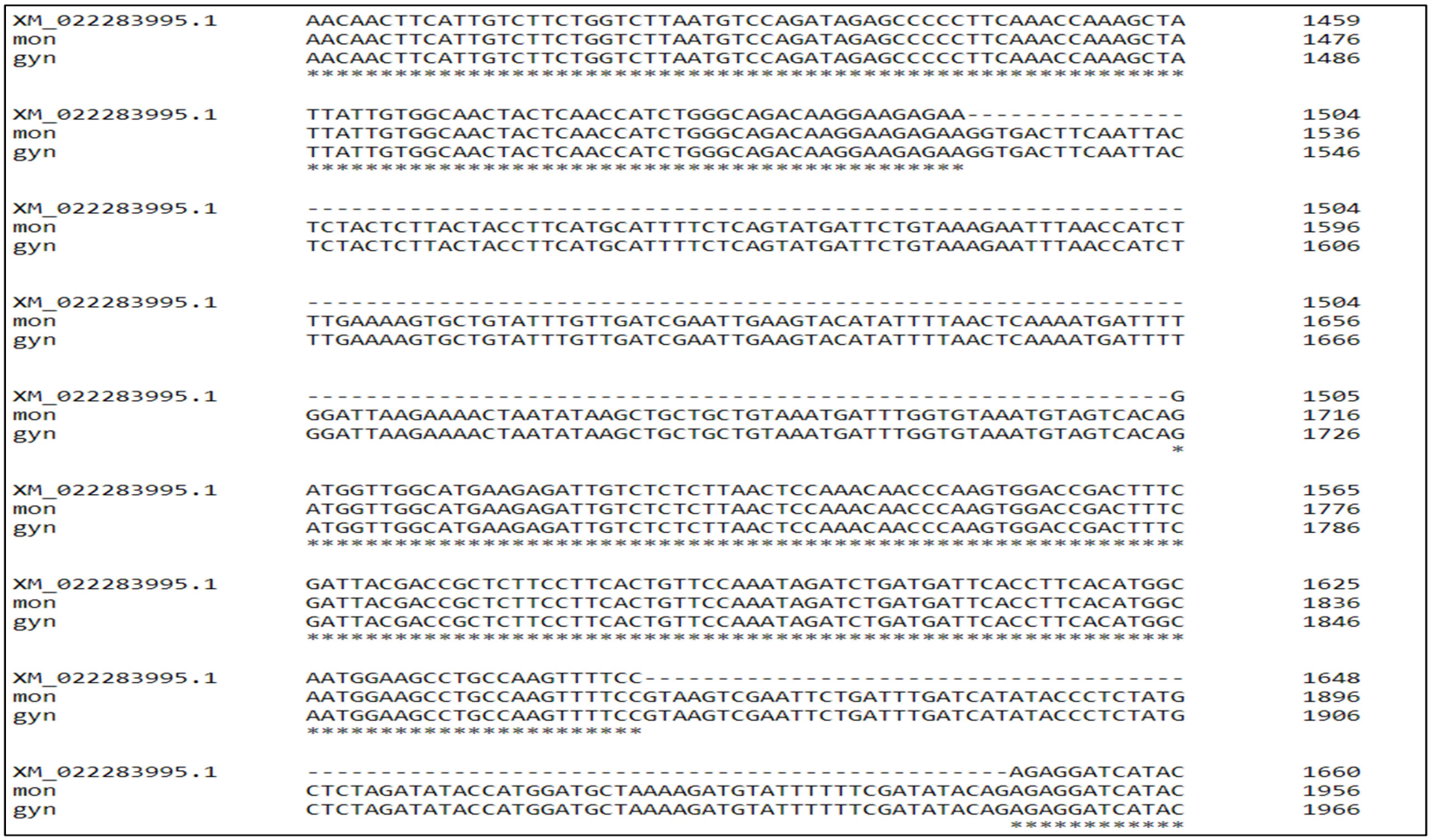


Fig. 4.6. Multiple sequence alignment of *SPL* gene

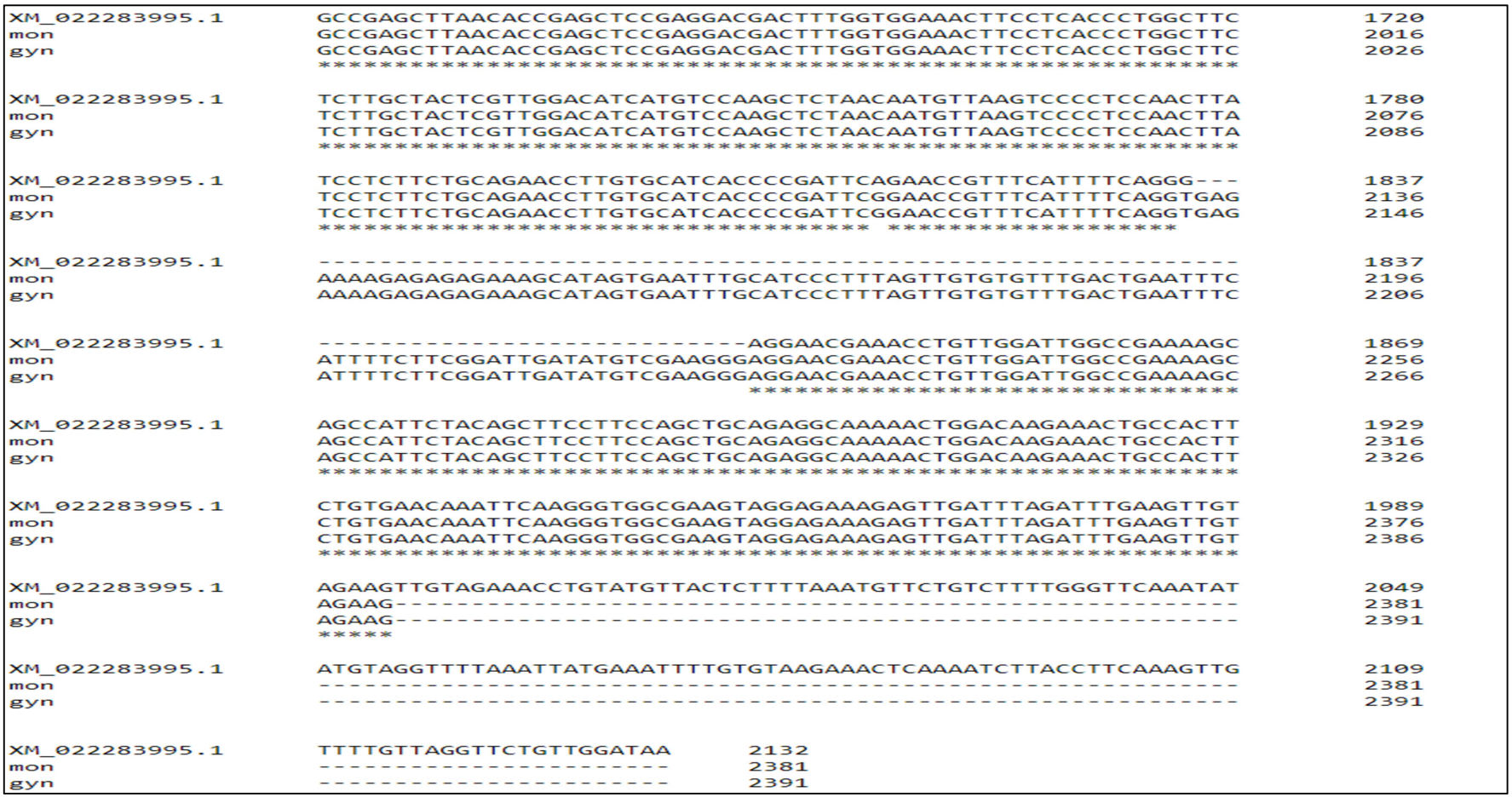


Fig. 4.7. Multiple sequence alignment of *SPL* gene

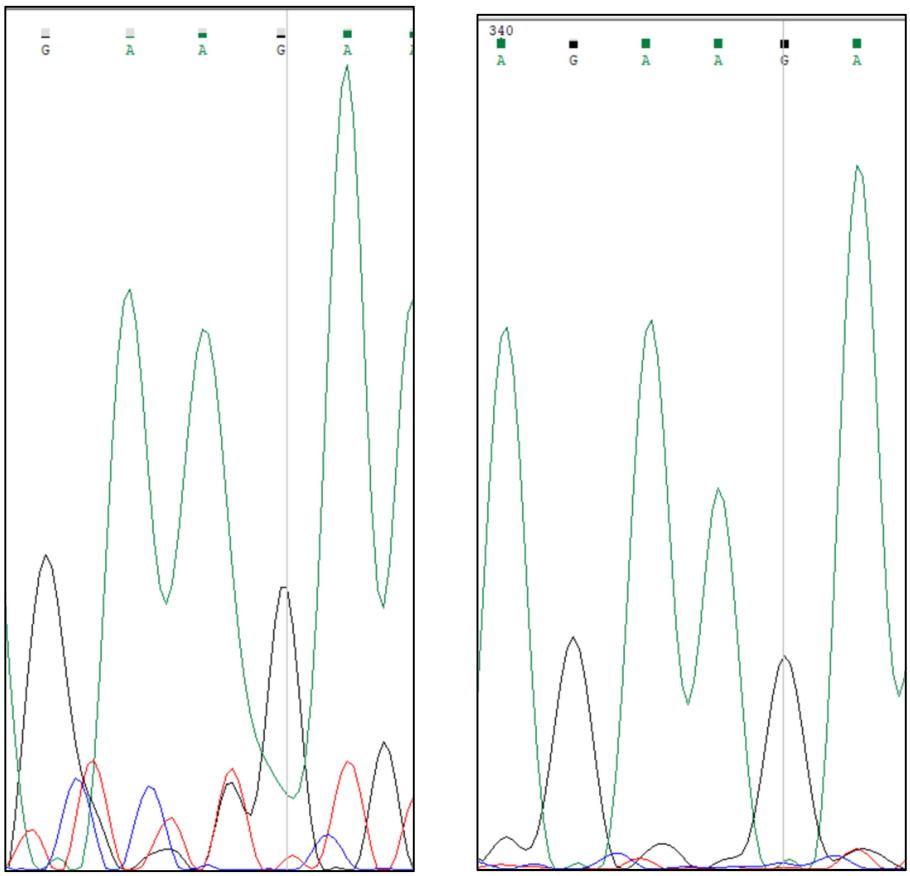


Fig. 4.8. Chromatogram peak images for Var 1 **1)** Monoecious sequence **2)** Gynoecious sequence

