



T.R.
EGE UNIVERSITY
Graduate School of Applied and Natural Science



**OPTIMIZING CRISPR/CAS9 METHOD TO RESOLVE
BOLTING IN SPINACH AND USE OF CYTOKININ
OXIDASE INHIBITORS FOR EFFICIENT PLANT
TRANSFORMATION OF 14 SPECIES**

MSc THESIS

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Department of Seed Science and Technology

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Erasmus Mundus Master Program in Plant Breeding Second Cycle Programme

İzmir
2020

Here we certify that this thesis entitled “**Optimizing CRISPR/Cas9 Method to Resolve Bolting in Spinach and Use of Cytokinin Oxidase Inhibitors for Efficient Plant Transformation of 14 Species**” conducted, prepared and submitted for the degree of Master of Science by Irum Saadia KHAN has been recommended for acceptance and approval for oral defence as it complies both with the "Ege University Graduate School policies" and "Ege University Graduate School of Natural and Applied Sciences' rules and regulations". This study is fully adequate, in scope and quality, as a thesis for the degree of Master of Science and the Candidate was found successful and the thesis was approved by the thesis committee by unanimity voting on 16 / 09 / 2020.

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16/09/2020



Irum Saadia KHAN

ÖZET**İSPANAKTA ÇİÇEKLENME SORUNUN ÇÖZÜMÜ İÇİN
CRISPR/CAS9 YÖNTEMİNİN OPTİMİZE EDİLMESİ VE 14
TÜRDE ETKİN BİTKİ TRANSFORMASYONU İÇİN SİTOKİNİN
OKSİDAZ İNHİBİTÖRLERİNİN KULLANIMI**

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Ekonomik olarak önemli bir ürün olan *Spinacia oleracea*, sağlık ve besin içeriği bakımından çok faydalıdır. Çiçeklenme, ıspanak yetiştiricileri ve ıslahçıların karşılaştığı ciddi bir sorundur ve kaliteyi ve verimi büyük ölçüde düşürür. Yeni CRISPR/Cas teknolojisi, araştırmacılara bitkilerdeki istenmeyen özelliklerden sorumlu olan genlerin devre dışı bırakılması konusunda yardımcı olmaktadır. Doku kültürü, transgenik bitkinin geliştirilmesinde büyük öneme sahiptir, ancak düşük transformasyon oranı bitki genom editing’de en önemli darboğaz olarak karşımıza çıkar. Özellikle transformasyona uğramış tek bir hücreden transgenik bir bitkinin rejenerasyonu, çoğu bitki türü için halen zor olmaya devam etmektedir. Bu nedenle, ıspanak ve önemli 13 üründe CRISPR/Cas ile genin devre dışı bırakılması, doku kültürü rejenerasyonu ile kombine edilerek gerçekleştirildi. Bunun için çalışmada, çıplak tohumlu bazı bitkilerde çiçeklenmeden sorumlu ana genler olan T (Ft) lokusu ve Ft ile etkileşen protein 1 (FtPI1) seçildi. CRISPR / Cas çalışmasının bir parçası olarak ıspanakta bu genlerin bulunabilmesi için primerler tasarlandı. Ispanakta *in vitro* tohum çimlenmesi ve sürgün rejenerasyonu farklı büyüme ortamlarında gerçekleştirildi. Yeni nesil *in silico* tasarlanmış sitokinin oksidaz inhibitörleri (CPPU, JN1 ve JN4) tek başına ve BA ve 2iP ile kombinasyon halinde, etkili yaprak rejenerasyonunun sağlanması amacıyla ekonomik bakımdan önemli on dört farklı ürün üzerinde

uygulanmış ve elde edilen sürgün sayısı ıspanak ile karşılaştırılmıştır. Elde edilen sonuçlar, ıspanak çeşidimizde her iki genin heterozigot formda bulunduğunu ortaya çıkardı. Uygulanan *in vitro* protokollerinde Ispanak tohumlarının sadece %25-30'u çimlendi. Ispanak rejenerasyonunun, temel MS ortamında MSmod3B ortamına göre nispeten daha iyi olduğu görüldü. Bununla birlikte, rejenerasyon her iki ortamda da %50'den daha az olarak gerçekleşti. Bitki türleri, farklı sitokinin oksidaz / dehidrojenazın (CKO / CK) uygulamalarına karşı farklı büyüme modelleri gösterdi. Bu 14 bitki türünden sadece 5 tanesi sürgün geliştirirken, ıspanak da dahil olmak üzere diğer 9 bitki türünde sadece kallus oluşumu gözlemlendi. Sitokinin oksidaz/dehidrojenaz inhibitörleri, BA ve 2iP ile kombinasyon halinde kullanıldığında en yüksek sayıda sürgün elde edildi. Bitki türlerinin rejenerasyonunun büyük ölçüde genotipe bağlı olduğu belirlendi. Etkin ıspanak doku kültürü koşullarının oluşturulması ve heterozigot bitkiler için CRISPR / Cas protokolünün geliştirilmesi için daha fazla araştırma yapılmasının gerekli olduğu sonucuna varıldı. Daha iyi bitki rejenerasyonu için yeni sitokinin inhibitörleri daha da optimize edilebilir. Bu araştırma gelecekte farklı bitkisel ürünlerde, hem CRISPR/Cas hem de doku kültürü çalışmaları için protokollerin geliştirilmesine yardımcı olabilir.

Anahtar kelimeler: Çiçeklenme, CRISPR/Cas, Sitokinin oksidaz, sürgün rejenerasyonu, transgenik, rejenerasyon, heterozigoti, in-vitro.

ABSTRACT**OPTIMIZING CRISPR/CAS9 METHOD TO RESOLVE BOLTING
IN SPINACH AND USE OF CYTOKININ OXIDASE INHIBITORS
FOR EFFICIENT PLANT TRANSFORMATION OF 14 SPECIES**

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Spinacia oleracea is an economically important crop and is full of health and nutritional benefits. Bolting is a serious problem in spinach faced by growers and plant breeders which reduces the quality and yield on large scale. The novel CRISPR/Cas technology assists researchers with knocking-out genes that are liable for unwanted attributes in plants. Tissue culture plays a vital role in developing a transgenic plant, but low plant transformation efficiency is the main bottleneck in plant genome editing. Especially regeneration of an entire transgenic plant from a single transformed cell remains difficult for most plant species. Therefore, combined study for gene knockout through CRISPR/Cas in spinach, tissue culture regeneration in spinach and 13 other important crops was conducted. Flowering Locus T (Ft) and Ft-interacting protein 1 (FtPI1) were selected as these are the major genes responsible for flowering in number of angiosperm plants. Primers were designed to find these genes in spinach as a part of CRISPR/Cas study. In-vitro seed germination and shoot regeneration of spinach was performed in different growth medias. New generation of in silico designed cytokinin oxidase inhibitors (CPPU, JN1 and JN4) were applied alone and in combination with BA and 2iP on fourteen different economically relevant crops to develop efficient leaf regeneration systems and number of shoots were compared with spinach. Results revealed that both genes are present in our spinach variety in heterozygous form. Among all *in-vitro* protocols only 25-30% seeds of Spinach were germinated. Regeneration of spinach was comparatively

better in simple MS media than in MSmod3B media. However, regeneration was less than 50% in both medias. Each plant species demonstrated different growth pattern to different treatments of cytokinin oxidase / dehydrogenase (CKO/CK). Among these 14 plant species only 5 plant species developed shoots, while in other 9 plant species including spinach only callus formation was observed. Highest number of shoots were observed when these cytokinin oxidase/dehydrogenase inhibitors were used in combination with BA and 2iP. Regeneration of plant species is highly dependent on genotype. More research needs to be conducted to establish efficient tissue culture of spinach and to develop CRISPR/Cas protocol for heterozygous plants. The new cytokinin inhibitors can be further optimized for better regeneration. This research can help in future to develop protocols for different crops both for CRISPR/Cas and tissue culture study.

Keywords: Bolting, CRISPR/Cas, Cytokinin oxidase, shoot regeneration, transgenic, regeneration, heterozygosity, in-vitro.

PREFACE

The world is in dire need to introduce new crop production technologies to accelerate the delivery of healthy food everywhere. There is no doubt that CRISPR/Cas9 has been a revolutionary technology in crop protection and production. In recent years, CRISPR/Cas9 has started to bring revolution to accelerate the potential of introducing new traits especially in terms of resistance to diseases, drought and increasing the percentage of yield in less number of years as compared to traditional plant breeding methods. Tissue culture plays an important role not only in multiplication of plants but also gene transformation in an effective way. Regeneration of a plant out of a single transformed cell is crucial step in CRISPR/Cas9 methodology for almost all plants. Spinach is an important leafy vegetable with full of health benefits. It is consumed all over the world because of its taste and nutritious nature. Early flowering (known as bolting) reduces overall quality of the leaves and farmers suffer from heavy loss because of low yield. I chose this topic because as a student of plant breeding focused on molecular science, I really wanted to play my part to help growers and farmers in an efficient way.

The main aim of this research is to optimize CRISPR/Cas9 knock-out procedure for the genes responsible for bolting in spinach. Recent advances in understanding the flowering physiology and progress in gene editing will allow knocking out key genes in this process without effecting other pathways. Precocious flowering in horticultural crops causes plenty of difficulties for growers. By transient expression of specific CRISPR/Cas9 endonucleases, essential flowering genes will be targeted, and Loss-of-function mutants will be created. For this purpose, successful establishment of tissue culture of spinach is important. Different protocols used in this study evaluate the percentage rate of spinach regeneration.

This study also aimed to evaluate the application of new generation of in silico designed cytokinin oxidase inhibitors to increase the efficiency of plant transformation in several economically important crops and their comparison with spinach. Cytokinin inhibitors play important role in plant regeneration. Hopefully,

this research will open more paths in study of flowering genes, bolting control, CRISPR/Cas9 optimization and tissue culture systems.

IZMIR

16 / 09 / 2020

Irum Saadia KHAN



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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Definition</u>
2,4,5-T	Trichlorophenoxyacetic corrosive
2,4-D	Dichlorophenoxyactic corrosive
2iP	Isopentenyl adenine
BA	Benzyl adenine
Cas	CRISPR associated
CCs	Companion cells
CKO/CKX	Cytokinin oxidase/dehydrogenase
CO	CONSTANT
Conc.	Concentration
CRISPR	Clustered regularly interspaced short palindromic repeat
DKW	Driver and Kuniyuki woody plant medium
DNA	Deoxyribonucleic acid
DSB	Double strand break
FAO	Food and Agriculture authority
FT	FLOWERING LOCUS T
FT	Flowering Locus T

LIST OF ABBREVIATIONS (continued)

<u>Abbreviation</u>	<u>Definition</u>
FTIP1	FT-INTERACTING PROTEIN 1
FTIP1	Ft-interacting protein 1
HDR/HR	Homology directed repair
IBA	Indole-3-butyric corrosive
MMS	Modified MS
mRNA	Messenger RNA
NAA	Naphthaleneacetic corrosive
NaKR1	Sodium Potassium Root Defective 1
NaKR1	Sodium potassium root defective1
NHEJ	Non-homologous end joining
NOA	Naphthoxy acidic corrosive
OD	Optical density
PAM	Protospacer adjacent motif
p-CPA	Para-chllorophenoxyacetic corrosive
PEBP	Globular, phosphatidylethanolamine-binding
PEBP	Phosphatidylethanolamine-binding

LIST OF ABBREVIATIONS (continued)

<u>Abbreviation</u>	<u>Definition</u>
RNA	Ribosomal nucleic acid
SAM	Shoot apical meristem
SAM	Shoot apical meristem
SEs	Sieve elements
SH	Schenk and Hildebrandt
Stat	Statistics
TYLCV	Tomato yellow leaf curl virus
VIB	Vlaams Instituut voor Biotechnologie
VW	Vacin and Went
WPM	Woody plant medium)

1. INTRODUCTION

Spinach, botanically called *Spinacia oleracea* is an important vegetable of family Amaranthaceae/ Chenopodiaceae. This family also includes some other important members for example chard, quinoa, and sugar beet. It is first produced in Iran and it was famous as herb of Persia by the early Chinese in 7th century. After then its domestication was considered in different parts of china, Europe, and USA through international trade system (Ribera et al., 2020). It was then used as canned food and its leaves became famous as mixed salad along with other green vegetables in late 13th century in all over Europe (Roughani and Miri, 2019). Spinach is consider as important vegetable crop because of its nutritional value and fast growing nature and from past few years its demand and production increases in United States and in different parts of Europe including Belgium (Correll et al., 2011a). It is known as superfood in various cultures. As spinach is full of nutritional benefits; therefore, it is famous as a functional food. Its nutritional list includes large number of phytochemicals, minerals, vitamins and bioactive. It includes sodium, potassium, calcium, and phosphorus as important minerals. It contains vitamin B, C, K and A. It is composed of 91.4% water, 3.6% carbohydrates, 0.4% fats, 2.9% proteins and 8.8% fibers. This leafy dark green vegetable is full of anticancer, anti-inflammatory, and anti-obesity properties and is famous for hypoglycemic activity (Roberts and Moreau, 2016). The healthy seeds of spinach are used to cure fever, and many other heart and liver diseases as they are therapeutic in nature. Adding spinach to your diet can prevent from diabetes and it lowers sugar level in blood, and it is also for healthy hair and skin. Moreover chlorophyll content present in its dark green leaves is beneficial to block the growth of carcinogenic cells (Roughani and Miri, 2019). It is also important source of calcium for bones. As spinach is not only tasty but is also full of health and nutritional benefits, it is regarded as power house of its family along with beet and chard. (Al-qumboz and Abu-Naser, 2019).

Bolting is described as the ability of the plants to produce flowers earlier than the harvesting season. Basically, it is rapid change of vegetative phase to reproductive phase naturally, and it is one of the important characteristics for spinach breeding. Now a days production of a spinach which bolts slowly is

consider an important objective of many breeding programs (Chitwood,). The change of a phase from vegetative to reproductive is just not associated with flower production but it is also responsible for many other morphological changes (Pouteau and Albertini, 2009). Apart from spinach bolting also plays significant role in other plant species for example in garlic, onion, lettuce, sugar beet etc. In leafy vegetables, bolting affects adversely as early flowering can reduce the quality of leaves significantly which lead to the loss of farmers as they will get lower price of their product. Thus bolting affects the phenotype of leafy vegetables as lettuce and spinach and becomes the reason of decrease in market cost especially in summer season (Liu et al., 2018). It is very important for plant breeders to consider the timing at which plant bolts as it is associated not only with seed production but also with the overall quality and life span of the crop. Bolted spinach cannot lead to good profit. It can also reduce the plant yield on a large scale. Thus when spinach bolts it produces long stem, flower head and lead to seed production and the leaves structure changes and it became bitter, tasteless or inedible so left with no choice except growing it again in a new season or either using it only for seeds (Kim et al., 2000a; Goreta and Leskovar, 2006a; Chen et al., 2019a). Therefore, it is important to study genes related to flowering in vegetables and how they work to find permanent solutions towards bolting (Abe et al.,). Therefore, useful research mainly focusing on causes and effects of bolting is the need of the time. Late-bolting spinach can be use whole year round. Studies have shown that bolting of spinach depends on the temperature and length of the light period (Chun et al., 2000). Hormones treatment have also influence on bolting. It seems that some vegetables try to reproduce next generation as soon weather conditions (light and temperature) fluctuate. Generally, bolting is divided into two types such as inflorescence axis elongation which is common in *Arabidopsis* and is only related to formation of flower. Other type of bolting is known as stem internode elongation which is seen in spinach and lettuce and it involves not only flower differentiation but also stem internode elongation as its names suggests. So, the factors influencing the bolting depends mainly on the type. Spinach can bolt because of many reasons including temperature extremes, water stress, photoperiod. Bolting is difficult to control in spinach as its inflorescence is tiny in structure (Chen et al., 2019a).

Climate is changing drastically, and world population is increasing on a very rapid rate. Growing enough food crops that can feed this global world and are climate resilient as well is a need of an hour. At present, this is a big challenge faced by farmers, agriculturists, researchers, biotechnologists, and plant breeders (Scheben et al., 2017a). Traditional way of breeding used for crop quality improvement is a laborious method and it alone cannot withstand with modern agriculture requirements. To increase the genetic variability through cross breeding takes a lot of time as big parts of genomes in large number of crops has been fixed due to evolution of millions of years in a breeding sector. Mutation breeding and transgenic breeding are playing a big role in quality improvement, but these methods are also as laborious as cross breeding when compared to newly introduced precise genome modifications through CRISPRCas. It is demonstrated well in the figure 1.1 (Pacher et Puchta, 2017).

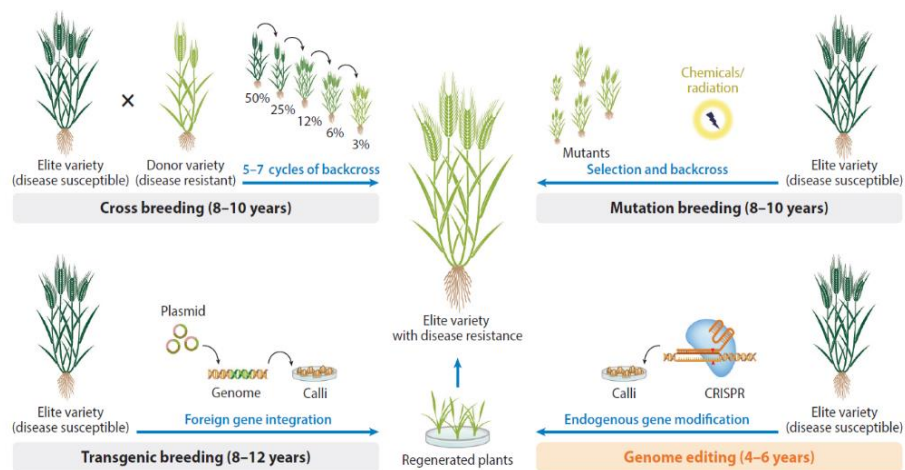


Figure 1.1. Comparison of breeding systems Source: (Pacher and Puchta, 2017).

CRISPR/Cas9 is bringing revolution not only in the field of animal sciences but also in plant sciences. The world is in dire need to introduce innovative plant production technologies to increase the delivery of healthy food everywhere under the pressure of changing climatic conditions. The field of crop science is now moving towards new advance technology such as use of RNA-programmable CRISPR-Cas9 to compete with new challenge of improving quality, increasing the yield and developing resistant varieties to feed the increasing population (Doudna and Charpentier, 2014a). In recent years, CRISPR/Cas has started to play an important role to accelerate the potential of introducing new traits in terms of resistant varieties, high percentage of yield in less number of years as compared to traditional plant science approaches (Scheben et al., 2017b).

Clustered regularly interspaced short palindromic repeat CRISPR/Cas systems are very important immunity systems and they are present in archaea and bacteria (Xing et al., 2014a; Tycko et al., 2016; Ishino et al., 2018). CRISPR/Cas system was first discovered in *Escherichia coli* in early 1987 while analyzing its DNA (Ishino et al., 2018). Biological life is characterized by biomolecules called DNA and DNA is made up of chains of nucleic acids. The characteristics of an organisms are coded by DNA molecules. The process of transcription and translation of DNA helps to synthesize proteins which are involved in almost all the cellular mechanisms. Mutational changes in genome of organism can occur naturally or can be induced through artificial mechanisms. These changes can be positive or negative (Morange, 2015b). These CRISPR regions were soon discovered in many other organisms i.e. bacteria and archaea, defined as their defense mechanism (Morange, 2015a). In recent 5 to 10 years CRISPR regions took the attention of scientists when they discovered it to be a revolutionary technology because its target specific genome editing character (Mao et al., 2013a; Morange, 2015a; b; Ishino et al., 2018; Javed et al., 2018; Makarova et al., 2020). Since then, this novel technology has gained interest by both manufacturers and scholars. It is very attractive and precise genome editing tool for the scientific community both in animal and plant sciences (Doudna and Charpentier, 2014a). In recent 10 years, many problems have been solved by using this precise tool. Some examples include development of disease resistant; drought tolerant and seedless varieties of some species have been developed. A lot

of work has been done on tomato. Resistance to the geminivirus, tomato yellow leaf curl virus (TYLCV) by targeting three regions of the viral genome (Tashkandi et al., 2018). Shelf life was also enhanced by delayed and permanently inhibited ripening (Ito et al., 2015). Seedless variety and tomato variety resistant to powdery mildew was also developed via CRISPR/Cas genome editing (Nekrasov et al., 2017; Ueta et al., 2017). Similar work has been done in many other economical important crops for example tobacco resistant variety against bean yellow dwarf virus (Baltes et al., 2015), variety of soybean which has low polyunsaturated fats (Demorest et al., 2016), bacterial blight resistant variety of rice (Jiang et al., 2013) and a variety with larger and more number of grains of rice (Li et al., 2017). potato variety with low concentration of reducing sugars (Clasen et al., 2016), maize drought tolerant variety (Shi et al., 2017) and cucumber variety resistant to broad virus (Chandrasekaran et al., 2016) has been achieved. In short, CRISPR/Cas has revolutionized the field of biotechnology and plant breeding especially in terms of crop improvement (Xing et al., 2014b).

No doubt, in past few year, CRISPR Cas technique has been widely used in plant sciences for crop improvement, resistance to stress, drought and increase in yield. But this technique in improving crop quality requires the molecular genetics, bioinformatics, and a well-established tissue culture of that crop as well. Almost all the procedures for crop gene modifications depends on the tissue culture, a long and laborious process. Low plant transformation efficiency is the main bottleneck in plant genome editing (CRISPR CAS). Especially regeneration of an entire transgenic plant out of a single transformed cell remains difficult for most plant species (Chen et al., 2019b). Plant transformation and plant regeneration capability depends on the genotype of the specie and it is also influences by many other factors such as type of explants and the selective agents used in the culture media (Rakosy-Tican et al., 2007; Rai and Shekhawat, 2014). The explant tissue used for regeneration can be sensitive because of the presence of the antibiotic in the selective media and it can effect the transformation efficiency on a large scale. The most common observation is the programmed cell death of the tissue because of its non suitability with the culture media and agrobacterium mediated transformation. Browning of tissues also has been observed in some of the species because of the production of reactive oxygen after

agrobacterium induction (Li et al., 2006; Zhou et al., 2014; Sabbadini et al., 2019). Plant hormones are essential to regenerate a complete plant from a single cell or a tissue as they play a vital role in the process of organogenesis (Sales et al., 2002). It has been observed the high regeneration effectiveness can be achieved by using cytokinin in an increased amount in a lot of species but Cytokinin oxidase/dehydrogenase (CKO/CKX) because it interrupts the amount of cytokinin by its degradation activity (Bilyeu et al., 2001). Cytokinin is an important plant hormone which has its role in cell division. In organogenesis, it help in the process of both root and shoot development (Galuszka et al., 1998; Zatloukal et al., 2008). Almost 40 to 50 years back, Miller and Skoog collaborated with different laboratories. The team worked on purification of cytokinin. They were successful in purifying the first cytokinin crystal and they checked its activity in the tissue culture development of tobacco where they found its role in number of activities (Muller and Sheen, 2007b). CK is made up of class of adenine-derived molecules involved in the signaling of number of different processes in plant growth and development such as control of stem elongation, differentiation of vascular tissues, root formation, shoot development, biogenesis of chloroplast, seed germination, inflorescence growth and stress tolerance (Sakakibara, 2006; Muller and Sheen, 2007a).

The main aim of this study is to analyze the genes in spinach responsible for bolting to optimize a CRISPR/Cas protocol to knock out those genes. Recent advances in understanding the flowering physiology and progress in gene editing will allow knocking out key genes in this process without effecting other pathways. Other necessary techniques to be mastered are gene delivery systems and adventitious shoot regeneration. Precocious flowering in horticultural crops causes plenty of difficulties for growers. For instance, in spinach, early flowering induced by long days ('bolting') reduces the quality of the final product. The aim of this research is to stop the onset of the flowering process by using genome editing techniques. By transient expression of specific CRISPR CAS9 endonucleases, essential flowering genes will be targeted, and Loss-of-function mutants will be created.

The secondary aim is to establish successful tissue culture protocol for spinach and to check the application of New generation of in silico designed cytokinin oxidase inhibitors to increase the efficiency of Plant transformation in number of economical important crops and their comparison with spinach it as also a major problem in plant genome editing (CRISPR CAS). Both CRISPR/Cas and tissue culture are important part of biotechnology and biosciences engineering department.



2. LITERATURE REVIEW

Generally, spinach (*Spinacia oleracea* L.) is a dioecious plant but monoecious plants can also be found in spinach specie. The use of strict male lines with better degree of homozygosity is beneficial for plant breeders. Therefore, monoecious plants can be self-pollinated, and these lines can be obtained efficiently from them (Onodera et al., 2008; Yamamoto et al., 2014). During its vegetative phase of growth, spinach produces a rosette in it happens usually in winter season. The seed stalk emerges from the bunch of the leaves that are arranged in the form of flower like rosette. The leaves of spinach look like a triangle (pointed) or somewhat ovate(round). They can be flat or savoy or crinkled. The flowers of spinach are very visible and attractive as they are very tiny in structure. The flower produces small dry fruits as shown figure 2.1 (Sherry et al., 1993). As spinach is an annual crop, farmers must harvest it within a short period of time otherwise it will start bolting. Two types of spinach can be harvested mainly such as baby leaf spinach and mature leaf spinach. Baby leaf spinach takes 3-5 weeks and 5-8 weeks for mature spinach which is mainly for fresh consumption and frozen market. Processing spinach can be grown for longer period as compared to baby leaf and mature spinach as it will give more yield in the form of bigger and thick leaf. Baby leaf can be harvested soon but it gives less yield, although it can be sold on higher rates as salads (Yamauchi et al., 2011) . The planting densities have increased over the years to increase yield; for baby leaf production the sowing density is between 8.6 - 9.9 million seeds per hectare (Lester et al., 2010; Correll et al., 2011b).



Figure 2.1. Inflorescence of spinach source: Sherry et al.,1993.

Cultivation of spinach is possible in many types of climates but suitable weather for seed production of spinach is cool maritime season with long days. The main seed producing country of spinach seed is Denmark because of its perfect climatic conditions. Its geographical location with long summer days but mild coastal climate is perfectly suitable for the high yield seed production of spinach. It is in a record of 2012 when Seed production from Denmark covered the 75% of the global market (Deleuran et al., 2013). In past, the seed of spinach had small spikes but currently the seed is but now its smooth in texture (Shetty et al., 2012). Spinach production graph has shown the top ten producing countries from year 2012 to 2018. China is the top producer of spinach as shown in figure 2.2. Belgium is also in the top ten producers of spinach. Its production area and yield has been shown figure 2.3.

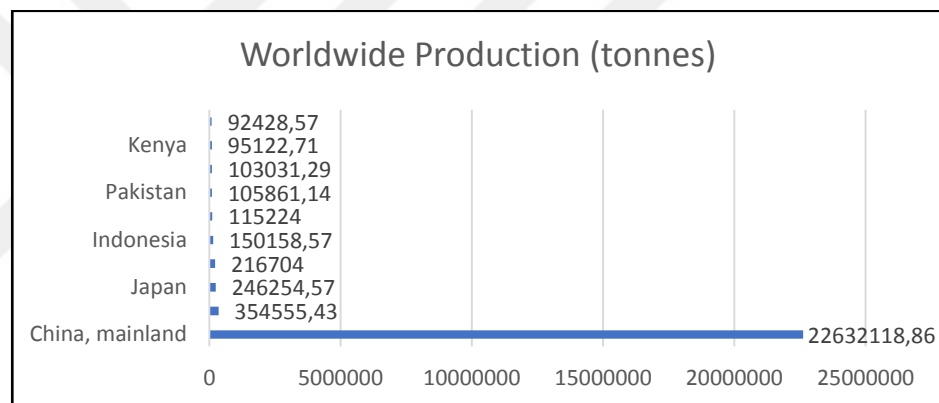


Figure 2.2. Worldwide production of spinach - Source: FAOSTAT.