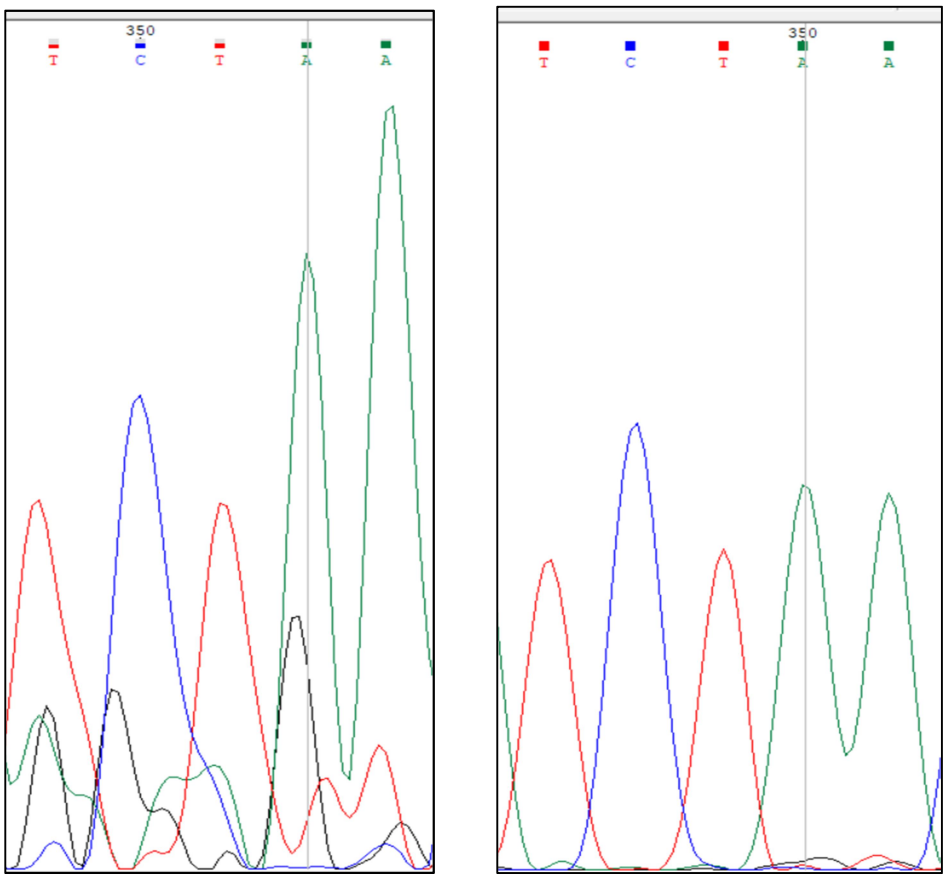


Fig. 4.9. Chromatogram peak images for Var 2 **1)** Monoecious sequence **2)** Gynoecious sequence

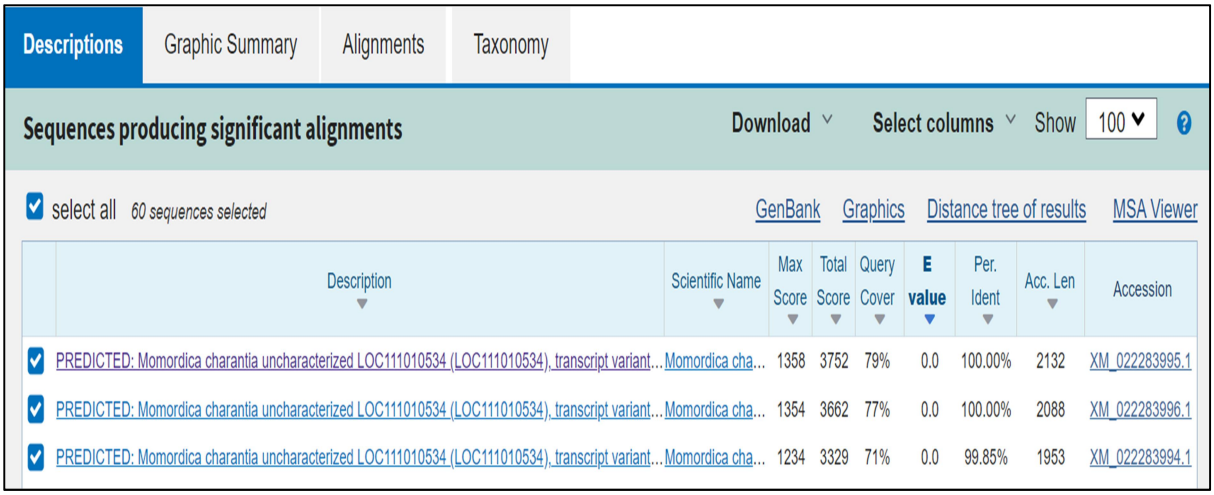
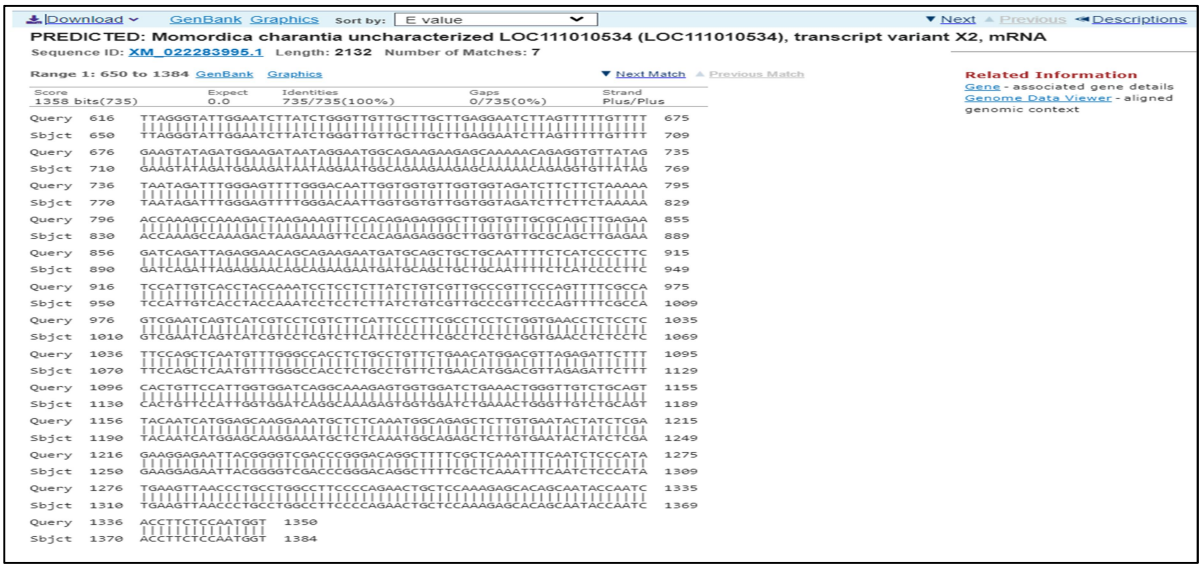


Fig. 4.10. BLASTn analysis in monoecious line **a)** Sequences showing significant identity with query sequence in monoecious line



b) Results of BLASTn analysis showing alignment of the sequencpe with *Momordica charantia* (Accession no XM_O2283995.1) in monoecious line

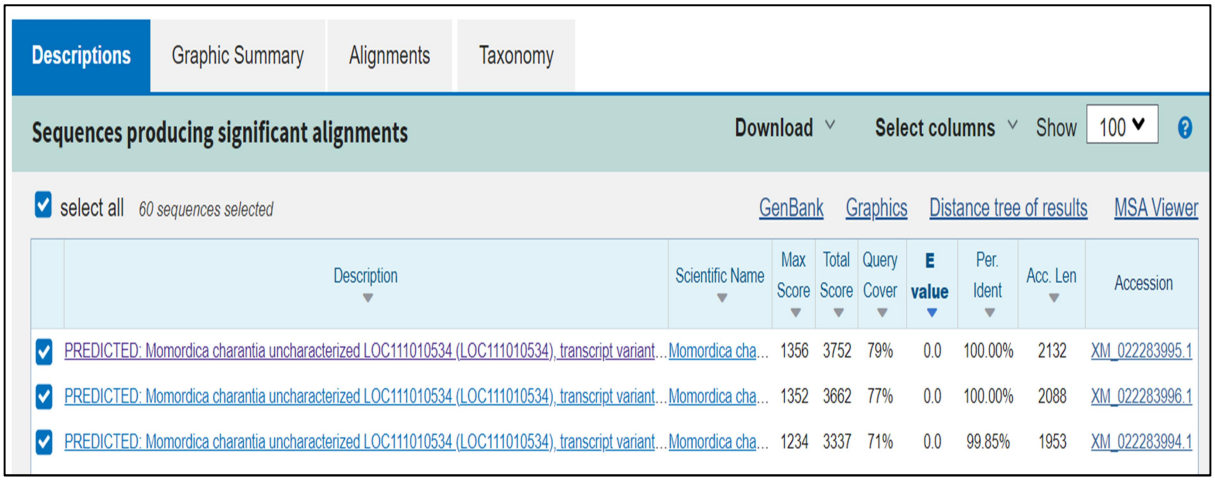
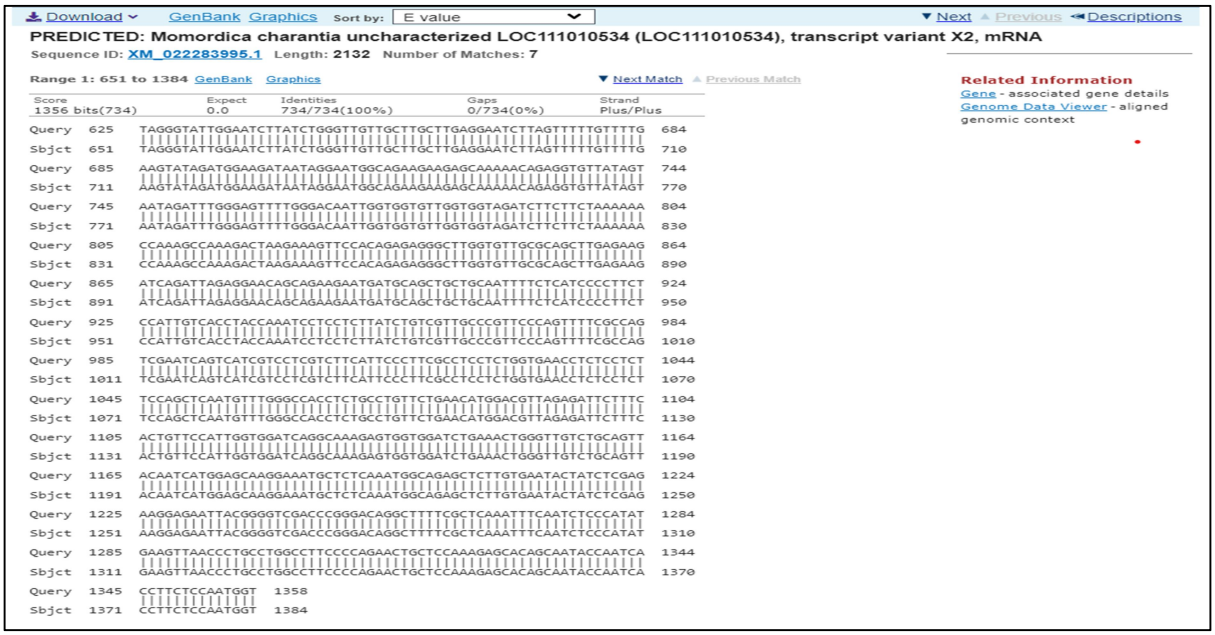
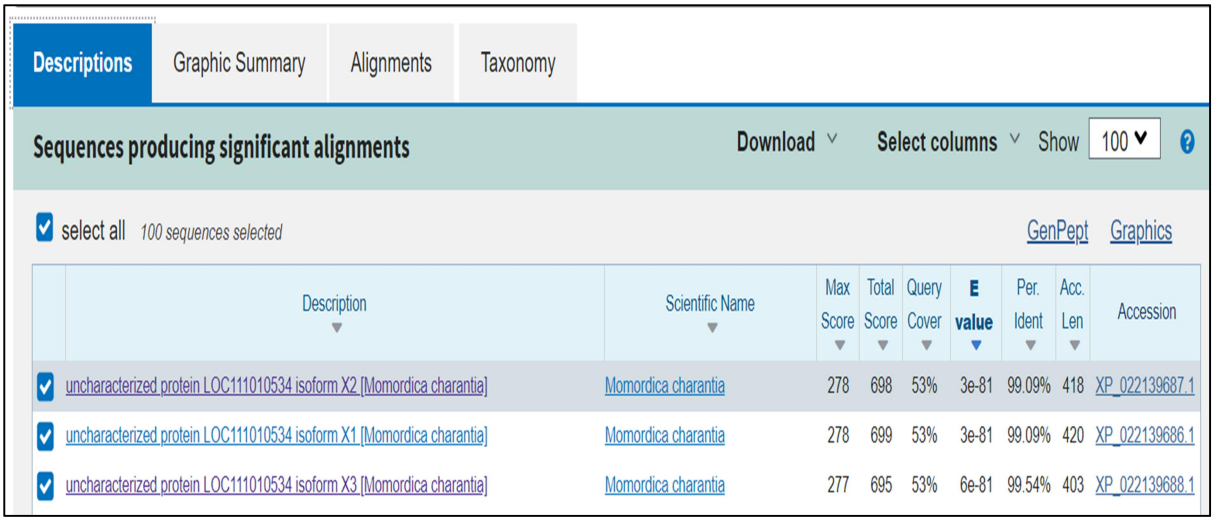