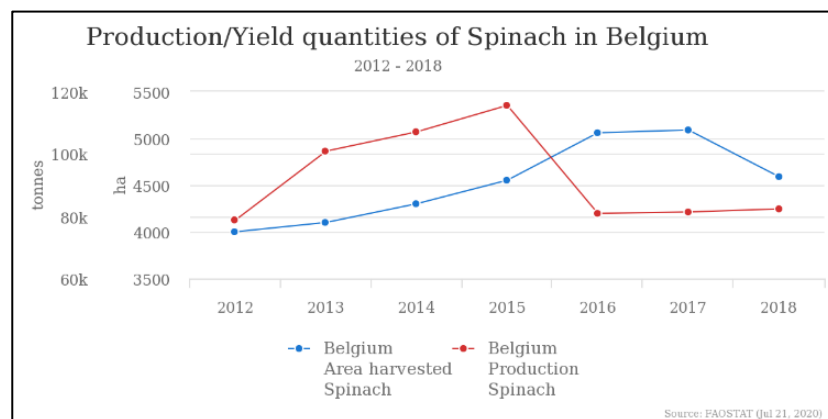


Figure 2.3. Production of Spinach in Belgium - Source: FAOSTAT.

Spinach is an important crop and bolting is a serious problem for spinach growers (Goreta and Leskovar, 2006b). Bolting damages the quality of spinach as it causes early flowering and seed formation in spinach making it inedible (Kwon et al., 2016). The main cause for bolting discovered until now is the cool season and change in the light period. Day length induces this behavior of early flowering (Mutasa-Göttgens et al., 2010). In annual crops like spinach, bolting occurs much earlier than their maturity state as compared to biennials for example in carrot, it occurs because of over-wintering (Kim et al., 2000b). Bolting can reduce its yield on a large scale. When spinach bolts it becomes bitter in taste and inedible so farmers left with no choice except using this bolting spinach for seed purpose (Chitwood,).

To comprehend the mechanism of bolting in spinach, the study of flowering genes is essential. Physiological investigations on flowering time control have indicated that plants incorporate numerous ecological signals. Predictable elements, for instance, day length and vernalization, are viewed as 'primary', yet certainly intrude with, or can even be subbed by, less *predictable* components (Bernier and Périlleux, 2005). All plant parts partake in the detection of these interacting factors. A significant long distance signaling is discerned to happen through the phloem between the leaves and the shoot apical meristem (SAM) on account of floral initiation by photoperiod (An, 2004). From vegetative to regenerative development in several plant species, the modification is constrained by day length. In leaves day length is apprehended and prompts a fundamental signal, called florigen, that travels through the phloem to the shoot pinnacle. Florigen causes transition in gene articulation at the shoot apical meristem (SAM), that reconstruct the SAM to form flowers rather than leaves (Turck et al., 2008). The incredible hunt for florigen, the ubiquitous, long distance flowering regulator propounded by Chailakhan during the 1930s, emanated the finding a decade prior that FT-like proteins contented the florigen prediction. They are little globular, phosphatidylethanolamine-binding (PEBP) proteins, phloem-expressed, graft-transmissible, and apt to transport to the shoot pinnacle to exhibit as vigorous stimulator of flowering in numerous plants (Putterill and Varkonyi-Gasic, 2016). Genes that control Arabidopsis FT protein activity and a certain features of Arabidopsis FT protein that prompt it a viable florigen have erstwhile been

distinguished (Andrés et al., 2020). Generally various FT-like genes that render overlapping and/or disparate functions in advancing flowering are present in plants (Jung and Müller, 2009). For instance, FT and TSF advance *Arabidopsis* flowering, as do GmFT2a and GmFT5a in soybean and Hd3a and RFT1 in rice. (« Activation Tagging of the FT.pdf »; Abe, 2005; Choi, 2012). The long-distance development of FLOWERING LOCUS T (FT) is controlled by NaKR1 gene. In companion cells (CCs) of the vasculature, articulation of NaKR1(SODIUM POTASSIUM ROOT DEFECTIVE1) and FT is actuated by Photoperiodic control of CONSTANS (CO). NaKR1 is imperative for long distance movement of FT through sieve elements (SEs) from leaves to the SAM, while FTIP1 (FT-INTERACTING PROTEIN 1) intercedes the movement of FT protein from CCs to SEs as appeared in figure 2.4. (Zhu et al., 2016a).

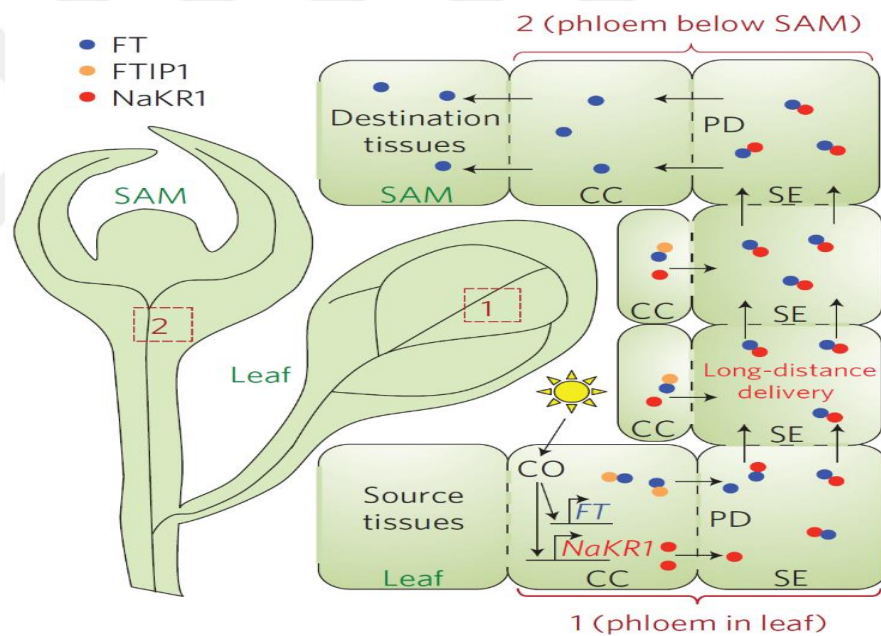


Figure 2.4. Role of FT and FTIP1 genes - Source: (Zhu et al., 2016) (Putterill and Varkonyi-Gasic, 2016).

FT protein satisfies most of the criteria of florigen. From induced leaves, it locomotes toward the shoot pinnacle and athwart graft associations from donor to receptor for flowering initiation. The extraction of florigen from flowering plants and initiation of flowering in vegetative plants with applied concentrates is anticipated that cannot be accomplished attributable to the proteinaceous nature of

FT. An essential supposition of the florigen speculation is that florigen is a universal signal for flowering. Consequences of interspecific grafting tests and of grafts between various photoperiodic reaction types bolster this notion (Wigge, 2011). Subsequent scrutinizing 3 million such yeast tests, the scientists recognized a molecule whose messenger RNA (mRNA) articulation designs resemble those of FT, later named FT-INTERACTING PROTEIN 1 (FTIP1). Plants bloomed a lot later in reaction of long days having mutant, non-functional variants of the FTIP1 gene. The flowering time of such mutants was reestablished to a great extent back to typical when these were provided a working adaptation of gene. These discoveries recommended FTIP1 was critical to how flowering was constrained over time. FTIP1 controlled the transmission of FT from companion cells to sieve elements and along these lines to the shoot. FTIP1 is a basic regulator for the mechanism of FT. (Zeevaart, 2008; Choi, 2012; Liu et al., 2012; Wickland and Hanzawa, 2015a; Ahn, 2016; Zhu et al., 2016b).

The function of FT gene and FTIP1 gene has been discovered in many plants. FT gene helps in floral induction in many plants but the process of repression of flowering has been also discovered in many plants. Although the role of FT gene in sugar beet which is closely related specie with spinach is repression of flowering but from this we cannot conclude that it can also repress flowering in spinach because it has been observed that FT functions differently even in closely related species for example tobacco and tomato belongs to same family but role of FT gene is different in both of them, in fact, there are four types of FT genes in tobacco, out of which one is playing its role for the repression of floral induction while other three aid in flowering (Wickland and Hanzawa, 2015b) Role of FT gene and its pathway in spinach is not discovered yet and with CRISPR/Cas knockout, it can be revealed and aids in controlling bolting permanently.

CRISPR/Cas can assist researchers with knocking out genes that are liable for unwanted attributes in plants particularly to instigate resistance in plants against pathogens (Andolfo et al., 2016a). CRISPR can assist in obliterating the gene liable for bolting in spinach. CRISPR/Cas is potent genetic tool. The mechanism and structure of CRISPR/Cas is intriguing yet convoluted and a

similar time (Mao et al., 2013b; Xing et al., 2014c; Belhaj et al., 2015a; Liu et al., 2017).). In clarification CRISPR can be excessively convoluted or too basic relying upon the details given. CRISPR was first studied in *E. coli*, as referenced previously. In the CRISPR two fundamental segments are identified; firstly, there are the repeats that are short sections of DNA which are between 20 to 30 nucleotides long which are called palindromes. A palindrome is a chain of nucleotides that repeat the similar left to right on the two strands of DNA. These palindromes are indistinguishable and in the middle of these, there are DNA pieces called "spacer DNA" (Amitai and Sorek, 2016). The spacer DNA has been intriguing because it is not homogeneous, and each bit of this is unrepeated. Researchers discovered afterwards that the spacer DNA is totally comparative with viral DNA and primarily with bacteriophage DNA (Ran et al., 2013; Maeder and Gersbach, 2016). They likewise recognized a few genes connected with CRISPR, so these are the CRISPR-linked or Cas-genes. These Cas-genes code for Cas-proteins which usually are called helicases. These proteins uncoil and cut the DNA. In this way, the idea is that this is basically bacterial organisms' immune system, a protective approach to battle the bacteriophages. When a bacteriophage embeds its DNA into *E. coli* typically it should assume control over the cell components and execute it (Schaeffer and Nakata, 2015). Nevertheless, the Cas-proteins are encoded and the CRISPR RNA is translated, since the cell has CRISPR framework. Just before viral DNA begins the infection, the cell deactivates it. On the off chance that another virus assaults the bacteria, the microorganisms encodes another sort of protein called cas1 (Doudna and Charpentier, 2014b; Xing et al., 2014c; Belhaj et al., 2015a; Scheben et al., 2017c). This cas1 takes the viral DNA, disintegrate it, and more critically duplicates it as another spacer DNA to the CRISPR arrangement of the bacterial genome. So, all these spacer DNAs are basically the historical backdrop of old viral infections (Jiang and Doudna, 2017). The entire strategy of CRISPR/Cas is clarified in the figure 2.5. for the most part with bacteriophage DNA (Pennisi, 2013)

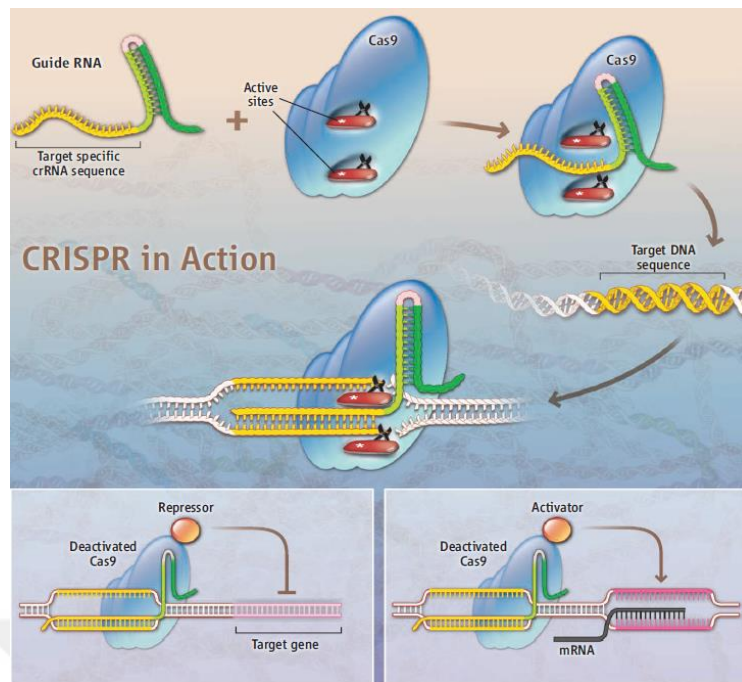


Figure 2.5. CRISPR/Cas CRISPR system can knock in and cut specific c DNA, knocking out a gene or enabling part of it to be replaced by substitute DNA. More recently, Cas9 modifications have made possible the repression (lower left) or activation (lower right) of specific c genes. (Pennisi, 2013)

Gene knockout To cause exact mutations, take out or to embed a gene, scientists worked a similar way and utilized CRISPR. This CRISPR framework was first found in the research center of J. Doudna and E. Charpentier when they were considering *Streptococcus pyogenes*. They made a CRISPR-cas9 framework with their own guide RNA (gRNA) explicit to the piece of DNA of their interest (Doudna and Charpentier, 2014b).

The gRNA has two sections, first is the CRISPR part which has the RNA direction of where to make the cut and second is the protein which makes the cuts in the DNA (Bouché, 2001). Protospacer adjacent motif (PAM) is likewise another sequence which encourages protein to discover precise location where it is intended to cut on the DNA. PAM is consistently close to the sequence that is targeted. It comprises of a short succession of at least 3 nucleotides and it regularly begins as NGG, NRG, NGCG (and there are numerous different kinds of PAM) where N could be any nucleotide. The entire DNA strand is checked by the guide RNA and marks a double stranded cut into the DNA, once it finds the

coordinating PAM sequence (Tian et al., 2017). Thus, when the cut is fabricated, the DNA dismantles, and the cell attempts to restore it which results in mutation. Along these lines, thusly a gene can be silenced/taken out. The reverse way around is the point at which a gene is to be annexed to a DNA strand. All things considered a host nucleotide sequence is given to cell which fits in a gene is embedded. The gRNA alongside Cas9 sits on the targeted DNA at the PAM site and makes a double strand break (DSB). This disintegration is then fixed by the cell by two different methods. The cell fixes it by non-homologous end joining (NHEJ) technique by presenting indels, in the event of gene knock out. The introducing DNA donor template is given to the cell while in the event of gene knock-in and is restored by homology directed repair (HDR/HR repair) technique. The two strategies are utilized among scientific network for different objectives of gene knock out or gene addition (Tsien et al., 1996; Belhaj et al., 2015b; Ma et al., 2016; Andolfo et al., 2016b). Entire procedure of gene knockout and gene knock-in is appeared in the figure 2.6 (Fernandez i Marti et Dodd, 2018).