Fig. 4.11. BLASTn analysis in gynoecious line **a)** Sequences showing significant identity with query sequence in gynoecious line



b) BLASTn result window showing alignment of the sequence with *Momordica charantia* (Accession no XM_O2283995.1) in gynoecious line



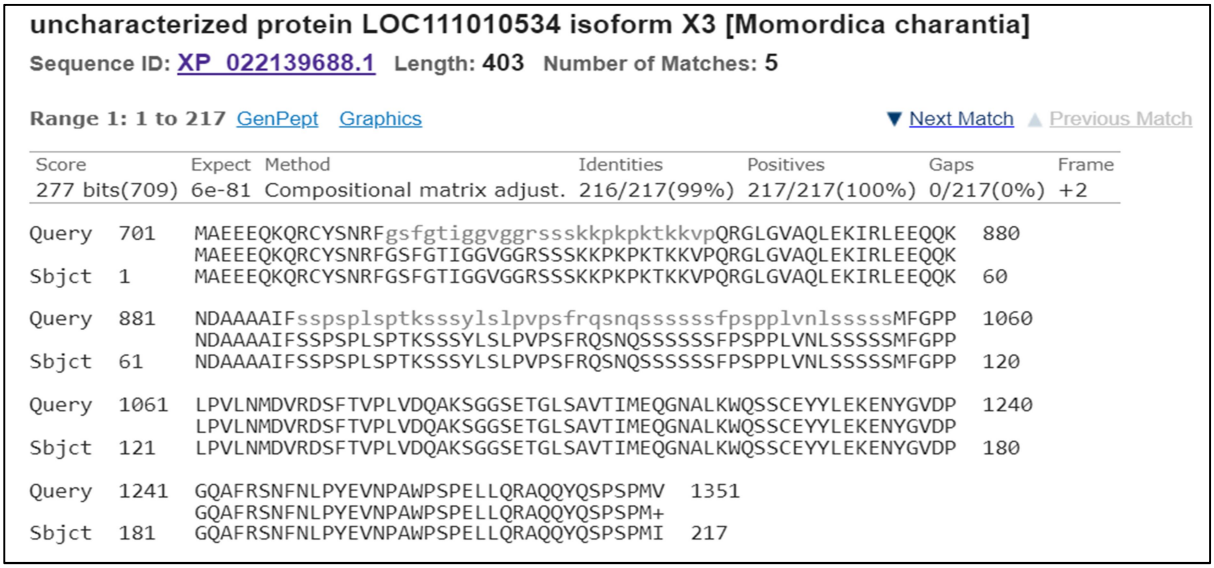
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenPept Graphics

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
uncharacterized protein LOC111010534 isoform X2 [Momordica charantia]	Momordica charantia	278	698	53%	3e-81	99.09%	418	XP_022139687.1
uncharacterized protein LOC111010534 isoform X1 [Momordica charantia]	Momordica charantia	278	699	53%	3e-81	99.09%	420	XP_022139686.1
uncharacterized protein LOC111010534 isoform X3 [Momordica charantia]	Momordica charantia	277	695	53%	6e-81	99.54%	403	XP_022139688.1

Fig. 4.12. BLASTx analysis in monoecious line **a)** Sequences showing significant identity with query sequence in monoecious line



uncharacterized protein LOC111010534 isoform X3 [Momordica charantia]

Sequence ID: XP_022139688.1 **Length:** 403 **Number of Matches:** 5

Range 1: 1 to 217 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
277 bits(709)	6e-81	Compositional matrix adjust.	216/217(99%)	217/217(100%)	0/217(0%)	+2

```
Query  701   MAEEEQKQRCYSNRFgsfgtiggvggrssskkpkpktkkvpQRGLGVAQLEKIRLEEQQK  880
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Sbjct  1     MAEEEQKQRCYSNRFGSFGTIGGVGGRSSSKKPKPKTKKVPQRGLGVAQLEKIRLEEQQK  60

Query  881   NDAAAAIFsspsplsptkssslslpvpsfrqsnqsssssfpspplvnlssssMFGPP  1060
             NDAAAAIFSSPSPLSPTKSSSYLSLPVPSFRQSNQSSSSSFPSPPLVNLSSSSMFGPP
Sbjct  61    NDAAAAIFSSPSPLSPTKSSSYLSLPVPSFRQSNQSSSSSFPSPPLVNLSSSSMFGPP  120

Query  1061  LPVLNMDVRDSFTVPLVDQAKSGGSETGLSAVTIMEQGNALKWQSSCEYYLEKENYGVDP  1240
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Sbjct  121   LPVLNMDVRDSFTVPLVDQAKSGGSETGLSAVTIMEQGNALKWQSSCEYYLEKENYGVDP  180

Query  1241  GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPMV  1351
             GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPM+
Sbjct  181   GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPMI  217
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b) BLASTp result window showing alignment of the sequence with *Momordica charantia* (Accession no XP_O22139687.1) in monoecious line

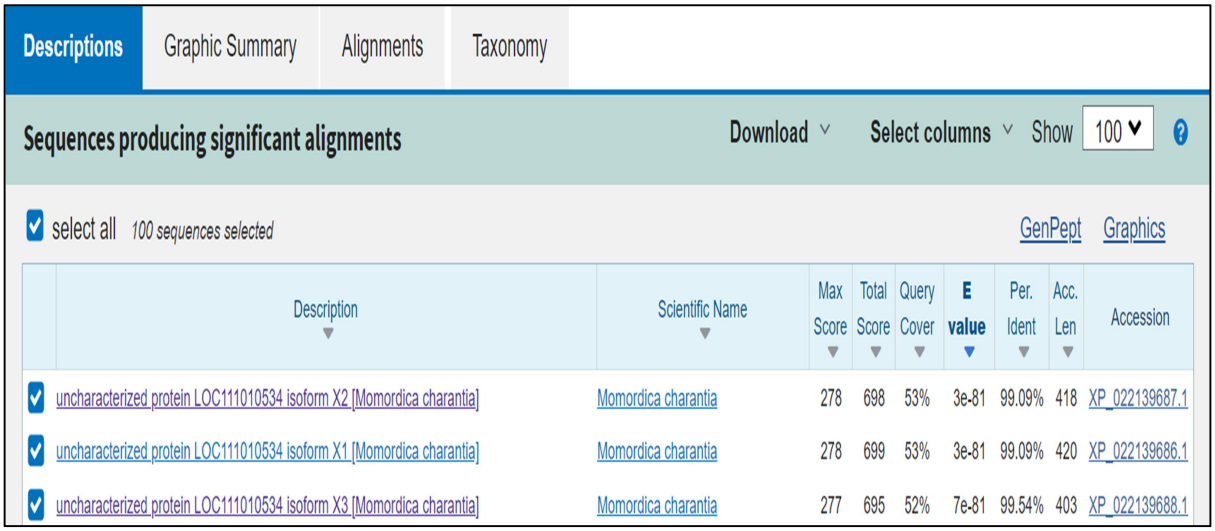

Fig. 4.13. BLASTx analysis in gynoecious line **a)** Sequences showing significant identity with query sequence in gynoecious line

uncharacterized protein LOC111010534 isoform X3 [Momordica charantia]

Sequence ID: XP_022139688.1 **Length:** 403 **Number of Matches:** 5

Range 1: 1 to 217 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
277 bits(709)	7e-81	Compositional matrix adjust.	216/217(99%)	217/217(100%)	0/217(0%)	+1

```
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Sbjct  1     MAEEEQKQRCYSNRFGSFGTIGGVGGRSSSKKPKPKTKKVPQRGLGVAQLEKIRLEEQQK  60

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Sbjct  61    NDAAAAIFSSPSPLSPTKSSSYLSLPVPSFRQSNQSSSSSSFPSPPLVNLSSSSSMFGPP  120

Query  1069  LPVLNMDVRDSFTVPLVDQAKSGGSETGLSAVTIMEQGNALKWQSSCEYYLEKENYGVDP  1248
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Sbjct  121   LPVLNMDVRDSFTVPLVDQAKSGGSETGLSAVTIMEQGNALKWQSSCEYYLEKENYGVDP  180

Query  1249  GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPMV  1359
             GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPM+
Sbjct  181   GQAFRSNFNLPYEVNPAWPSPELLQRAQQYQSPSPMI  217
```

b) BLASTX result window showing alignment of the sequence with *Momordica charantia* (Accession no XP_O22139687.1) in gynoecious line

Fig. 4.14 Result window of ExPASy translate tool showing the amino acid sequence corresponding to SPL gene in monoecious line

Fig. 4.15. Result window of ExPASy translate tool showing the amino acid sequence corresponding to *SPL* gene in gynoecious line

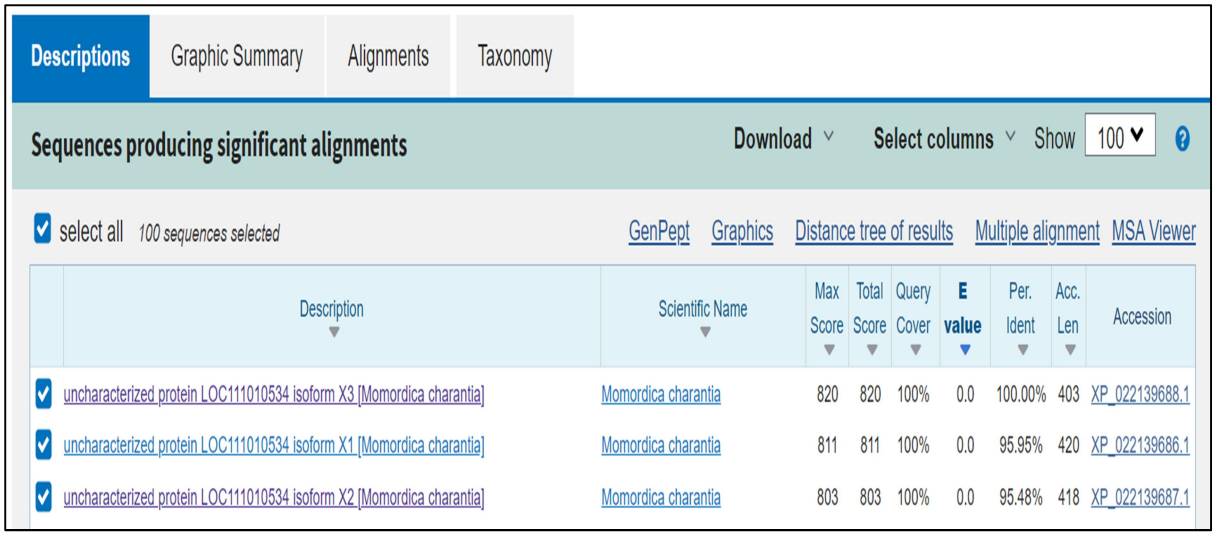
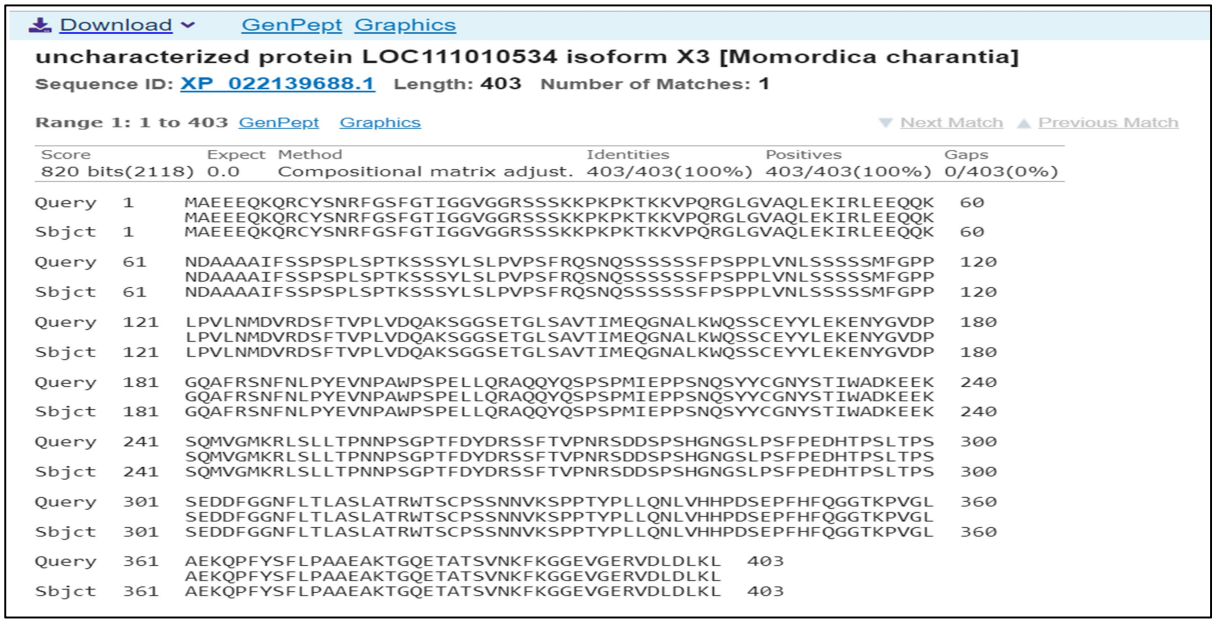


Fig. 4.16. BLASTp analysis in monoecious line **a)** Sequences showing significant identity with query sequence in monoecious line



b) BLASTp result window showing alignment of the sequence with *Momordica charantia* (Accession no. XP_022139688.1) in monoecious line

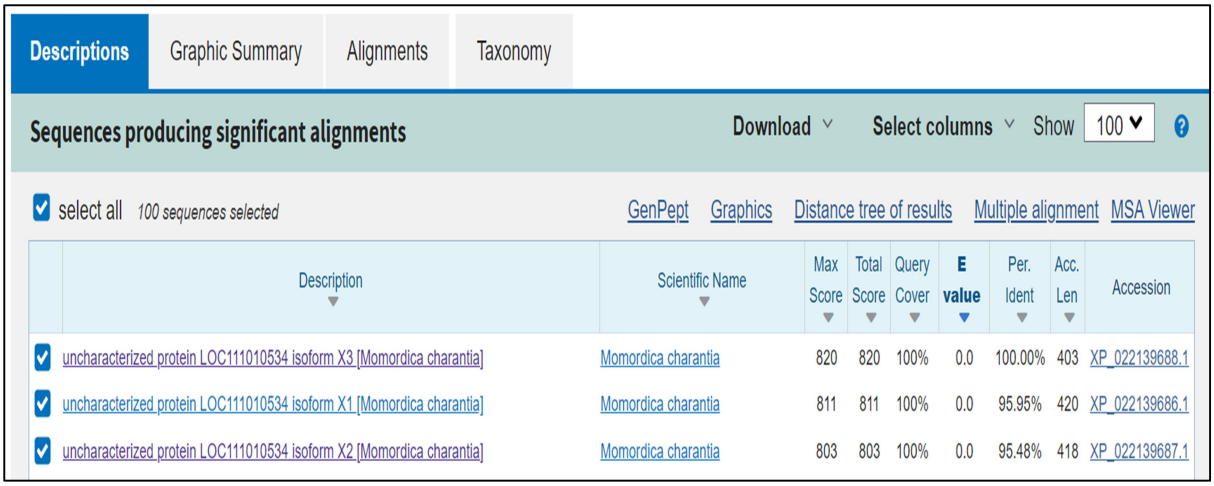
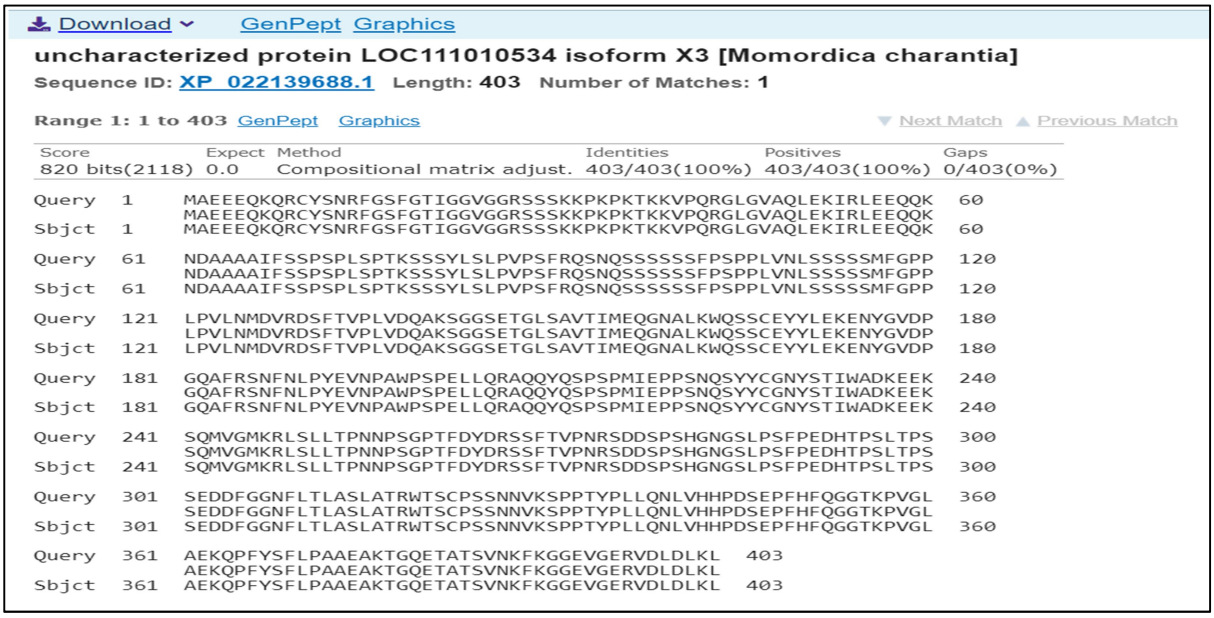


Fig. 4.17. BLASTp analysis in gynoecious line **a)** Sequences showing significant identity with query sequence in gynoecious line



b) BLASTp result window showing alignment of the sequence with *Momordica charantia* (Accession no. XP_022139688.1) in gynoecious line

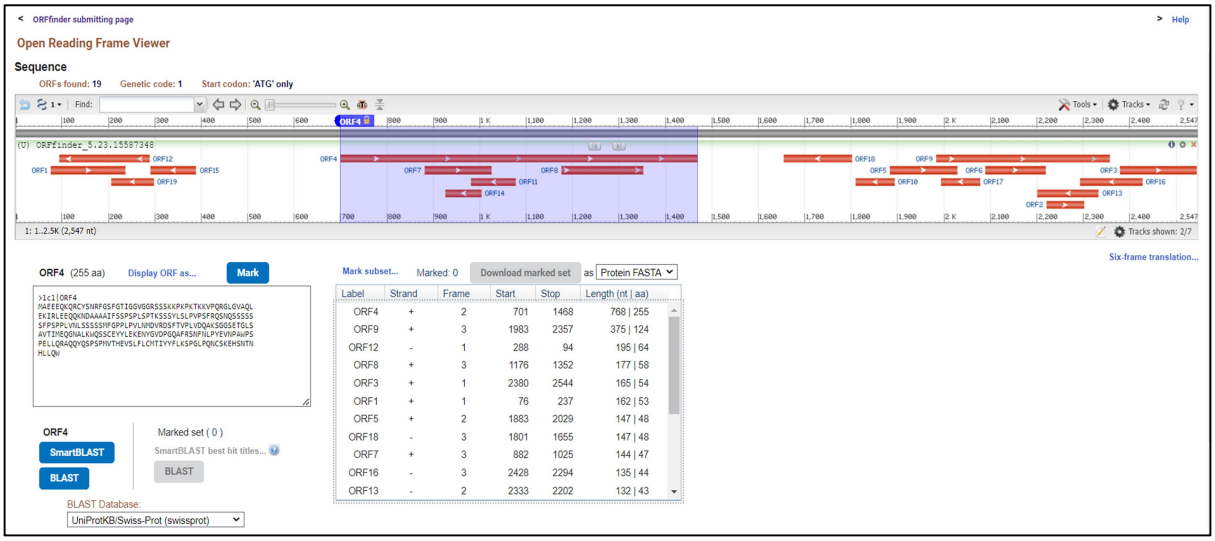


Fig. 4.18. ORFs in *SPL* gene identified using ORF finder in monoecious line

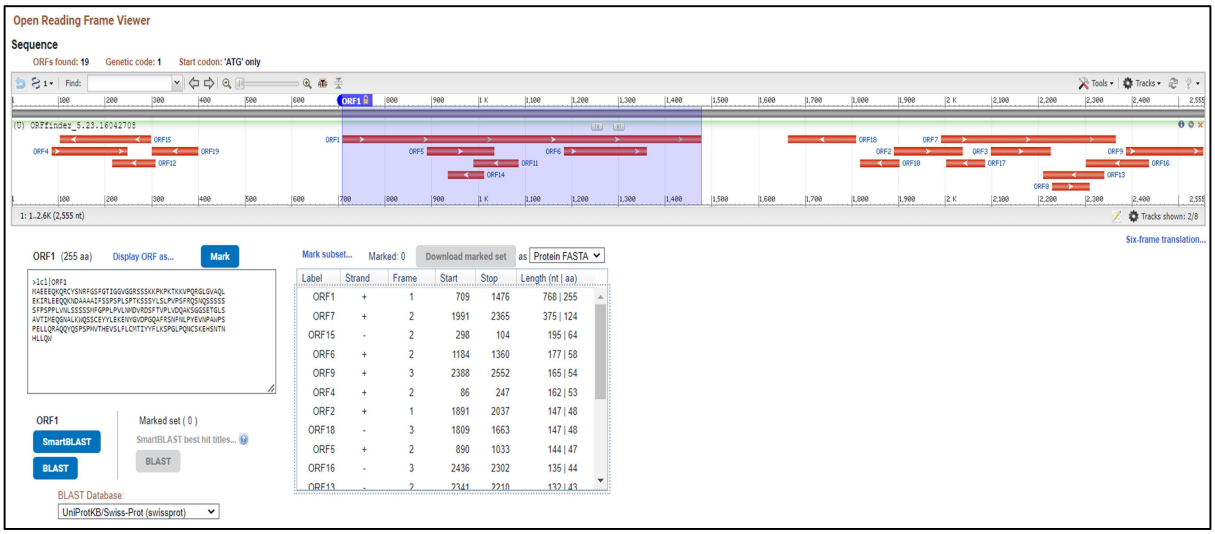


Fig. 4.19. ORFs in *SPL* gene identified using ORF finder in gynoecious line

4.4. Real Time – quantitative Polymerase Chain Reaction (RTqPCR) analysis for *SPOROCTLESS* expression

To study the differential expression of *SPOROCYTELESS* gene, RT-qPCR analysis was performed in the floral buds of male and female flowers of monoecious and female flowers of gynoecious lines (Plate 4.4).

4.4.1 RNA isolation

Quantity and quality of the RNA isolated from the floral buds was assessed using a spectrophotometer. The A_{260}/A_{280} ratio was between 1.80 to 1.92, suggesting pure RNA. The concentration was between 769 and 851 ng/µL (Table 4.3 and Plate 4.5).

Table 4.3. Concentration and purity of RNA samples

Sample	Concentration(ng/µL)	A_{260}/A_{280}
Gynoecious	769.03	1.832
Monoceious - Male	851.99	1.921
Monoecious - Female	781.70	1.811

4.4.2 Synthesis and quantification of cDNA first strand

When the quantification of cDNA was done using nano volume spectrophotometer, concentration was found adequate with A_{260}/A_{280} ratio close to 1.8 (Table 4.4).

Table 4.4. Concentration and purity of cDNA samples

Sample	Concentration (ng/µL)	A_{260}/A_{280}
Gynoecious	1121.1	1.743
Monoecious-Male	1092.8	1.789
Monoecious-Female	1715.2	1.761

4.4.3 Designing primers for RT-qPCR analysis

Details on the primers designed for RT-qPCR analysis are given Table 4.5.

Table 4.5. Features of the RT-qPCR primers designed

Orientation	Name of primer	Sequence (5' to 3')	Target gene	Product size (bp)
Forward	SPL-RT	ACAGAGAGGGCTTGGTGTTG		
Reverse	SPL-RT	AGGCGAAGGGAATGAAGACG	*SPL*	193
Forward	ACT-F	TACTCTTTCACCACCACCGC		
Reverse	ACT-R	AGGGCATCGGAACCTTTCAG	*ACT*	196

4.4.4 Confirmation of first strand cDNA synthesis

The cDNA samples were thermal cycled using the primers for the housekeeping gene *actin* and amplification of bands at 196 bp has confirmed the synthesis of cDNA first strand in all samples (Plate 4.6).

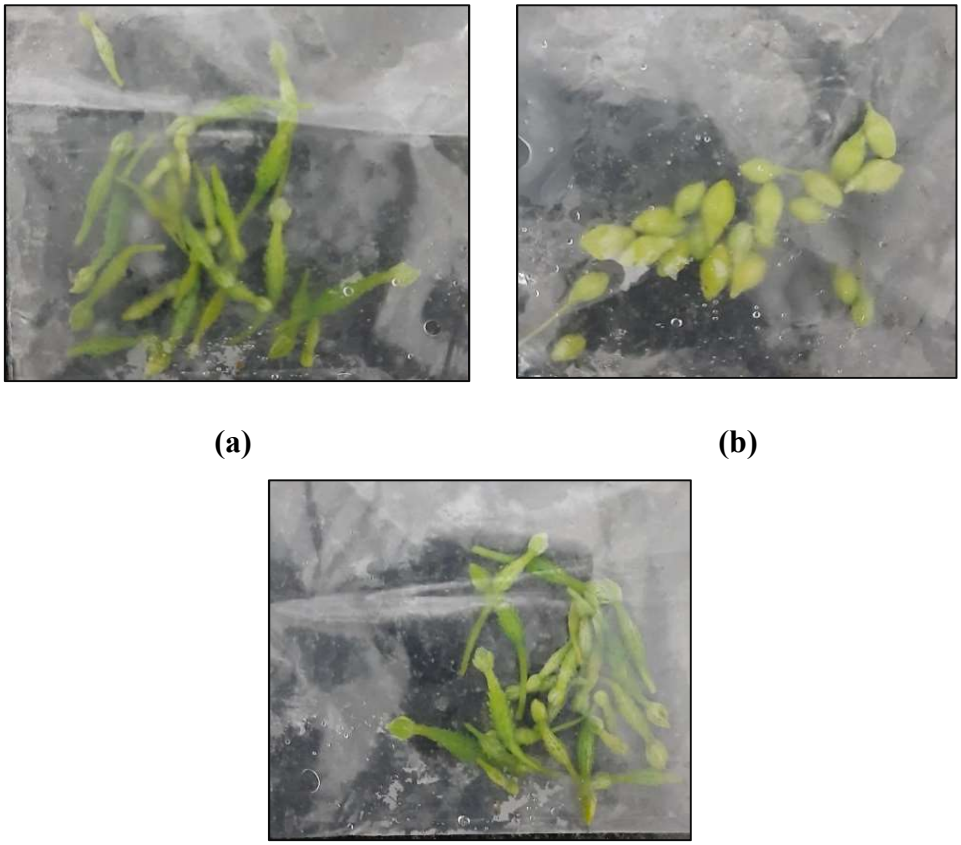


Plate 4.4. Buds used for RNA isolation for *SPL* expression analysis **a)** Monoecious female buds **b)** Monoecious male buds **c)** Gynoecious buds

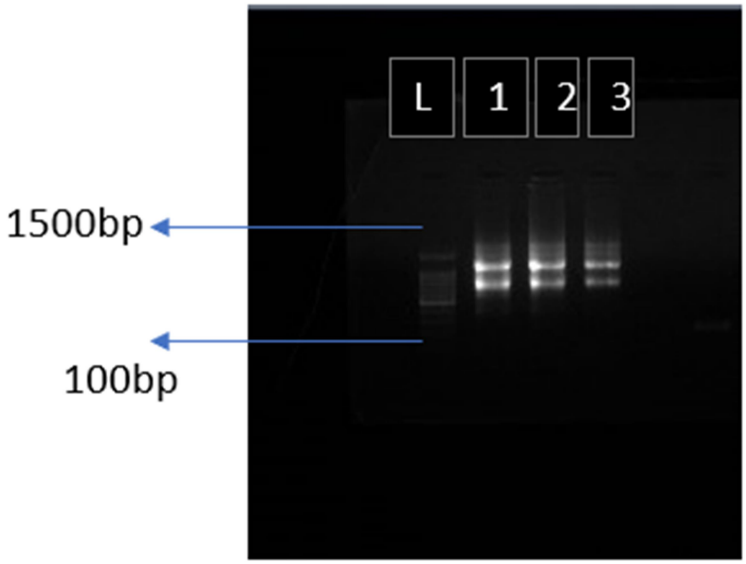


Plate 4.5. Agarose gel profile of RNA isolated L: Ladder, 1- Gynoecious, 2- Monoecious male, 3- Monoecious female

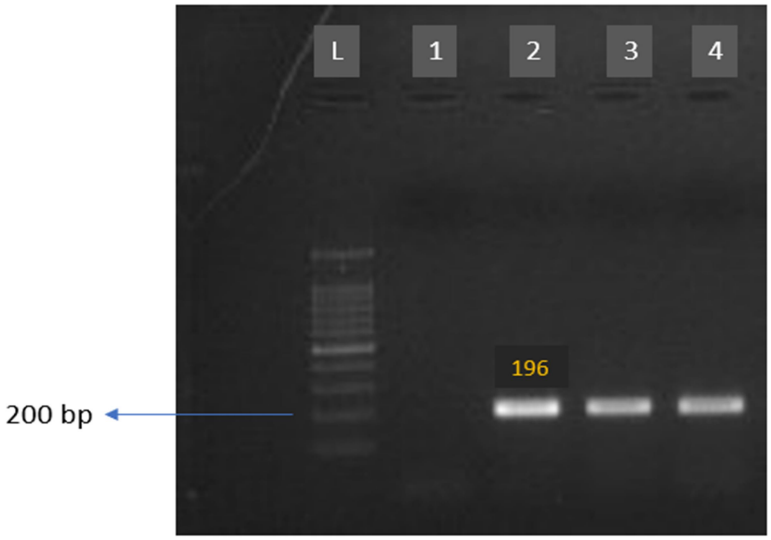


Plate 4.6. Confirmation of synthesis of first strand cDNA. L: 1kb Ladder, 1: NTC, 2: Gynoecious sample, 3: Monoecious male, 4: Monoecious female

4.4.5 RT-qPCR analysis

Differential expression of *SPL* gene in the floral buds of monoecious and gynoecious plants was studied using RT-qPCR. In the melt-curve analysis, the melting temperature was found to be 84.67 °C for *SPL* amplicons (Fig. 4.20). The relative expression levels of *SPL* were normalised with the expression of endogenous control (*actin*) and fold change in expression was calculated using the formula $2^{-\Delta\Delta Ct}$ taking male as the standard (Table 4.6). The relative expression levels of SPL in different floral buds is graphically represented in Fig. 4.21.

Table 4.6. Relative expression of *SPL* gene in three floral buds

Name of sample	**Ct Mean of *SPL***	**Ct Mean of *Actin***	**ΔCt**	**ΔΔCt**	**2^-ΔΔCt (Relative expression)**
Male	40.26	17.57	22.69	0	1.00
Female	38.02	19.77	18.25	-4.44	21.69
Gynoecious	40.37	25.79	14.58	-8.11	276.75

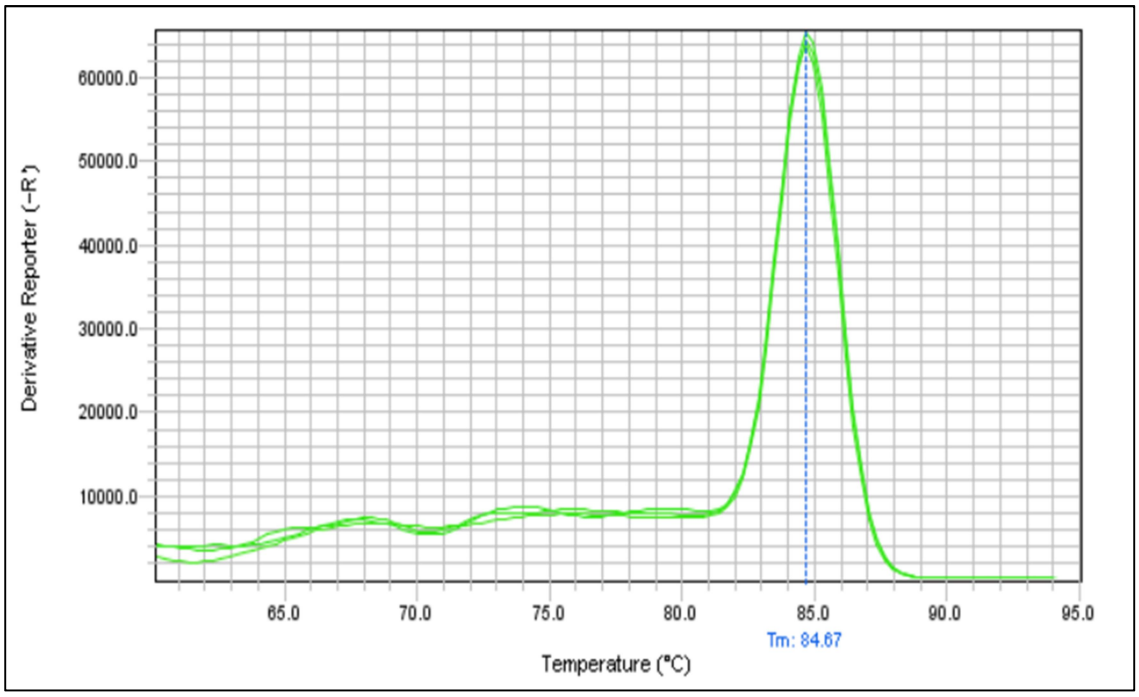


Fig. 4.20 Melt curve analysis. Unique melting peak of 84.67 ºC obtained for *SPL* amplicons

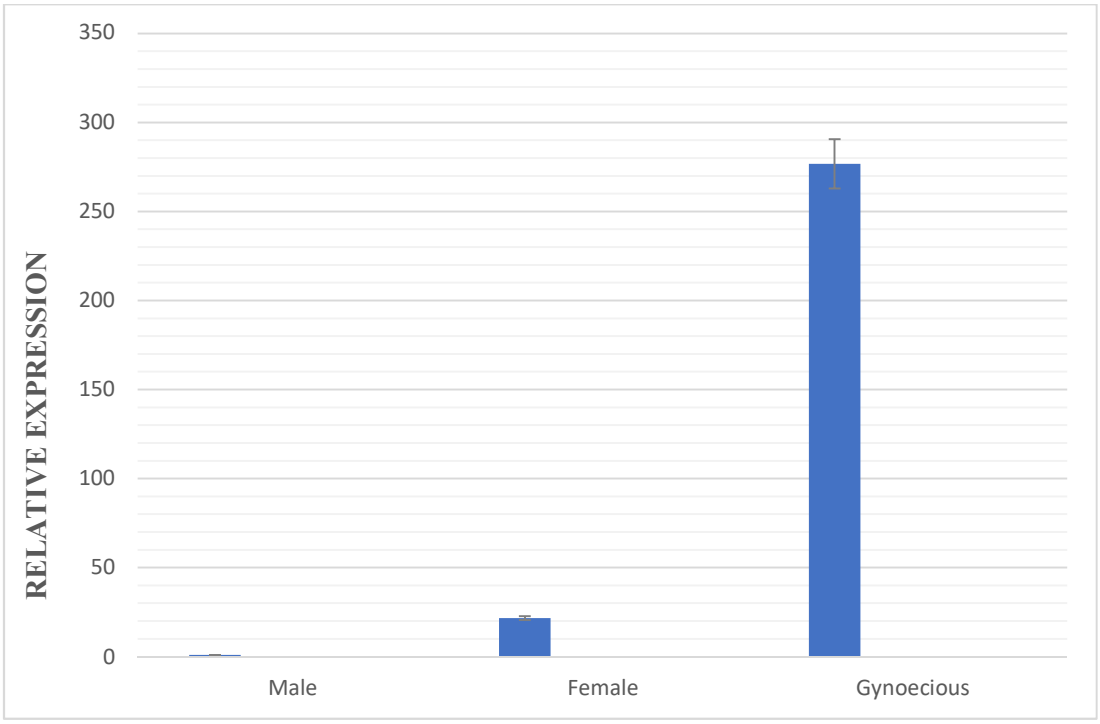


Fig. 4. 21 Relative expression of *SPL* gene in the floral buds

Discussion

5. DISCUSSION

The research work entitled "Characterization and expression analysis of *SPOROCYTELESS* gene in monoecious and gynoecious lines of bitter gourd" was carried out at Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Thrissur, during 2020-2023. The results of the study are briefly discussed in this chapter.