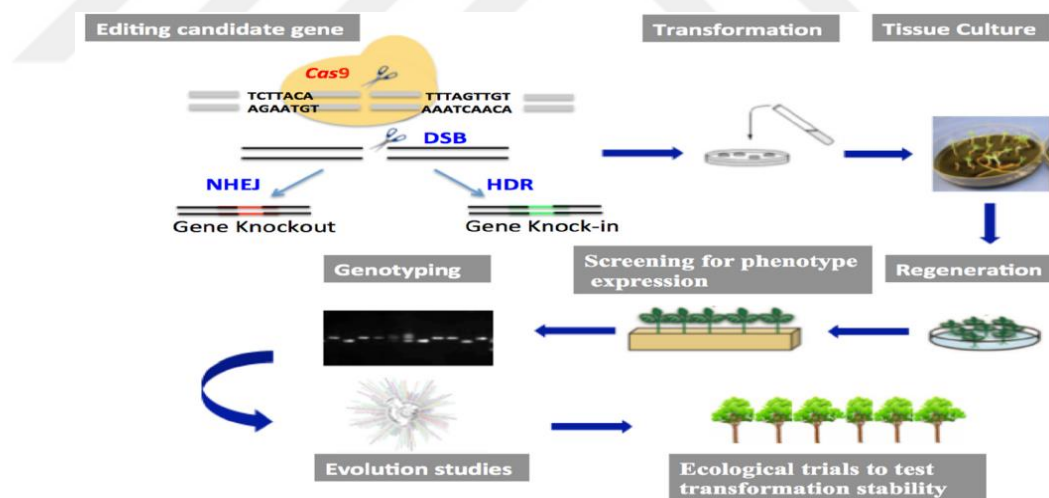


Figure 2.6. Gene knock-out procedure Source: (Fernandez i Marti and Dodd, 2018).

Agrobacterium Transformation is an important procedure to be followed in CRISPR/Cas9. After the finding, genetic changes procured advertence of researcher. *Agrobacterium tumefaciens* that is a bacterium with a capacity to move its vector to the plant's cells (Rashid et al.,). An agrobacterium interceded

modification implies that a gene of intrigue is secluded from the source plant, it is embedded in a vector which has the promotor with the goal that the gene could be transcribed in the plant (Pickardt et al., 1991). Then vector is embedded into a bacterium for instance, *Agrobacterium* by utilizing diverse modification techniques that is, electroporation, heat shock and so on. The microscopic organisms (bacteria) entailing the transgenic vector are then permitted to separate in a growth media (LB media) (Ding et al., 2009). For some period at that point, with this bacterial media the leaf areas are cultivated permitting the bacteria to move the vector to the leaf cells and afterward leaf segments are then developed in dark on a co-culture medium permitting the bacteria to move the vector to plant cells (Walkerpeach and Velten, 1994). The leaves afterwards transposed to another media that contains antibiotic with the purpose that no bacteria develop later at the present stage and the transformed leaves remain sterile (Hensel et al., 2009). The gene transfer is discerned later when the plants grow up and the leaves moreover grown like normal in-vitro plants (Ding et al., 2009).

There are two kinds of method to introduce CRISPR/Cas protocol in plants such as leaf infiltration and co-culture growth. **Leaf infiltration** is famous now a days for quick interpretation of results. The method of co-culture growth must be followed for the efficient transformation of *agrobacterium*, but it is time taking process to visualize and observe the results and check whether it has been successful or not. Transformation through transient mechanism to add or delete the genes on a temporary basis. This method is more flexible, labor saving, and fast in comparison with traditional stable co-culture growth transformation. This method is followed by direct injection of transformed *agrobacterium* into the leaves. A syringe with no needle is used for this purpose (figure 2.7). *Agrobacterium* solution is filled into this syringe. *Agrobacterium* suspension is then injected into the plant through the tip of the injection by using little pressure. The air spaces of the leaves then take up this suspension. Several strains of *agrobacterium* can be transferred by using this method. Multiple genes can be compared using this easy method (Berestovoy et al., 2018).



Figure 2.7. Example of infiltration Source: (Berestovoy et al., 2018).

Plant tissue culture: Primarily because it is brisk, costs less, keeps up the genetic make-up and countless plants can be developed in brief timeframe, the asexual propagation is intriguing for analysts just as for cultivators. In numerous asexual propagation strategies like cutting, grafting, layering and budding, tissue culturing has brought about advancements (Kumar and Loh, 2012). These strategies nevertheless are increasingly utilized in cultivating, agricultural lands, breeding, and other field related activities. It is significant for the producers to keep up a sterile plant culture in laboratories, as different exploration tests have been conducted on such plants and any contaminant may meddle in the validness of the outcomes or it might crater the examination. The growth compartments (plastic and glassware and so on.), place to prepare, sterilize and reserve the media, cultivation chambers (controlled condition of light, force, temperature, and dampness), and other observatory offices must have present in plant tissue culture laboratories. The overall methodology of developing a plant in tissue culture includes preparing growth media in a growth compartment and sanitizing it, after coagulating of the media, shifting the plants into the media in a laminar flow to reduce the menace of adulterant. It is constantly prompted to utilizing sterile forceps and scalpels. Appropriately, covering the top of the compartment and afterward placing it in a growth chamber as per the light, temperature conditions and humidity of the plant (Nguyen et al.,). In media plant hormones may and may not be incorporated (it is reliant on the plant which is developed and the objective behind the tissue culture). Cytokinins, Gibberellins and Auxins are the three significant hormones which are being utilized. Cytokinins regulate apical dominance variation, shoot differentiation, cell division and so forth. It is utilized

for the initiation of differentiation and callus cell division for the in-vitro culture. Additionally, for the multiplication of the shoot, cytokinins is utilized. Benzylamino purine (BAP), Isopentenyl-adenine (2-ip) and furfuryl amino purine (kinetin) are mostly used cytokinins (Bari and Jones, 2009). Auxins are typically liable for the abscission, prolongation of the internodes, apical dominance, establishing and tropism and so forth. Auxins are utilized for cell division and separation of the root cells in invitro- culture. Dichlorophenoxyacetic corrosive (2,4-D), Indole-3-butyric corrosive (IBA), Naphthaleneacetic corrosive (NAA), Naphthoxy acidic corrosive (NOA), Trichlorophenoxyacetic corrosive (2,4,5-T), Para-chllorophenoxyacetic corrosive (p-CPA), are majorly utilized auxins (Yu et al., 2017). Gibberellins are utilized occasionally and there are more than 20 diverse sorts of gibberelins. The most widely recognized one is GA3 (Gaspar et al., 1996a).

Under various conditions for various plants, an assortment of growth medias have been utilized throughout the years. There are numerous growth medias including, SH media (Schenk and Hildebrandt), MMS (Modified MS), DKW (Driver and Kuniyuki woody plant medium), WPM (Woody plant medium), B5 medium, VW (Vacin and Went) media. The Murashige and Skoog (MS) media is the most well-known growth media and has been generally utilized. All these medias are majorly utilized for supplying several nutrients to the plants. The media gives macronutrients which incorporate CHO (carbon, hydrogen and oxygen) just as Calcium, Phosphorus, Nitrogen, Potassium, Sulfur and so forth. In micronutrients it supplies Iron, Molybdenum, Copper, Iodine, Zinc, Cobalt Manganese, Chlorine and so on. Additionally, to the plants the media transmit fundamental nutrients, amino acids and other inorganic and organic compounds (Phillips and Garda, 2019). A coagulating catalyst is another significant media component. Several coagulating catalysts are accessible, all of these are originated from various sources, for instance agarose, micro agarose, agar, gellan gum, gelatin and so on. Out of all gelling agents, agar acquired from seaweed is generally utilized. Chemically agar is a polysaccharide. As it effectively dissolved in water and solidified at 45°C, the melting temperature ranges from 60-100°C, agar turned out as a better utilizing supplement contrasted with different agent. This agent does not respond with different substances added to the gel. In

addition, agar is stable under practically all the developmental conditions and is not processed by the plant enzymes. Along these lines, these qualities molds agar substantially more functional and solidifying agent for plant growth media and prior option.(Adaoha Mbanaso and Roscoe, 1982). While propounding about all these constituents of a growth media, pH is additionally vital. It is balanced before the media sterilization. It might differ in specific cases, generally pH of a media ranges from 5.0 to 6.0. It gives invariability to the supplements, gel and causes the plant to develop in an ideal environment. An overview of plant tissue culture types is given in the figure 2.8 (Kumar and Loh, 2012).

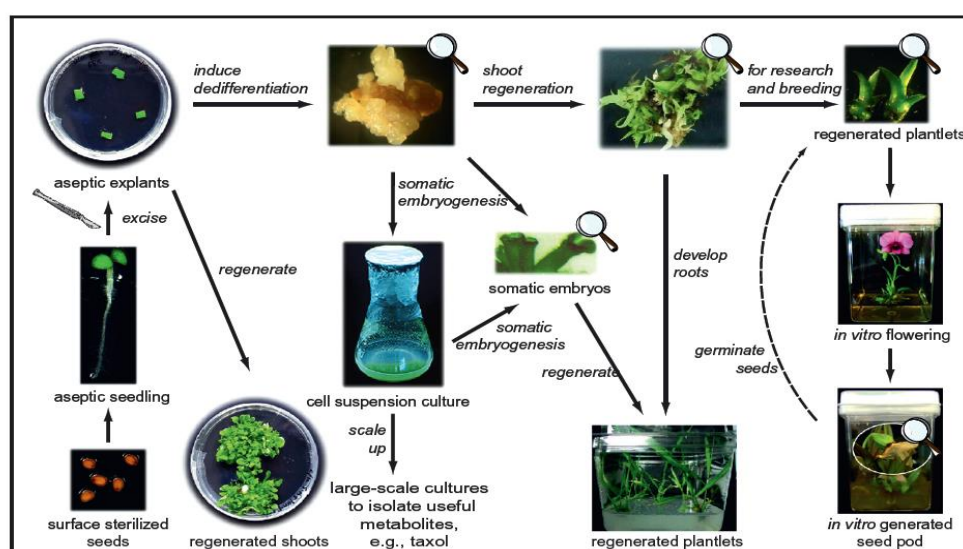


Figure 2.8. Various form of PTC Source: Kumar and Loh, 2012

A completely developed plant has practically the entirety of its cells in peculiar structure, which implies every cell has developed into performing a function. This is named as cell differentiation (Quiroz-Figueroa et al., 2006). When discussing tissue culture, there are two different ways, former is to regenerate the plant by its shoot whilst later is leaf inception where another plant is cultivated from a slight division of the leaf. As the plant's shoot bears buds and it regenerate through these buds producing another plant anew, renders the former kind of development more convenient. As the subsequent case entails the cells of the leaf section to turn out to be least particular and proceed as meristematic cell it is therefore quite distinctive. Meristematic cells are the differentiated cells in the

plant. Transforming differentiated cells into slightly specified cells is called dedifferentiation (Grafi and Avivi, 2004).

This procedure compels a reasonable measure of PGRs (**Plant development hormones**). These PGRs are given to the plant from the development media and the cells are coordinated to form into roots or shoots relying upon the reason for the analysis and sort of the hormones utilized. On the off chance that it happens effectively, the plant produces callus and afterward it begins to develop into typical plant (Budke et al., 2018). The plant hormones are a fundamentally random assortment of small molecules procured from different crucial metabolic pathways (Jiménez, 2005). These compounds are significant plant development regulators and intervene reactions to both abiotic and biotic stresses. During the most recent ten years there have been many energizing breakthroughs in our comprehension of plant hormone biology, comprising new findings for the areas of hormone biosynthesis, transport, discernment, and reactions. Receptors for huge numbers of the significant hormones have now been distinguished, giving new chances to examine the chemical explicitness of signaling of hormones. Unexpectedly significant role for the ubiquitin-proteasome pathway in hormone signaling are likewise uncovered through these investigations. Also, recent work affirms that hormone signaling collaborates at numerous levels during plant development and improvement. Later, a significant challenge will be to comprehend how the data emanated on by these simple compounds is coordinated during plant growth and development (Davies, 2012, 2013). Initially found during the 1950s via Carlos Miller, the cytokinins are N6-subbed adenine-based molecules that are dependent on their capacity to advance plant cell division⁴⁴. Since they have been embroiled in a wide scope of developmental procedures comprising germination, root and shoot meristem capacity and leaf senescence. Moreover, cytokinin has a significant job in the arrangement of nitrogen-fixing nodules and other plant-microbe associations. Cytokinin oxidase (CKOx) is the main known plant catalyst efficient for degenerating naturally prevailing cytokinins. It was found first in an unrefined extract of refined tobacco tissue and has since been exhibited in a few higher and lower plants (Amasino, 2005). In Plant tissue culture, cytokinin-active urea derivatives are used to inhibit the activity of cytokinin oxidase reaction. The compounds play vital role in cell

regeneration as they inhibit cytokinin oxidation and allow plants to regenerate its shoots, roots and develop into a complete plant (Hare and Staden, 1994).