The Cucurbitaceae family accommodates several important vegetable crops, including bitter gourd (*Momordica charantia* L.; 2n=2x=22). Bitter gourd is also known as bitter melon, balsam pear, bitter cucumber, African cucumber, and *Karela* (Heiser, 1979). India produces roughly 1.2 million metric tonnes of bitter gourd from an area of 99,000 hectares (NHB database, 2018-19). Major states growing bitter gourds are Tamil Nadu, Kerala, Maharashtra, Uttar Pradesh, Andhra Pradesh, and Gujarat. Only 12 of the 47 species in the genus *Momordica*, which originated in tropical Africa, are found in Asia and Australia (Schaefer and Renner, 2010). The *Momordica c.* var. *charantia*, which yields large fruits, and *Momordica c.* var. *muricata*, a wild variety with small, rounded fruits, are the main botanical varieties of *Momordica charantia* (Chakravarthy, 1990; John and Antony, 2008). It is a trailing viny plant that grows quickly and is extremely valuable in nutritional and therapeutic terms. The bitter gourd's immature fruits are often eaten as vegetables and known for its anti-diabetic and other therapeutic effects (Robinson and Decker-Walters, 1997). Given that its fruits are high in vitamin A, vitamin C, iron, phosphorus, and carbohydrates, it is regarded as most nutrient-dense among the cucurbits (Miniraj *et al*., 1993; Desai and Musmade, 1998; Behera, 2004). Its enormous therapeutic benefits are attributable to the presence of advantageous phytochemicals, which are known to have immune-boosting, antiviral, anti-diabetic, antibiotic, antimutagenic, antihelmenthic and cancer-preventive characteristics. Phytochemicals such as momordin and charantin in the fruits are clinically proven for its hypoglycemic activity (Grover and Yadav, 2004). Monoecy, having separate male and female flowers in a plant, is the main sex form of bitter gourd. However, gynoecious sex form with only female flowers, is reported from India and Japan (Ram *et al*., 2002; Iwamoto and Ishida, 2006). Use of gynoecious lines as female parent in the hybridization programme is a cost-effective strategy since the manual labour required for emasculation can be avoided. Though many gynoecious lines are reported in

major cucurbits such as cucumber, initial gynoecious bitter gourd lines were reported from KAU (KAU-Mc-Gy101 and KAU-Mc-Gy102; Minnu *et al*., 2022; Sunny *et al*., 2022; Baluchamy *et al*., 2023), IIVR (Gy-323, Gy-333, Gy263B, Gy-2116, Gy-2135, Gy-318, Gy-144; Ram *et al*., 2002; Moharana *et al*., 2022), IIHR (IIHRBTGy-491, IIHRBTGy-492; Varalakshmi *et al*., 2014) and IARI (DBGy-201 and DBGy-202; Behera *et al*., 2006; Behera *et al*., 2009) only. A gynoecious inbred, OHB61-5 with high combining ability was also reported from Japan (Iwamoto and Ishida, 2006; Iwamoto *et al*., 2009).

Flowering in plants is a complicated process involving multiple genes and pathways. In the ABC model of flowering, stamen formation is the result of interaction between B and C genes (Bowman *et al*., 1991). The specification of stamen identity in *Arabidopsis thaliana* is controlled by a combination of the B genes *APETALA3* (*AP3*) and *PISTILLATA*, the C gene *AGAMOUS* (*AG*), and the E genes *SEPALLATA* (*SEP1* to *SEP4*). *AG* is understood to activate the transcription of the "floral organ-building" gene *SPOROCYTELESS/NOZZLE* (*SPL*/*NZZ*). *SPL* plays a central role in regulating microsporangium differentiation, because SPL/NZZ mutant anthers fail to form archesporial cells (Schiefthaler *et al*., 1999; Yang *et al*., 1999). Ectopic expression of *SPL*/*NZZ* has resulted in ectopic expression of *AG* and *SEP3* in the outer whorls of flowers, indicating that this gene is engaged in controlling stamen identity via interacting with genes required for stamen identity (Liu *et al*., 2009). In wild-type *Arabidopsis* ovules, megaspore mother cell differentiation required *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*), as demonstrated by the *spl/nzz* mutant failing to develop megaspore mother cell (Zhao *et al*., 2017; Mendes *et al*., 2020).

The chances for the isolation of a gynoecious line from a monoecious population is often rare since gynoecy in bitter gourd is under the control of a single, recessive gene *gy-1* (Ram *et al*., 2006). Thus, with the increasing demand for the development of gynoecious lines in crops such as bitter gourd, genes identified to have definite roles in sex expression assumes importance. The *SPL* genes is shown to have pronounced role in stamen and pistil development in flowers. To proceed with the alteration of the sex forms in any crop by genetic engineering, the candidate gene has to be thoroughly characterized. Also, its expression levels in different sex forms has to be well understood. Thus, the objective of this study was to characterize the *SPL* gene in bitter gourd and to study its expression in the male and female flowers in

monoecious and gynoecious sex forms, to formulate the strategies to develop the gynoecious lines through genome engineering.

In this study, characterization of the *SPL* gene was carried out by isolating the genomic DNA from the bitter gourd leaves of both monoecious and gynoecious lines. DNA was isolated from the first or second bitter gourd leaf from the tip, which is pale green and delicate. With a small adjustment, the Rogers and Bendich (1994) CTAB technique of DNA isolation methodology produced DNA of high quality and yield. The alteration employed was the direct addition of PVP while pre-warming in a water bath to the CTAB buffer.

The generation of modified forms of protein, polyphenols, and secondary metabolites is the main issue that arises during the isolation of DNA from plants (Weising et al., 1995; Matasyoh et al., 2008). To avoid the oxidation of polyphenols to polyquinones, which bind to the DNA and make extraction challenging, samples were collected in ice. Liquid nitrogen was used to grind the samples, making the cell walls brittle and to inactivate the enzymes, which also helped in reducing phenol oxidation. Addition of 50 µL of 1-mercapto ethanol helped in controlling protein contamination by denaturation of proteins. RNAse A (Ribonuclease A) was used in eliminating RNA contamination (Gallego and Martinez, 1996; Wettasinghe and Peffley 1998). The quantity and quality of the DNA used during molecular analysis are key factors in deciding the outcome of subsequent procedures such as PCR and sequencing. The quantity and quality were assessed using spectrophotometric techniques and agarose gel electrophoresis, respectively.

DNA samples were electrophoresed on a 0.8 % agarose gel to confirm that the DNA used in this study was of high-quality. Since the NanoDrop ND-1000 spectrophotometer accurately and consistently detects the absorbance from one uL of sample, the quantity analysis performed with it was proven to be effective. In contrast to the typical UV spectrophotometer, it does not use cuvettes or any other sample-contaminating equipment. The maximum absorbance of nucleic acids occurs at 260 nm, while that of proteins occurs at 280 nm. For high-quality DNA samples, the A_{260}/A_{280} ratio will fall between 1.8 and 2.0 (Meena, 2014; Thakur et al., 2014). The isolated samples' absorbance ratio fell within this range.