Tissue culture of spinach is not well established till date. A very limited work had been done until now. The other problem is tissue culture varies a lot from genotype to genotype and for each plant specie, you must develop a specific tissue culture protocol. A little mistake in the procedure can make a big difference in growth culture. No doubt, spinach is an important horticulture growth and a lot of studies have been done on it. There is not a lot of studies available on tissue culture of spinach. To establish well developed tissue culture protocol of spinach, a lot of research need to be conducted. Only a limited work is done by Al-Khayri in 1992 (Al-Khayri et al., 1992).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Experiment year and place

The experiment place for this interesting research project was Laboratory of in-vitro plant biotechnology at Bioscience engineering department of Ghent University (HOGhent), Ghent, Belgium. The project for spinach is a part of PhD research conducted in Laboratory of in-vitro plant biotechnology in collaboration with Vlaams Instituut voor Biotechnologie (VIB) at Ghent, Belgium. The project for cytokinin oxidase inhibitors was a collaborated project with Laboratory of in-vitro plant biotechnology at Bioscience engineering department of Ghent University and the Institute of Experimental Botany in Czech Republic. The project was started on February 02, 2020 and it was finished on July 31, 2020.

3.1.2 Environment of the place of experiment

The whole project was conducted in the control environment of Laboratory of in-vitro plant biotechnology. Each growth chamber was specialized for different kind of experiments. Light, temperature and humidity were controlled in each chamber according to the type of the experiment performed. The lab was well equipped to perform tissue culture experiments. Laminar flow, autoclave, glassware and plastic ware, chemicals for the media preparation, growth chamber, physical balance, pH meter, Umonium (and/or alcohol), microscope were available for the establishment of tissue culture of spinach and application of new generation of in silico designed cytokinin oxidase inhibitors on plant regeneration of economical important crops. Most of the molecular tools such as PCR and gel electrophoresis were also available in the laboratory. Some of the molecular experiments were supposed to be done with help of VIB laboratory but it was closed due *Corona Virus Lockdown*. Therefore, some of the tools for molecular analysis were ordered to work in the Laboratory of in-vitro plant biotechnology such as nano drop and gene cloning kit.

3.1.3 Plant material used

For the experiment of spinach, spinach seeds used were F1 hybrids as shown in the figure 3.1 After germination of these seeds in test tubes, same plants were used for regeneration purpose. For application of new generation of in silico designed cytokinin oxidase inhibitors, 14 economical important plant species were taken from a PhD student working on these plant species in the laboratory. These plant species include *Spinacia oleracea*, *Tabebuia guayacan*, *Petunia hybrid*, *Ocimum basilicum*, *Dalbergia retusa*, *Coleus esculentum*, *Paulownia fortunei*, *Nicotiana tabacum*, *Tectona grandis*, *Drosera rotundifolia* and *Lobelia erinus*. All these plant species were in sterilized culture media. Only *Swietenia macrophylla* and *Swietenia mahogany* were taken from plants grown in soil.



Figure 3.1. Spinach seeds used in the experiment.

3.1.4 Chemicals, medias, and tools

For sterilization of seeds and plant material, 10% bleach and tween 20 available in the lab was used. MS and MS mode3B media were used for regeneration of the plants. Cytokinin oxidase inhibitors used for regeneration experiment came from Institute of Experimental Botany in Czech Republic. All necessary chemicals, hormones and tools for tissue culture and CRISPR/Cas protocol were arranged in the laboratory.

3.2 Methods

The whole project was divided into different tasks. Tissue culture and molecular part was done side by side to get good results on time.

3.2.1 Tissue culture establishment

For successful establishment of tissue culture of spinach both seed germination and shoot Regeneration were performed in *in-vitro* laboratory.

3.2.1.1 Initiation of spinach by seeds

Different experiments were performed for in-vitro seed germination of spinach. MS media was prepared. For its preparation, water was taken in a container with a magnetic stirrer in it. MS media and sucrose were added and dissolved. The pH was raised to 5.8 by adding KOH (1M) drop by drop. After Adding these chemicals and adjusting the pH, the media was transferred to a stove and heated. While heating the media, plant agar was added, and the media was heated until it became clear (indication that plant agar was dissolved properly). 20 racks of test tube with 24 test tube in each rack were filled with 10ml of media (figure 3.2). Test tubes were autoclaved for 15 minutes at 120°C. The test tubes were kept aside to cool down till the media became solid. Seeds were sterilized by using 10% bleach and different methods were being followed such as removal of seed coat, 0,5 % Chlorine for 15 min, 5 % Chlorine for 30 min and 24 hours of soaking in water. 100 seeds were sown for each treatment to grow them in test tubes with MS media under laminar flow. Data in percentage was collected after each 5 days until 1 month.

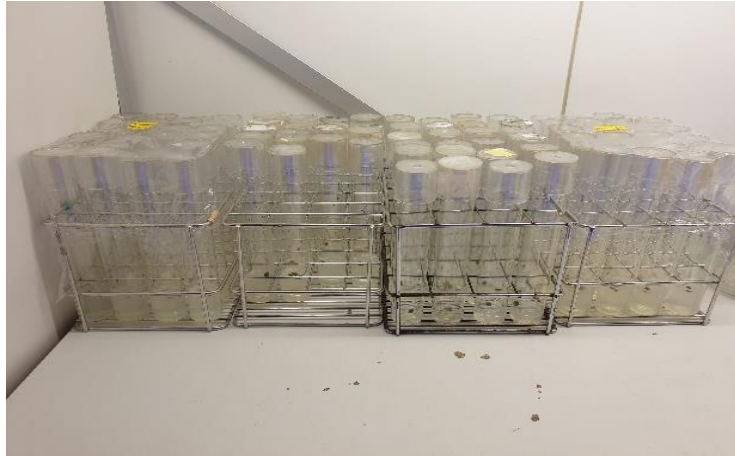


Figure 3.2. In vitro seed germination of spinach.

3.2.1.2 Regeneration of spinach

As in vitro seed germination of spinach was very slow, regeneration was done almost after 1 month of the germination experiment. The germinated plants were taken from the test tubes and were regenerated in two different medias described in the tables below (table 3.1 and table 3.2). These medias were prepared by following same procedure as described in section 3.2.1.1. Well developed plants were selected and shoots with two nodes were cut from these plants under laminar flow. 5 shoots were taken and were placed in equal distance in each jar containing these medias. 20 jars were prepared for each type of media. This experiment was conducted in far red and blue light chamber under controlled conditions (figure 3.3.). Data was collected after each 10 days until 40 days.



Figure 3.3. Regeneration experiment for spinach

Table 3.1. Media 1 for shoot regeneration

Chemical	Amount/ liter	Unit
MS including Vitamins	4.405	g
Sucrose	20	g
Agar	6	g
pH	5.8	

Table 3.2. Media 2 for shoot Regeneration

Chemical	Amount/ Liter	Unit
MS mod 3B (Vitamins)	2.63	g
Sucrose	30	g
Agar	6	g
pH	5.8	

3.2.1.3 Leaf regeneration of spinach

Leaf regeneration of spinach was done to check its progress rate before trying CRISPR/Cas gene knockout protocol. Simple MS media was prepared and used for this experiment as well. Petri dishes with 10ml of medias was being prepared for the experiment. These hormones (table 3.3) were used. Three repetitions were used for treatment. Petri dishes were places in far red and blue light (figure 3.4). Data was collected after 2 months of the experiment to observe callus using electron microscope available in the laboratory. This experiment was repeated twice in the laboratory because of the contamination in the first experiment.

Table 3.3. Hormones used for leaf regeneration of spinach

Hormones	Concentration (μM)	Amount added per 10ml (μl)
BA	10	22.5
TDZ	2	4.4
2iP	10	26
Z	10	22
control	0	0

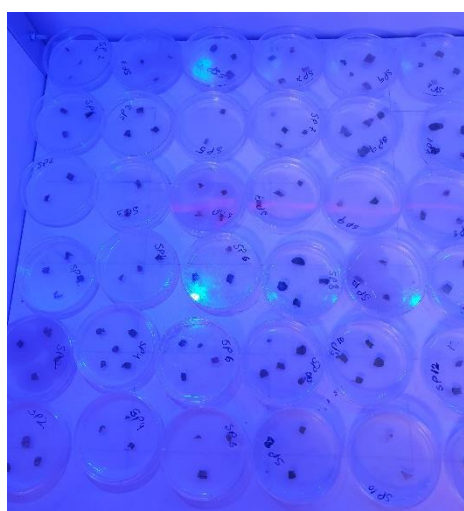


Figure 3.4. Leaf Regeneration experiment of spinach

3.2.2 Application of new generation of in silico designed cytokinin oxidase inhibitors on plant regeneration

New generation of in silico designed cytokinin oxidase inhibitors (CPPU, JN1, JN4) were applied on economic relevant crops to develop efficient shoot regeneration systems. These 14 different plant species were selected from in vitro lab (table 3.4). Experimental code was used to label each plant specie. There effect was checked on each plant and then were also compared with spinach. 0.5 cm² of leaf explants were cut and transferred to shoot induction medium (figure 3.5).The media used was MS mod3.B (2.63g/L) with sucrose (30g/L) and agar (7 g/L). The pH was maintained at 5.8 before autoclaving. These cytokinin oxidase inhibitors were added according to the concentrations protocol given in the table 3.5 and these compounds were combined with hormones BA and 2iP as both them are already tested hormones u for regeneration. Summary of the treatments is given in table 3.6. The experiments were done in different culture rooms according to the plant specie. Explants in three replicates per plant specie for each treatment were conducted. Data was evaluated in different time schedules depending on the growth of each plant specie to observe the callus formation and number of shoots.

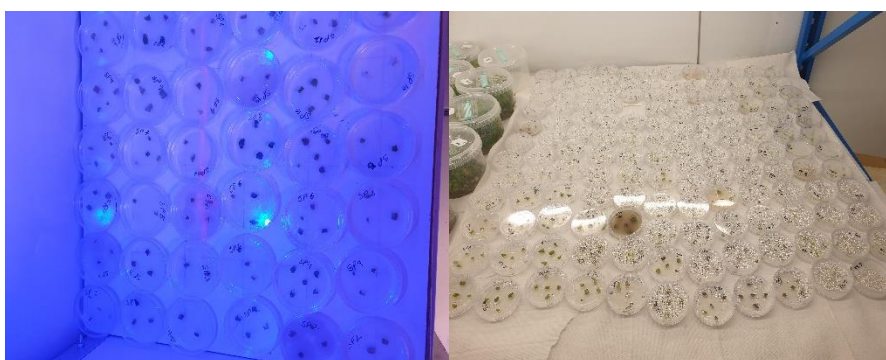


Figure 3.5. Experiment for cytokinin oxidase inhibitors

Table 3.4. Plant list used for cytokinin oxidase inhibitors

Code	Scientific Name	Family
Sp	<i>Spinacia oleracea</i>	Chenopodiaceae
Gu	<i>Tabebuia guayacan</i>	Bignoniaceae
Pe	<i>Petunia hybrid</i>	Solanaceae
Ba	<i>Ocimum basilicum</i>	Lamiaceae
Da	<i>Dalbergia retusa</i> ,	Dalbergia
Co	<i>Coleus esculentum</i>	Lamiaceae
Pou	<i>Paulownia fortunei</i>	Paulowniaceae
Tab	<i>Nicotiana tabacum</i>	Solanaceae
Te	<i>Tectona grandis</i>	Lamiaceae
Dr	<i>Drosera rotundifolia</i>	Droseraceae
L	<i>Lobelia erinus</i>	Campanulaceae
Ro	<i>Tabebuia rosea</i>	Bignoniaceae
swt mph	<i>Swietenia macrophylla</i>	Meliaceae
mgh	<i>Swietenia mahogany</i>	Meliaceae

Table 3.5. Treatment Design for Application of New generation of in silico designed cytokinin oxidase

					conc	0	0.5	0.5	0.5
					name	control	CPPU	JN1	JN4
					MM	0	248	419	354
					stock conc	1	1	1	1
					to add (μl)	0.0	1.2	2.1	1.8
conc (μM)	CK	stock conc	MM	to add					
0	Control	1	0	0.0		1	2	3	4
5	2iP	1	220	11.0		5	6	7	8
5	BA	1	226	11.3		9	10	11	12

Table 3.6. Summary for treatments used for cytokinin oxidase inhibitors

Treatment	Combination
T1	CONTROL
T2	CPPU
T3	JN1
T4	JN4
T5	2iP
T6	2iP + CPPU
T7	2iP + JN1
T8	2iP +JN4
T9	BA
T10	BA + CPPU
T11	BA + JN1
T12	BA +JN4

3.2.3 Leaf infiltration in spinach for gfp expression

To check the efficiency of spinach plant that how well it responds to agrobacterium transformation and at which rate it takes up foreign DNA, leaf infiltration method was used. The agrobacterium containing GFP gene was grown overnight in YEB media. The composition of the YEB media is given in table 3.7. YEB media was prepared and autoclaved. 25ml of YEB media was taken in two falcon tubes. Under sterile condition of vertical laminar flow, a small quantity of already growing GFP containing agrobacterium was added to one of the falcon tubes. The other tube was used as control. The falcon tubes were then closed remaining little open for the airflow and were placed at shaker at 200rpm at 28°C for over night.

Table 3.7. Composition of YEB media

Chemical	Amount per 1 liter of distilled water
Yeast extract	1g
Beef extract	5g
Peptone	5g
Sucrose	5g
MgSO ₄ .7H ₂ O	500mg
pH	7.2

Next day, the optical density (OD) of the bacteria was calculated. The bacteria could not be injected into the leaves while being in YEB media because the plant reacts to the media. So, a new media “MES buffer” was prepared. The concentration of MES buffer was 10mM MES and 10mM MgCl₂ maintained at a pH of 5.6-5.7. The MES buffer could be stored at 4°C. From the OD of bacteria in YEB media, the falcon tube was centrifuged, and all the supernatant YEB media was discarded. According to the OD, enough amount of MES buffer was added that the OD of this new solution becomes 1. To make the bacteria virulent, 150µM acetosyringone was added to the MES bacterial solution at the concentration of 20mg/l. The bacteria were then incubated at room temperature for 3 hours (Wydro et al., 2006). A sterile syringe was filled with 5ml this virulent bacterial MES solution and several leaves of spinach were injected very carefully. After two days of the injection, the leaves were examined under the UV fluorescence microscope from Leica company model MZFLIII to observe the expression of the GFP. Progress C10 plus cameras was used to capture the pictures.