The success of a PCR experiment depends critically on the design of the oligonucleotide primers. The selection of primers and the amplification of target sequences found in the template DNA depend on crucial factors such the primer's sequence, length, and melting temperature. Utilising the sequence that is present in the NCBI genome database, three sets of primers were created to amplify the *SPL* gene since the size of the gene is >2kb and the processivity of *Taq* was only 1500 bp. Primer3 software was used to choose the primers. Optimising the specificity of a PCR reaction requires careful consideration when choosing the ideal annealing temperature. PCR can be used to determine the ideal annealing temperature gradient (Yang et al., 2005; Sipos et al., 2007). To find the ideal annealing temperature for each of the three pairs, gradient PCR was carried out.

The PCR product of all the three primer pairs of both the lines were separately pooled to 50 µL each and sequenced on Sanger platform. All the sequences had a quality value of more than 20, which suggested that they are of good quality.

The forward and reverse sequences obtained for the three primer pairs were assembled into single contig. The final contig lengths of 2381 and 2391 bp were obtained for the monoecious gynoecious lines. When compared to monoecious genotypes, there were no variations in the sequence of the gynoecious sample for the *SPL* gene. The *SPL* gene is not characterised in any of the cucurbits.

Using BLASTn, sequences of monoecious and gynoecious lines developed in this study were found to have 100% identity with 79% query coverage with sequence of *Momordica charantia* (LOC111010534) (Accession no. XM 022283995.1). In BLASTx, the sequences had 99.09% similarity with the protein sequence of *Momordica charantia* (Accession no. XP_022139687.1). Similarly, BLASTp of the deduced protein sequences in both lines showed that the sequence is 100% identical to the protein sequence of *Momordica charantia* (Accession no. XP 022139688.1). Analysis using ORF Finder showed that the nucleotide sequence had 19 ORFs in both lines with ORF4 as longest with 768 bp coding for 255 amino acids.

To study the expression profile of the *SPL* gene in the monoecious and gynoecious flowers, RNA was isolated from the floral buds at the earliest stage. As *SPL* is a C class gene, its expression will be more in the floral buds (Schiefthaler *et*

al., 1999; Yang *et al*., 1999) and hence the floral buds were chosen for studying the expression of this gene using qRT-PCR.

Prior to RNA isolation, the samples were kept in RNA Later (Sigma). While working with RNA, special precautions must be taken to assure an RNase-free environment. It is essential to utilise RNase-free tools and glassware in the lab and to prevent the unintentional entry of RNases from any other sources (Krieg, 1996; Loomis, 1974). Diethyl pyrocarbonate (DEPC) was applied to the microtips, microcentrifuge tubes, mortar, and pestle employed in RNA isolation. TAE buffer and 75 % ethanol were made using double autoclaved DEPC-treated water. According to Robert and Farrell (2010), the DEPC is a strong, non-specific chemical inhibitor of RNase. The work bench and all of the apparatus, including the gel electrophoresis machine, were cleaned with 75% alcohol before RNA isolation. In this present study RNA isolation was done TRIZOL reagent (Rio *et al*., 2010), which is an effective method for RNA extraction (Kansal *et al*., 2008). Agarose gel electrophoresis was used to examine the extracted total RNA. Good quality RNA with intact bands corresponding to 28S and 18S rRNA were seen in the agarose gel profile. RNA was subjected to quality analysis and quantified using a Nanodrop Spectrophotometer. The AA ratio for the samples ranged between 1.80 and 1.92, indicating that the RNA isolated using the TRI reagent is of good quality and has a decent yield. The concentration of the RNA extracted was between 769 and 851 ng/µL. Using the Revert Aid first strand cDNA synthesis kit, the first strand cDNA was created from the total RNA that had been extracted.

Errors in qRT-PCR studies might arise from minor differences in the initial amount of RNA utilised, the quality of the RNA, variances in the efficiency of cDNA synthesis, and PCR amplification. A specific gene is amplified alongside the target gene in order to eliminate mistakes and correct sample variance. This gene will act as an internal reference against which other gene expression values can be normalised. Housekeeping genes, such as those encoding actin, ribosomal RNA, a cytoskeletal protein, a glycolytic enzyme, and glyceraldehyde-3-phosphate dehydrogenase, are frequently utilised for normalisation (Gilliland et al., 1989). The expression of housekeeping genes should be abundant, with minimal inherent variability, and not co-regulated with target genes (Chervonova et al., 2010). As an endogenous regulator, the actin gene was utilised in the present RT-qPCR experiment.

One pair of primers were designed for *SPL* gene (test gene) and *actin* gene (endogenous control) based on the available sequences in NCBI database using Primer3 software. Real-time PCR employing SYBR Green chemistry was used for the gene expression research. Utilising Bio-Rad's SoAdvanced Universal SYBR Green Supermix, real-time PCR tests were carried out. once it has attached to double-stranded DNA, SYBR Green I's fluorescence is increased by more than 1,000-fold (Dragan et al. 2012). Because SYBR-Green I is more user-friendly and less expensive than TaqMan probes, it is preferable for measurement of nucleic acids (Ponchel et al., 2003). According to Makkouk and Kumari (2006), the amount of amplified product produced after each amplification cycle directly correlates with the amount of fluorescence produced during that cycle. In the current research work, the relative gene expression study was conducted using the Comparative Ct technique (Livak, 1997). The fractional PCR cycle number at which the fluorescence exceeds the threshold level is known as the cycle threshold (Ct) value. According to Arya et al. (2005), a fluorescent signal that is detected above the threshold is considered a true signal and is utilised to determine the threshold cycle for a sample. In the current work, melt curve analysis was also carried out to verify the fidelity of the amplicons. Melt curve is produced when fluorescence is plotted as a function of temperature. Gel electrophoresis is not necessary because desired and unwanted products can be differentiated based on melting temperature (Ririe et al., 1997). When melt curve analysis was carried out, it was found that *SPL* amplicons has unique melting temperature of 84.67 °C.