3.2.4 Cris.pr/cas protocol

If the problem of bolting in Spinach can be resolved, it will be a great advantage for the growers. To achieve this goal, the genes which are involved in controlling the flower production in Spinach were studied in literature. The identified genes were then amplified in the Spinach and results were analyzed. Following is the detailed overview of the workflow.

3.2.4.1. Flowering gene identification

A detailed literature study was carried out to find out any citation, experiment or closely related information that provides some insight on the flower controlling genes in spinach. No literature was found which discussed about the flower controlling genes in spinach or its closely related genes such as Sugar beet, Chard or Quinoa. So, flower controlling genes in general were studied. As Arabidopsis is the model plant, flower controlling genes in Arabidopsis were studied in detail. FT genes in Arabidopsis and many other plant species were found to be involved in controlling flowering. These genes showed varying

expressions, as in some plants these genes favored flowering while in some plants these genes restricted flowering. Based on these evidences, five FT genes were selected, and the sequence homology was checked in Spinach by [NCBI BLAST tool](#). The reason of selecting five genes was to target all the genes, as it was not sure if all or none of these genes were present in Spinach under experiment in the lab. Table 3.8 shows the homology and web-links of the FT genes in Spinach by NCBI BLAST Tool. FT-PEBP gene from Arabidopsis (NCBI ref. [NM_118156.2](#)) and FTIP gene of Arabidopsis (NCBI ref. [NM_120768.3](#)) was run in BLAST tool to check the homology in Spinach. From the BLAST results of FT-PEBP, four FT-like genes, FtIG1, FtIG2, FtIG3 and FtIG4 were selected based on the sequence homology. One gene FTIP was selected from the BLAST results of FTIP of Arabidopsis. It was hypothesized that these genes might also have a role in the flower production in Spinach and by knocking-out these genes, a difference in flower production will be observed.

Table 3.8. List of targeted flowering genes and their sequence homology in Spinach.

Gene name	NCBI ref. in Arabidopsis	NCBI ref. in Spinach	Sequence homology	E-value
FtIG1	NM_118156.2	XM_021997171.1	62%	7e-79
FtIG2	NM_118156.2	XM_021987745.1	62%	1e-76
FtIG3	NM_118156.2	XM_021987744.1	64%	2e-79
FtIG4	NM_118156.2	XM_021987747.1	62%	2e-67
FTIP	NM_120768.3	XM_021987254.1	70%	0.0

3.2.4.2. Primer designing for the flowering genes

Primer pairs were designed to check the presence of the FT genes in Spinach under experiment in the lab. In the **1st set of primer pairs**, primers were designed for the mRNA sequences of the FTIG1, FTIG2, FTIG3 and FTIG4 genes by using [NCBI Primer Tool](#). Table 3.9 shows the details of the primers for the FTIG1, FTIG2, FTIG3 and FTIG4 genes. Several factors were considered while designing the primers such as:

- The primer length is between 18-25bp
- The primer does not form hair-pin structure
- The primer does not have off-targets
- The primers do not make primer-dimers
- The primer pairs have annealing temperature close to each other
- The G-C content of the primers is between 40-80%
- The primers do not have repetition of more than 4 single nucleotide bases
i.e. AAAAA or CCCCC
- The primers amplify a good size of the target gene

Table 3.9. Details of 1st set of primer pairs

Gene name	Sequence (5'→3')	Template strand	Length	Tm	GC%	Self-complementarity	Self-3' complementarity	Product length
FtIG1	GCATCAACAAGTGCGCCAA	Plus	19	60.01	52.63	4	0	576
	GCGAAATTAAGGAGCTGCCA	Minus	20	58.91	50	4	3	
FtIG2	ACACCTGGCTTAGTTTTCCA	Plus	21	57.69	42.86	3	0	1588
	ACTTTTATTCAAGACCCTCTGAGAT	Minus	25	57.74	36	5	3	
FtIG3	GTCGGGTTGTCGGTGATGTT	Plus	20	60.6	55	2	0	784
	AGGCCAATACCTCATCGGTAGA	Minus	22	60.43	50	4	2	
FtIG4	CATTGGGTATCTTCTCTCTACAAC	Plus	25	57.22	40	3	0	1131
	ATTGGGCCGGGCTGATATG	Minus	19	59.92	57.89	5	2	

Key: Forward primer; Reverse primer

FltG1

GTCAAAAAATATGCTAGGATA**GCATCAACAAGTGGCCAA**AGAGATCCATTAGAAATAGGTAGAGTTATCGGAGATGTTTGGAT
CCCTTCAATAGGCTCTGTAGCTCTTAGAATCAGCTATAACAATAGAGTGGTTACTACTGGAGGAGAATTTAGGCCATCTGAAGTTGTT
AGCCAACTAGAGTCGAGATTGGAGGTGATGACCTTAGAAGTTCTACACCTTGGTAATGGTGGATCCCGATGCTCTAGCCCAAGT
AACCCTGACCTGAGGGAGTACTTGCCTGGCTGGTACTGATATTTCTGGGACCACTGGTGCATCCTTGGTCAAGAGGTGGTTTGC
TATGAAAGTCCACGACCACCAATGGGGATACACCGATTGTATTTGTGTTTCAACAATTAGGAAGGCAAACTGTTTATCCACCA
GGATGGCTCCAACTTCATAACTAGAGACTTTGCTGAACCTTATAATCTTGGACTACCAGTTGCTGCTCTATTTCATTTGCAAAA
GGGAAGGAGTTGTGGTGAAGGAGGTGTAATTCGATCTCCATCTTATGTTTCT**GGCAGCTCTAATTCGG**ACTTAATTAAG
ATATTCATAATTTAGTACACCTT

FltG2

TTAAGCCAAATTTTCATCTTCTATGTTTTTGTCTACCGCTTCTACTTTATTCTATTTTGGCATTCATTTACTATCTTACCCTTAT
TCACATAATAATTACTCTCCCTGTTATTACCCATTAAATTT**ACACCTGGCTTAGTTTTCCA**TCATTTCTACACTTACCCTTCTTAAA
ACAAGAGTCTACAATAAATGGGTATTTAAAAAAGGAGGGAGTTTTGTATAAGAATGCAATTCATCTCAAAACACCTTAAA
TATTCCCCACATGCCCCACCCCTGCCCACACCCCATCCCCACCTCTGCCCGCGCAGAAAAAGAGAAGAATAAGCCAAAAACGATA
GTGAGGCATTAAAGCTTCATGTTCTTGTAAAAACATTTAGCTTTCACTTACAACCTCTCTACATCGTCCAATGTTCTAACTTCTTCAA
GAATCTAACCCATTATAGTTATGAAAGTAAGTGGACCTCTAGTCGTTTTAATCACAGAAAAAGATTTGGTAGGCCAAAAATTGGA
AAATGAGATAAAACAAAAACATAGAAATAGAGAAATATAGTTCTACACCGTACTAACGGGGTAGCAACTGATGCAAGATGAGA
TGCGTTGAAAAGATGATGGATACCTTCACTTACCTTAGGTTGATGAGTTACTATATAAGTCCACATTGGGTACCTTCTCTCCACAACCT
TAGTAACCTCTCTTTATTTATCCAAATAGTAAGTTGAGGAAAAAGAAACATTTTTTACCATTCTCTATAAATGAGGAGAGCAA
CAAGAGATCCCTCTCGTGGTGGTGGGTGAGTTTGGATCCCTCAATAGGAGTGTAATCTGAGAGTAAGATTACCA
ATAGAGATGTTTTAATGGATGTGAGTTTAGGCCATCTGAGGTTGTTAATCAGCAAGAGTTGAGGTTGGAGGTGATGACCTTAGGA
CTTTCTACACCTGGGTGATGGTGGACCTTATGGTCAAGAGGTGGTTTGTCTATGAGAACCCAGACCCCTCAGTTGGAATTCTCGTTTCA
ATATTCCCGGACACAGGAACATCTTATGGTCAAGAGGTGGTTTGTCTATGAGAACCCAGACCCCTCAGTTGGAATTCTCGTTTCA
TATTGTGTTGTTTCGACAAATGGGAAGGCAAAACCTTTACGACCCCGGTGGCGCCAAACCTTCAATACTAGAGACTTTGCTGAAG
TGTACAACCTCGGTTTACCGTTGCTTCACTTATTCAATTGCCAAAGGAGGAGGTTCCGGTGGGAAGAGTTCGTAAGTGGC
ACACTTTTTTACTCTACCGTCTAAGAGACCGGTGTCAAAACATCTACCGCGCTTTTAACTTCTGTTTAAATAGTAATAATAAAGTTG
TCATTATTAATAAAGGAATCGATGCTCTTCAAAACAAAGTACCTTATAAATTTGTCACAAAAACCTGCTTTTAGCCATTTTGTGA
GAGTAATGCAATATTGTAATGATCCACATAGCAGTGAATTTAGAAATCAGAGTGAATTAATCTGACATTTTATAGGGAATTAAC
TGTTTTGTTACTCTAATAATAAATGTAACATTAATTTACCTAAGTTATATTATAAGGCAATTATAAAGCTTATAAATCTTAAT
ACTTTGATAGTTTTTCTTGTAAATGTACA**ATCTCAGAGGGTCTTGAATAAAGT**TAAGCTTTTTTGTTTTCA

FltG3

CTCTACAACCTCTTAGTAACCTCATCTTCTATTTTCTACATAGCAGCTTATAGAAAAATTATAGCACGCTTAGAAAAAAGAAAAACA
ACTTCTTAACCATCTCTATCATGTCTAGAACACATAGAGATGCACTTGTGCTCG**GTGGGTCTGCGGTGATGT**TTGGATCCCTTCA
ATAAGAGTGTAAATATGGGTGTAGATACGCCAATAGAGATGTTAGTAATGGATGTGAATTTAGGCCATCTGAGGTTGTTAGCCAAC
CAAGAGTAGAGATTGGAGGTGACGACCTTGAACCTTCTATACCTTGGTGATGGTGGACCCCGATGCTCCTAGCCCAAGTAACCCAC
ACTTGAGGGAGTACTTGCATTTGGTGGTGAATGATATTCGGGGACACAGATGTTTCTATGTCGAAGAGTTGGTTGCTATGAGA
GCCCAAGGCCAACAGCAGGGATACATCGAATTTGATTCTGCTATATCGACAACCTTGAAGACAAACCGTTTATCTCTCGGTTGGC
GCCAAACCTCAAGACTAGAGACTTGTCTGAAATATACAACCTCGGCTTACCGTTGCTGCGGTTTATTTCAACTGCCAGAGGGAGG
GAGGCTCTGGTGGAAGAAGACTGTGAATCTGAAATTTAGGTGCACTCGAATACGCCGTGCACTATGAGTATTTTAACTC
ATTGGTGATCCCTATACATTTTCTCTCGCTTATAAAGGGAATGTGAGAAGGTGCACGATGCACCTACTATGTTTGGTGAAATC
CTCGACATTTCTTACTCTACCGTAAAGAGCGCTACCCAAACATCTATATATGTACCACACTTTTAAATGCTTGGCTAATACTCG
CAGTAATCAACACTTCTAAAAATAAATACAT**CTACCGATGAGGTATTGGCT**TA

FltG4

AGGACTTAGTATATAAGTGTA**CATTGGGTATCTTCTCTACAA**CTTCTTAGTAACACGCTTTTATTTTCTAAATAGCCGTATC
TTAAAAAATAAATATTTTCTTAACCCATTCCCTATCAATGCGTAGAGCAACAAGAGATCTCTCGTGGTGGTGGGTTGTCGGT
GAAGTTTTGGACCCCTTCAATAGGAGTGTAAATCTGAGAGTAAGATTTACCAATAGAGATGTTTTAATGGATGTGAATTCAGGCCA
TCTGAGGTTGTTAATCAACCAAGAGTGAATTTGGAGGTGACGACCTTAGGACTTTCTACACCTTGGTGATGGTGGACCCCTGATGCT
CCTAGCCCAAGTAATCCACATCTGAGGGAGTACTTGCCTGTTGGTACTGATATTCGGGGACAAACAGGTGTATCATACGGTCAA
GAGGTGGTTTGTCTATGAGAACCCAGACCTTCAAGTTGGAATTCATCGTTTCAATTTTGTGTTTTCGACAAATGGGAAGGCAAAACC
GTTTACGCTCCCGGTGGCGCCAGAACTTCAACACTCGAGACTTTGCCGAAGTGTACAACCTCGGTTTACCGGTGCTTGGTCTATT
TCAATTGCCAAAGGAGGGAGGTTTCGGGTGGAAGAAGGTGCTAAACAGTGCACACTTTTCACTCTACCGTCTAAGAACCGGTG
CTAAACATCTGCTGCGCTTTTAAACATCTGTCTAATAGTAATAATAAAAAATTTGTCATTATTAATAAGGAATGTAGCTCTTCTAAA
ACAAGTACCTTATAAATTTGCTACTAAAACTGCTCTTTAGACATTTTGTAGAGTAATAACAATAAGTAATGATCCACATAGCA
GTAATTTGACTATTGAGAATCAGAGTAGTAATTAAGGCAATTTTATAGGGAATTTACTGTTTGTACTCTAATAAATGTAATAA
TTATTTACCAAGTATATTATAAGGCACCTATAATAGTCTTAATCTTTTATGAGTTTATTTTATTTTGAATGTACAATCTCA
GAGAGTCAGAGGGGCTTGAATAAAAAGTTGAGCTTTTGTGTTTGTATGAATGTTGCTTCTGTTAAATGTTATGGTT**CATATCA**
GCCCGGCCCAATAGTATTTGGGCCACAGGTATTTAAATTTGCAAGGTT

Figure 3.6 Visual representation of the 1st pair of primers on the sequences downloaded from NCBI

Figure 3.6. Shows the visual representation of the primer pairs on their respective mRNA sequences.

Above primers were checked on the spinach plant under experiment in the lab. In the **2nd set of primer pairs** the complete sequences of all the genes including FTIP were acquired from the Spinach genome. The homology of all the above given mRNA sequences which were acquired from NCBI was checked by BLAST tool available at Spinach BLAST. The complete genomic sequences (including introns and exons) of all the

mRNA sequences were found in Spinach's genome. All the sequences showed homology on chromosome 4 of Spinach. These sequences were downloaded, and new primers were designed from NCBI Primer Tool to amplify them. The sequences of each gene were downloaded from [FtIG1 Jbrowse](#), [FtIG2 Jbrowse](#), [FtIG3 Jbrowse](#), [FtIG4 Jbrowse](#) and [FTIP Jbrowse](#). Table 3.10 shows the details of the 2nd set of primer pairs primers. All the primers were designed very carefully. Multiple primers were designed for a FtG1 and FtG3 genes so that in case there is any change in the sequence such as single nucleotide polymorphism (SNP) at one target site of the primer, the other primer can still be used to amplify the gene. FTIP gene was had a long sequence and very short exons, so four primer pairs were designed to amplify this gene from different regions.

Table 3.10 Details of 2nd set of primer pairs.

Gene name	Template strand	Sequence (5'→3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity	Product length
FtIG1.1	Plus	ATACGGAGTTCATGCGGAGTT	21	59.52	47.62	4	0	1294bp
	Minus	GGTCTACTCACCAGTGCAAG	20	57.91	55	4	0	
FtIG1.2	Plus	TGCAGTAGGCAACAATGGAGT	21	59.93	47.62	4	1	1228bp
	Minus	TGAGTGAGAACTGAGCATGAC	21	57.69	47.62	4	2	
FtIG2	Plus	GGTGAACATAAACATAACCACG GG	24	60.14	45.83	3	2	1359bp
	Minus	CTGACTTAGCATCTATCTGCCCT	23	59.42	47.83	4	0	
FtIG3.1	Plus	TGGGCCTTAATGGATGGCTG	20	60.11	55	4	1	1391bp
	Minus	GACGGTGGTTGGTACTTGGT	20	59.89	55	4	1	
FtIG3.2	Plus	CCTTGAGGTGTGAGCCTTGG	20	60.61	60	4	2	1156bp
	Minus	AAGTGTGGGTTACTTGGGCT	20	59.15	50	4	0	
FtIG4	Plus	CACCACTCCTCGAAACAAGA	20	57.47	50	4	0	1013bp
	Minus	GGGTCCACCATCACCTACAAT	21	59.44	52.38	4	2	
FTIP1	Plus	TGTCAAGTTTGCTGGTGGCT	20	60.4	50	4	0	1380bp
	Minus	CTCCTCGGCACAAAAAGAAGC	21	60.07	52.38	3	2	
FTIP2	Plus	CGCAGCTCACAGTAACGACA	20	60.66	55	4	0	1286bp
	Minus	AACTTGGCTTGCCTCTTCGTA	21	59.93	47.62	4	2	
FTIP3	Plus	CGTGGCGCTTTAGCCATTAG	20	59.7	55	4	1	1687bp
	Minus	GCGAACAGGAGTGCAGATTC	20	59.28	55	4	3	
FTIP4	Plus	TACGAAGAGGCAAGCCAAGT	20	59.31	50	4	1	1545bp
	Minus	CACCCTCAGCAAGGAGTGAT	20	59.38	55	4	2	

Figure 3.7. Show the visual representation of each primer on its specific target sequence in each gene. The untranslated regions (UTRs) of each exon are also highlighted. All the primers were checked by PCR to see the amplification.

3.2.4.3 Pcr amplifications of the primer pairs

The primers of all the genes were subjected to polymerase chain reaction (PCR) to check whether the primer sequences were specific to the genomic sequences or not and if the amplifications were of expected size. All the primer were ordered from IDT DNA Technologies. The primers were received in lyophilized form and were diluted by adding nuclease-free sterile water 10X than the nmol amount written in each primer tube. This dilution was made in sterile environment and 100 μ M stock solution was prepared. Further dilution was made for working solution (1 μ M) of each primer by adding 99 μ l nuclease-free sterile water and 1 μ l of stock solution. The stock solution of primers was then stored at -20°C and the working solution pf primers was used for further experimentation. The standard PCR protocol was followed. The annealing temperatures for primer pairs were set according to the specific annealing temperature of each primer. PCR kit (dNTPs, Buffer, Taq polymerase) was used from Promega company.

PCR amplification of 1st set of primers of FtIG1, FtIG2, FtIG3 and FtIG4:

For the amplification of the primers of FtIG1, FtIG2, FtIG3 and FtIG4 genes, PCR master mix was prepared according to Table.3.11. To avoid any mistake or human error, two repetitions of each primer pair were placed for PCR. So, total 10 samples were prepared. PCR protocol was set to be 95°C for 1 minute, 30 cycles of denaturation at 95°C for 2 minutes, annealing at a gradient of 55°C - 62°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The annealing temperature was used in gradient because each primer pair had a different temperature so the PCR mix of each primer pair could be run in the same PCR run at once. The samples were prepared in PCR tubes. The PCR mix of FtIG1 was placed at 59.1°C, FtIG2 was placed at 57°C, FtIG3 was placed at 60.4°C and FtIG4 was placed at 58.1°C annealing temperature.

Table 3.11. PCR mix for the 1st set of primer pairs

Chemical name	1x PCR mix volume
Taq buffer (5x)	5 μ l
dNTPs (10mM)	0.5 μ l
Forward primer (1 μ M)	1 μ l
Reverse primer (1 μ M)	1 μ l
H ₂ O (nuclease-free)	16.25 μ l
Taq polymerase (5U/ μ l)	0.25 μ l
DNA (10ng/ μ l)	1 μ l
Total	25μl

A second PCR was run with the same primer pairs, but the PCR mix composition and the PCR protocol were further optimized. Table 3.12 shows the optimized composition of PCR mix. The primer concentration was raised from 1 μ M to 10 μ M. The PCR protocol was changed to 95°C for 2 minute, 30 cycles of denaturation at 95°C for 40s, annealing at a gradient of 55°C - 62°C for 40s, extension at 72°C for 1 minutes and a final extension at 72°C for 5 minutes. For this PCR, three repetitions for each primer pair were run and total 12 samples were prepared. The annealing temperature for each primer pair remained the same. This PCR composition and PCR protocol was repeated to get more PCR product to send for sequencing.

Table 3.12. PCR mix for the 2nd set of primer pairs

Chemical name	1x PCR mix volume
Taq buffer (5x)	5 μ l
dNTPs (10mM)	0.5 μ l
Forward primer (10 μ M)	0.5 μ l
Reverse primer (10 μ M)	0.5 μ l
H ₂ O (nuclease-free)	17.875 μ l
Taq polymerase (5U/ μ l)	0.125 μ l
DNA (10ng/ μ l)	0.5 μ l
Total	25μl

PCR amplification of 2nd set of primers of FtIG1, FtIG2, FtIG3, FtIG4 and FTIP

The new primers were also ordered from IDT technologies and were diluted the same way to a working concentration of 10 μ M. For this PCR amplifications, primer mix were also prepared to amplify a larger or reasonable part of the gene to later send for sequencing. Table 3.13 shows the details of original primer pairs, the primer mixes, and the product size of all the amplifications. All the possible primer mixtures were tried so that later, if a few mixtures show primer-dimer formation or non-specificity, other mixtures could be used for amplification and sequencing. For further experimentation, the primer no. given to each primer pair and primer mixtures will be used in this study.

Table 3.13. Mixture of forward and reverse primers of 2nd set of primer pairs

Primer no.	Forward Primer	Reverse Primer	Product length
1	FtIG1.1	FtIG1.1	1294 bp
2	FtIG1.2	FtIG1.2	1228 bp
3	FtIG1.2	FtIG1.1	1149 bp
5	FtIG2	FtIG2	1359 bp
6	FtIG3.1	FtIG3.1	1391 bp
7	FtIG3.2	FtIG3.2	1156 bp
8	FtIG3.1	FtIG3.2	1076 bp
9	FtIG3.2	FtIG3.1	1471 bp
11	FtIG4	FtIG4	1014 bp
12	FtIP1	FtIP1	1381 bp
13	FtIP2	FtIP2	1287 bp
14	FtIP3	FtIP3	1688 bp
15	FtIP4	FtIP4	1546 bp
16	FtIP3	FtIP2	644 bp
17	FtIP4	FtIP3	1065 bp
18	FtIP1	FtIP3	2389 bp
20	FtIP2	FtIP1	1329 bp

Two repetitions of each primer pair and primer mixture were used. Total 34 test samples were run along with 4 control tubes. Same PCR composition was used as in table 3.11. Based on the annealing temperature of all the primers, an average temperature was used for each repetition. Table 3.14 shows the arrangement of the PCR tubes in the PCR along with the respective temperature gradient. The letter “C” in the table represents the “control sample tube” while all the red colored numbers represent the samples of primer pairs and mixtures. On the left side of the table the gradient temperature is mentioned and on the top of the table the column number of the PCR machine is mentioned.

Table 3.14. Arrangement of the PCR tubes according to the respective temperature of each primer no.

12	11	10	9	8	7	6	5	4	3	2	1	
												62°C
												61.5°C
												60.6°C
14	13	12	11	9	8	7	6	5	3	2	1	59.3°C
15	16	17	18	20				C	C			57.7°C
14	13	12	11	9	8	7	6	5	3	2	1	56.4°C
15	16	17	18	20	C	C						55.5°C
												55°C

Based on the results of the above PCR, primer no. 1, 2, 3, 5, 6, 9, 11, 12, 13, 14, 15 and 20 were selected to be further used for amplifications and sequencing. Primer no. 1, 2 and 3 were for gene FtIG1 and were repeated to test the amplification. Primer no. 5 was for gene FtIG2, primer no. 6 and 9 were for gene FTIG3, primer no. 11 was for gene FtIG4, primer no. 12, 13, 14, 15 and 20 were for gene FTIP. These primer no. were repeated to get more PCR product to be sent for sequencing. For these PCR amplifications, same PCR composition was used as in table 3. 11 and same PCR protocol was used as previous one.

3.2.4.4 Gel electrophoresis

Gel electrophoresis was run to check the results of the PCR amplifications. Agarose gel was used, and TAE buffer was prepared according to the composition given in Table 3.15. 1.5-2% agarose gel was prepared in 1X TAE buffer. Different sizes of combs were used to make larger or smaller wells for loading DNA. Different sized electrophoresis machines were used depending upon the sizes of the amplified fragments. 6X blue/orange loading dye, 100bp ladder (conc. of 0.13µg/µl) and GelRed (staining agent) were used from Promega company.

Table 3.15. Composition of TAE buffer

Chemical name	Amount (for 10X conc.)
Tris (400mM)	24.2 g
Na ₂ EDTA.2H ₂ O (20mM)	3.7 g
NaAc.3H ₂ O (200mM)	13.6 g
Hac (296mM)	8.5 g
H ₂ O (distilled)	Raise the final volume to 500 ml
pH of the final solution	Approx. 8.2

In each 25µl of PCR sample, 3µl of loading dye and 1µl of GelRed was added. 5µl DNA ladder +1µl GelRed was run for each electrophoresis. Each electrophoresis was run for 20-60 minutes depending upon the size of the fragments. The voltage ranged from 135V to 250V depending upon the size of the container and the size of the fragment. The DNA bands were observed by placing the gel on UV transilluminator as GelRed gives fluorescence in UV light.

3.2.4.5 Dna Excision From Gel

There are two ways to excise the amplified DNA product from a PCR mix. The first way is to directly excise the DNA from PCR mix by using DNA excision. While second way is to run the PCR product on gel, cut the bands of the fragments and then excise the DNA by using DNA excision kit. In this study second method was used as it was more efficient. The Promega DNA excision kit was used. The extraction manual was carefully followed the same way except that, due to lyophilization problem (discussed in the next section), 35µl nuclease-free water was used for elution of DNA from SV minicolumn instead of 50µl.

After excision of DNA from the gel, a small quantity of the excised DNA was always run on the gel to confirm that the excision was successful and there was no contamination or DNA damage.

3.2.4.6 Sequencing of the amplified PCR fragments

The sequencing of the amplified regions of each gene was necessary to confirm the sequences of each gene that if the sequences in the plant under experiment were same or if there were deviations (SNPs). Sequencing services from BioSearch LGC Genomics were used. The company uses Next Generation Sequencing (NGS) method for the sequencing of the DNA, which is very robust and accurate. All the samples for the sequencing were prepared according to the instructions' manual of the company. Extra care was taken about the concentration of the DNA and the primer pairs. Several samples were sent to this company for sequencing.

Some problems were faced during the sample preparation of PCR products of 1st set of primer pairs which are discussed in the results section. From the 2nd set of primer pairs, the PCR products of sample no. 5, 6, 9, 11, 12, 13, 14 and 15 were sent for the sequencing. Each PCR product of primer no. 5, 6, 11, 12, 13, 14 and 15 was sent separately with its forward primer while the PCR product of primer no. 9 was sent with only forward primer.

3.2.4.7 Pcr cloning of the Pcr product

PCR cloning technique was used to separately grow heterozygous alleles in agrobacterium and to send the fragments separately for sequencing. CloneJET PCR Cloning Kit from ThermoFisher Scientific Company was used. All the necessary enzymes, buffers, control, primers for the cloning were provided in the kit. *Agrobacterium tumefaciens* was taken from the already growing culture in the lab and was used for the cloning. It was important to convert the Agrobacteria to competent cells. A protocol exhibited by (Debler, 2019) and (Wang, 2006) was followed to make the cells competent. Competent agrobacterium cells were then stored at -80°C.