The relative expression levels of the *SPL* gene were normalized with the expression of the endogenous control *actin* gene. The fold change in expression of the *SPL* gene was calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). In the current study, the relative expression was normalized using male flower of monoecious as the standard and the fold change was compared. The *SPL* gene was had a higher expression in gynoecious flowers, compared with male and female flowers of monoecious plant, proving that this C class gene is involved in the development of megaspores.

Expression studies on *SPL* gene is reported in *Cucumis sativus (CsSPL)* by Xiaofeng Liu *et al.* (2018) who found that *SPOROCYTELESS (SPL)/NOZZLE (NZZ)* expression was nearly abolished in a mutant with severely defective anther and ovule

development. *CsSPL* was expressed specifically in the developing anthers and ovules. Additionally, *CsSPL* knock-down plants had less auxin buildup in their reproductive organs. Biochemical studies also revealed that *CsSPL* stimulated the expression of *AUXIN RESPONSE FACTOR 3 (CsARF3)* and was positively regulated by *CsARF13* during the development of reproductive organs, indicating sequential interactions of *CsSPL* with auxin signalling components in directing the development of the anther and ovule. As bitter gourd also belongs to the Cucurbitaceae family, this may be considered as base for the expression of *SPL* gene with respect to the floral organ development.

Summary

6. SUMMARY

The research work entitled "Characterization and expression analysis of *SPOROCYTELESS* gene in monoecious and gynoecious lines of bitter gourd" was undertaken at Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Thrissur. The objective of the study was sequence and characterize the *SPL* (*SPOROCYTELESS*) gene from monoecious and gynoecious bitter gourd lines and to study its differential expression in both the lines. The salient findings of the study are presented below.

- Seeds of monoecious bitter gourd variety Priyanka and gynoecious line KAU-Mc-Gy-102 were collected from the Department of Vegetable Science, College of Agriculture, Thrissur and plants were raised in pots
- Emerging leaves were collected from the monoecious and gynoecious plants and total genomic DNA was extracted. For all of the samples, absorbance ratio $A_{260/280}$ was in the range of 1.80-2.0, indicating that the DNA isolated is of high quality.
- Using the sequence of *SPOROCYTELESS* gene (XM_022283995.1) retrieved from GenBank, three sets of overlapping PCR primer combinations SPL-BG-1, SPL-BG-2 and SPL-BG-3, were designed to amplify the full length of *SPOROCYTELESS* gene
- Using three sets of primers, full length of *SPL* gene was PCR amplified at an annealing temperature of 65º C. Product sizes for SPL-BG-1, SPL-BG-2 and SPL-BG-3, were 718, 912 and 1000 bp, respectively in both the lines of bitter gourd.
- PCR products were paired-end sequenced and the overlapping sequences were aligned to generate 2381 and 2391 bp *SPL* gene sequence in monoecious and gynoecious lines, respectively.
- The sequences from monoecious and gynoecious bitter gourd lines had no valid difference.
- BLASTn analysis showed 100 % identity with 79 % query coverage with *Momordica charantia* transcript variant (LOC111010534) (Accession no XM_O2283995.1)

- BLASTx showed 99.09% identity with the sequence of *Momordica charantia* transcript variant (LOC111010534) (Accession no XP_022139687.1)
- The amino acid sequence of the *SPL* gene was deduced for both the monoecious and gynoecious lines using ExPASy translate tool
- Analysis using ORF finder showed that the nucleotide sequence had 19 ORFs in both lines with ORF4 being the longest with 768 bp coding region for 255 amino acids
- Differential expression of the *SPL* gene in the flower buds of monoecious and gynoecious lines was studied using RT-qPCR analysis
- One pair of primers each for *SPL* gene (test gene) and *actin* gene (control gene) was designed using mRNA sequences of the respective genes
- RNA was isolated from the emerging buds using TRI reagent and converted to cDNA using Oligo(dT)primers
- Synthesis of first strand of the cDNA was confirmed by PCR amplification using the primer sets specific to housekeeping gene, *actin*. Amplification of band at the expected size of 196 bp was observed in all the samples.
- In real time expression analysis, relative expression levels of the *SPL* gene was normalized with the expression level of endogenous control *actin*
- Three technical replicates and two biological replicates were used for each sample and he fold change was calculated through $2^{-\Delta\Delta Ct}$
- The relative expression of *SPL* gene was more in gynoecious flower buds compared with that in monoecious female and male buds, using male flower bud as standard.

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CHARACTERIZATION AND EXPRESSION ANALYSIS OF *SPOROCYTELESS (SPL)* GENE IN MONOECIOUS AND GYNOECIOUS LINES OF BITTER GOURD

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(2020-11-160)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the
requirement for the degree of

Master of Science in Agriculture
(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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2023

ABSTRACT

Bitter gourd (*Momordica charantia* L.; 2n=2x=22) is an important vegetable crop. India produces roughly 1.2 million metric tonnes of bitter gourd from an area of 99,000 hectares. The bitter gourd's immature fruits are often eaten as vegetables, which is known for its anti-diabetic and other therapeutic properties. It is rich in Vitamin A, C, iron and phosphorous. Its enormous therapeutic benefits are attributed to the presence of phytochemicals, which are known to have immune-boosting, antiviral, anti-diabetic, antibiotic, antimutagenic, anthelminthic and cancer-preventive characteristics. Gynoecious lines have revolutionized the hybridization programmes in cucurbits. Though many gynoecious lines are reported in major cucurbits such as cucumber, gynoecious bitter gourd lines are reported from KAU, IIVR, IIHR and IARI only.

The *AGAMOUS* (*AG*) gene, leading C class gene in the ABC model of flowering, acts by activating the transcription of *SPOROCYTELESS* (*SPL/NZZ*) gene and hence *SPL* is considered very important for the megaspore development in flowers. For the silencing/ over expression of this gene, aimed at altering the sex form of bitter gourd, the gene must be sequence characterized. Its expression pattern in the male and female flowers can further reveal the roles of this gene in sex expression in this crop. In this background, the present study "Characterization and expression analysis of *SPOROCYTELESS* (*SPL*) gene in monoecious and gynoecious bitter gourd lines" was under taken during the period from 2020-2023 at the Department of Plant Biotechnology, College of Agriculture, Thrissur, with the objective of sequencing and analysis of *SPL* gene amplified from the cDNA of monoecious and gynoecious bitter gourd lines and expression analysis of the gene in the developing buds of the male and female flowers of monoecious plants and female flowers of gynoecious plants.

Monoecious bitter gourd cultivar Priyanka and gynoecious line KAU-Mc-Gy-102 were raised in pots. Initially, male and female flower buds, at differentiating phase, from the monoecious cultivar and female flowers from the gynoecious line were collected. The collected flower buds were quick frozen in liquid nitrogen, brought to laboratory, stored in -20°C for a short while and used for RNA isolation. First strand of cDNA was synthesised from the RNA isolated from each flower bud group. However, the real time PCR primers designed using the mRNA sequences of the gene has failed to amplify the gene from the cDNA. Since intronic region in the gene was found lesser, it was decided to sequence characterize the gene using the genomic DNA. DNA isolated from the leaves using CTAB method was used for PCR

amplification of the *SPL* gene from monoecious cultivar and gynoecious line, using three primers pairs. The PCR amplicons were sequenced and contigs covering the complete length of the gene in both lines were generated. The final contigs in monoecious and gynoecious lines were 2381 2391 bp long, respectively. The sequence alignment had shown no variation between the monoecious and gynoecious lines. The BLASTn showed 100% identity with 79% query coverage, with the predicted sequence of *M. charantia* transcript variant (LOC111010534) (Acc. no. XM02283995.1). The BLASTx showed 99.09% identity with the sequence of *M. charantia* transcript variant (LOC111010534) (Acc. no. XP022139687.1). Amino acid sequence of the gene was deduced from the nucleotide sequence using ExPASy translate tool. BLASTp showed 100% identity with protein sequence of *M. charantia* (Acc. no. XP022139688.1) in both lines. Analysis using ORF Finder yielded 19 ORFs, with ORF4 being the longest with 768 bp coding for 255 amino acids.

For the expression analysis, RNA was isolated from the developing buds of male and female flowers of monoecious line and female buds of gynoecious line, using TRI reagent. The RNA was converted to cDNA using Oligo$(dT)_{18}$ primer and reverse transcriptase enzyme. Primer pairs for *SPL* and *Actin* gene (endogenous control) were designed and real time PCR reaction based on SYBR Green chemistry was carried out. Expression of *SPL* gene in the developing buds of male and female flowers of monoecious cultivar and female buds of gynoecious line was analyzed. The relative expression levels of the gene was normalised with the expression of endogenous control *Actin* gene, following the $2^{-\Delta\Delta Ct}$ method. The relative expression of the *SPL* gene was analysed by taking the male flower as control. Compared to male flowers, gynoecious flowers had higher fold change (276.5) followed by monoecious female flowers (21.69). The present study has sequence characterized the *SPL/NZZ* gene in M. charantia. The amino acid sequences were deduced and annotated. Expression analysis had shown that *SPL* gene is upregulated in the gynoecious flowers. Similarly, expression of this gene was higher in the female flowers in the monoecious cultivar compared to that in male flowers, proving its role in the development of megaspores in the bitter gourd flowers. Based the results, it is predicted that the monoecious lines overexpressed with *SPL* gene may turn gynoecious or produce more female flowers, leading to higher fruit yield.

Annexures

ANNEXURE I

List of laboratory equipment used for the study

Refrigerated centrifuge	: Kubota 6500, Japan, Eppendorf
Horizontal Refrigerated centrifuge	: BIO-RAD, USA
Thermal cycler	: Sure Cycler 8800 (Agilent Technology), Veriti thermal cycler (Applied Biosystems, USA) and ProFlex PCR system (Applied Biosystems, USA)
Gel documentation system	: Gel Doc™ XR* (BIORAD)
Micropipettes	: Eppendorf
Spectrophotometer	: NanoDrop ND-1000

ANNEXURE II

Reagents required for DNA isolation

1.2X CTAB extraction buffer (100ml)

CTAB	: 2 g
Tris base	: 1.21 g
EDTA	: 0.745 g
NaCl	: 8.18 g
PVP	: 1.0 g

(Adjusted the pH to 8 and made up final volume to 100 ml)

2. 10 per cent CTAB solution

3. Chloroform- Isoamyl alcohol (24:1v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed thoroughly

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0°C and used for the study.

5. Ethanol (70%)

To 70 parts of absolute ethanol (100%), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100ml)

Tris HCI (10 mM)	: 0.1576 g
EDTA (1 mM)	: 0.0372g

(The solution was prepared, autoclaved and stored at room temperature)

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

a. TAE buffer (50X) (for 1 L)

Tris Acetic Acid : 242 g

Glacial Acetic Acid : 57.1 mL

0.5 M EDTA (pH 8) : 100 mL

(final volume made up to 1 L)

b. Loading dye (6X)

0.25 per cent bromophenol blue

0.25 per cent xylene cyanol 30 per cent glycerol in water

c. Ethidium Bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in dark bottle.