PCR product of FTIP3 was used for this purpose. As described in the instructions' manual sticky-end cloning protocol was used for ligation and the ligation mixture was stored at -20°C. The control experiment of ligation and

transformation was also followed side by side. For the transformation of agrobacterium heat shock method was used. The stored agrobacteria were taken on 0°C from -80°C. In each tube of the competent bacterial mixture 10µl of the ligation mixtures were added was incubated at 0°C for 15-20 minutes. The tubes were then placed water bath for 60-90 seconds at exactly 42°C for an abrupt heat shock. The quickly 500µl SOB media (Sambrook and Russell, 2006) was added to each tube making the total volume 600µl. The tubes placed on the shaker at 37°C for almost 40-50 minutes. Petri plates of LB media (30ml in each plate) were prepared with antibiotic ampicillin at a concentration of 100µg/ml in it. Out of total volume of 600µl, 1X concentration (60µl) of the bacterial mixture was spread on one LB plate. Remaining mixture was centrifuged to 10X concentration and was spread on another petri plate. All the petri plates were placed in the incubator at 37°C for 16 hours.

For plasmid isolation of induvial alleles, 12 single colonies of bacteria were selected. These colonies of bacteria were carefully transferred on another LB plate with ampicillin in it. This plate was incubated for 16 hours at 37°C. After 16 hours, when the bacterial growth was visible in each colony, the plate was stored in the cold chamber to be used later for plasmid isolation and sequencing.

4. RESULTS

4.1 Seed Germination Under Different Treatments

The results for seed germination under different treatments after each 5 days of interval as described in table 4.1. It has been observed that highest percentage of germination rate was for the seeds that were soaked in water for 24 hours before the experiment which is 34% and the lowest observed percentage was in 5% chlorine for 30 minutes. But overall germination rate was lower than 50% even after one month as observed in the figure 4.1.

Table 4.1. Percentage of seed germination of spinach

	Control	Removing coat	0,5 % Chlorine for 15 min.	5 % Chlorine for 30 min.	24 hours water soaking
0-5 days	13%	15%	15%	11%	14%
5-10 days	25%	27%	29%	20%	30%
10-15 days	27%	29%	32%	21%	34%
15-30 days	27%	29%	32%	21%	34%

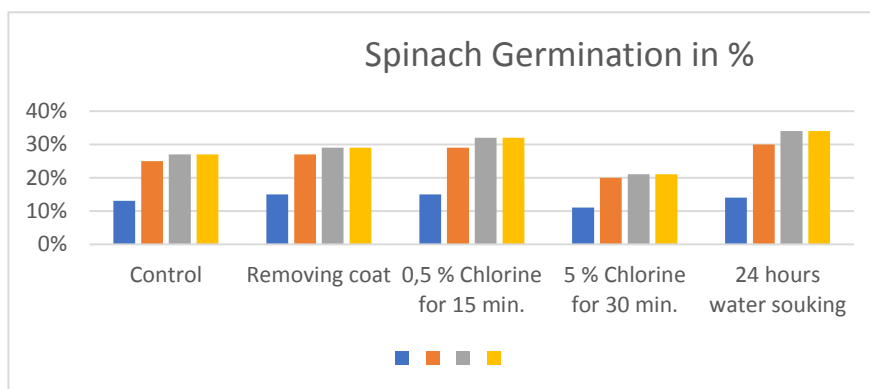


Figure 4.1. Spinach in vitro germination in percentage

4.2 Shoot Regeneration of Spinach

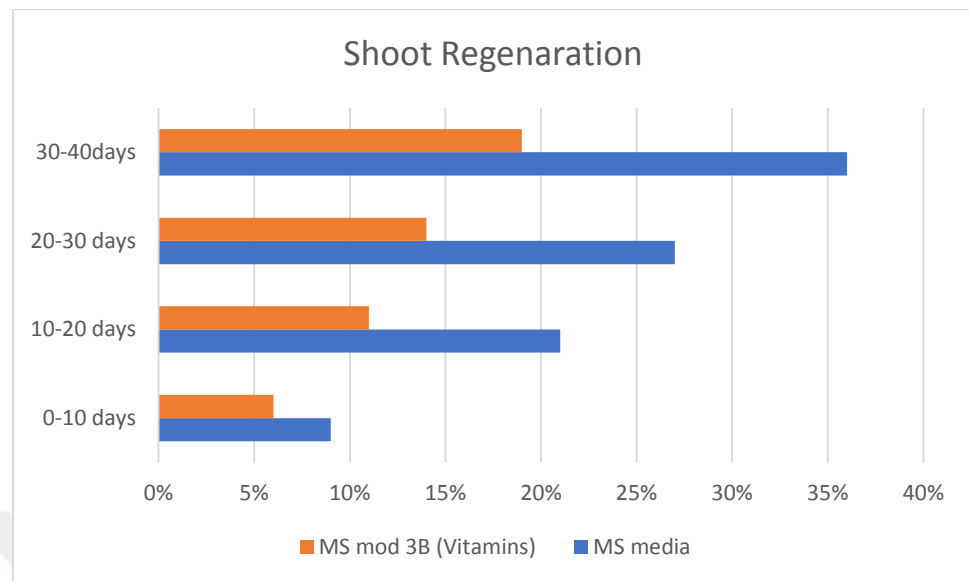


Figure 4.2. Spinach shoot regeneration in percentage

Shoot regeneration in both in simple MS and MS mod 3B (Vitamins) media showed that spinach regeneration was better in simple MS media than in MS mod 3B media (figure 4.2). After 10 days of the experiment there were almost 10% regenerated plants in MS media while it was only 6% in MS mod 3B media. After

40 days of the experiment, it was only around 20% of regenerative plants in MS mod 3B while it was around 40% in simple MS media. Spinach performed better in MS media, but it has been observed that overall experiment was slow, and the number of regenerative plants were less than 50% even after 40 days.

4.3 Effect Of Cytokinin Oxidase Inhibitors On Plant Regeneration

5 plants responded to the treatments, out of 14 plants used for this experiment. Other 9 plants did not show any shoots, they either formed callus or died after browning of the tissue. The results were collected for the 5 plants species. Mean number of shoots were calculated using SPSS statistical software and significant level was set as 0.05. Data was observed to compare the reaction of treatment in each plant and comparison was made between these 5 plant

species. Although our purpose was to compare the results specifically with spinach, but spinach did not respond to these compounds.

4.3.1 *Nicotiana tabacum*

Results in the table 4.2 show the highest number of mean in Treatment T6 and T11 which is around 30 and there is no significant difference in the mean number of shoots of both. Treatment T1, T3, shows 0 mean number of shoots and it can also be observed in figure 4.3 which shows that there is not callus formation in these treatments. In all other treatments, mean number of shoots is almost same as there is no significant difference in all of them except treatment T5 which is slightly less than others. These results give an idea that shoot regenerate well in combination of JN1 compound with 2iP and BA (T7 and T11).

Table 4.2. Comparison of mean number of shoots for tobacco

Treatments	Mean Shoot Number (0.05)
T1	0.000a
T2	22.4000c
T3	0.000a
T4	0.000a
T5	15.8000b
T6	32.8000d
T7	23.2667c
T8	22.1333c
T9	21.6000c
T10	21.6000c
T11	31.2667d
T12	21.8000c

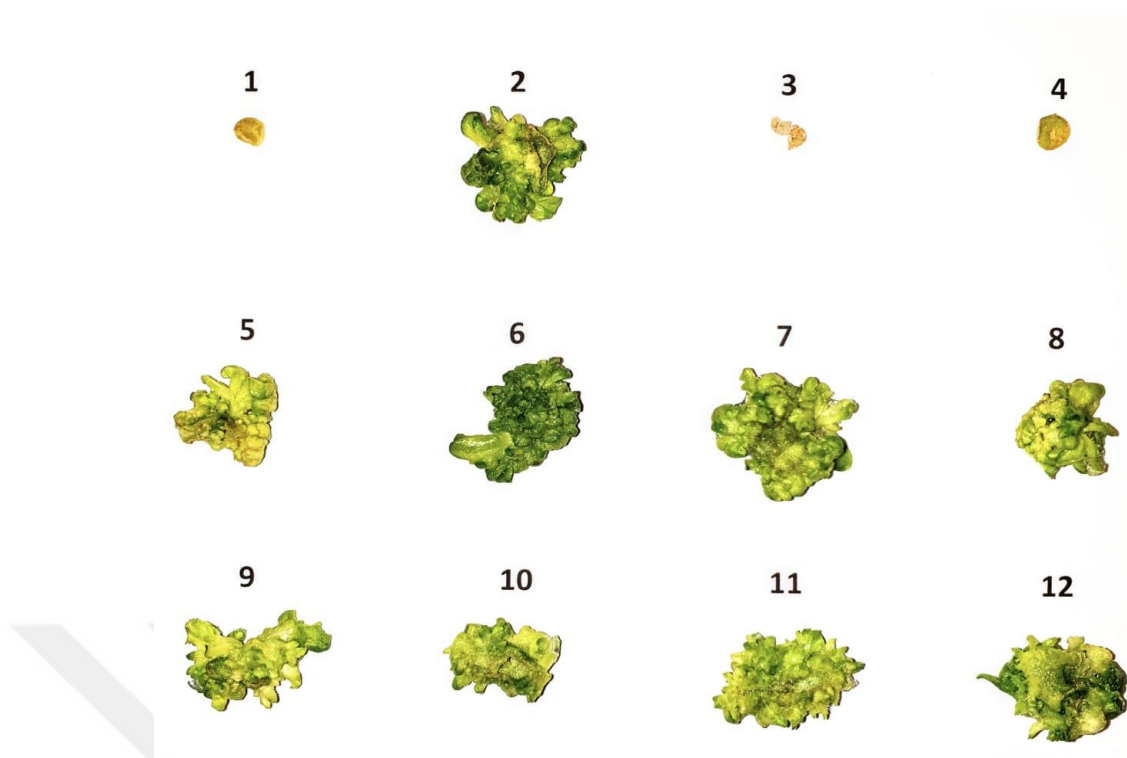


Figure 4.3. Visual comparison of 12 treatments in *Nicotiana tabacum*.

4.3.2 *Drosera rotundifolia*

In *Drosera*, results in table 4.3. show highest mean number of shoots in Treatment T2, T5 and T11 which is around 9 and there is slight difference in the mean number of shoots of these three treatments. Treatment T1, T7, T8, T10 shows 0 mean number of shoots and it can also be observed in figure 4.4. which shows that there is not even callus formation in these treatments. In all other treatments, mean number of shoots is almost same as there is no significant difference in all of them except treatment T2 which is slightly less than others. These results give an idea that shoot regenerate well in combination of JN1 compound with 2iP and BA (T5 and T11).

Table 4.3. Comparison of mean number of shoots for Drosera

Treatments	Mean shoot number (0.05)
T1	0.000a
T2	7.7333def
T3	4.2000bc
T4	2.667b
T5	9.8000f
T6	3.6667b
T7	0.000a
T8	0.000a
T9	6.6667cde
T10	0.000a
T11	8.5333ef
T12	5.1333bc

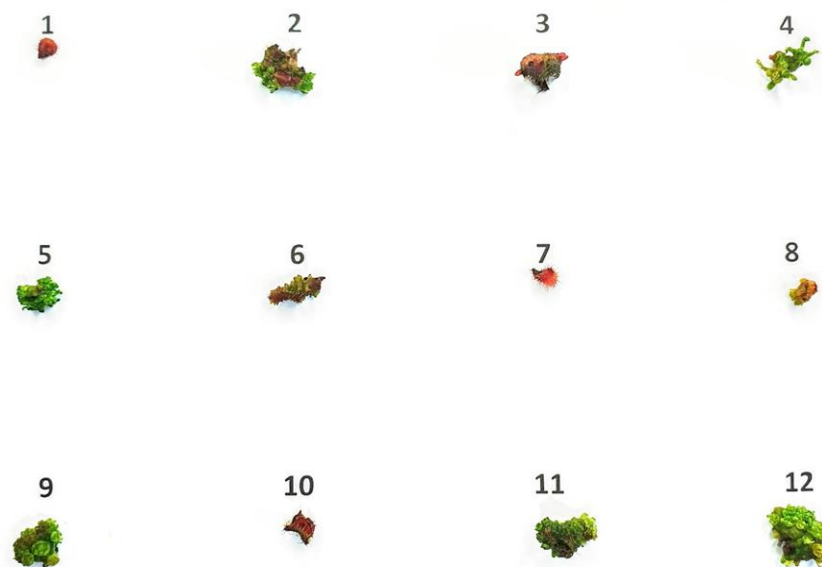


Figure 4.4. Visual comparison of 12 treatments in Drosera.

4.3.3 *Coleus esculentum*

Results in table 4.4 show highest number of mean in Treatment T6, T9 and T12 which is around 20 and there is no significant difference in the mean number of shoots of these treatments. Treatment T1, T5 and T11 shows 0 mean number of shoots and it can also be observed in figure 4.5 which shows that there is not callus formation in T1 and T11. There is callus formation in T5, but no shoots were observed. In other treatments T4, T7, T8, T10, there is no significant difference in mean number of shoots. These results give an idea that shoot regenerate well in combination of these compounds as compared to when applied alone.

Table 4.4. Comparison of mean number of shoots for *Coleus*

Treatments	Mean Number of shoots (0.05)
T1	0.000a
T2	8.1333bc
T3	2.0667ab
T4	15.8667de
T5	0.000a
T6	23.4667f
T7	15.3333cde
T8	9.8667cd
T9	17.0667def
T10	14.6000cde
T11	0.000a
T12	21.3478ef



Figure 4.5. Visual comparison of 12 treatments in Coleus.

4.3.4 *Lobelia erinus*

Results in table 4.5 show highest mean of shoot number in Treatment T8 which is 35.200 and there is significant difference in the mean number of shoots with other treatments. Most of the Treatments such as T1, T2, T3, T4, T9, T10, T11 and T12 shows 0 mean number of shoots and it can also be observed in figure 4.6 which shows that there is not callus formation in these treatments. In all other treatments, mean number of shoots is almost same which is around 20. These results give an idea that shoot regenerate in Lobelia well in combination of these compounds with 2iP and BA as compared to when applied alone.

Table 4.5. Comparison of mean number of shoots for Lobelia

Treatments	Mean Number of shoots
T1	0.000a
T2	0.000a
T3	0.000a
T4	0.000a
T5	22.2667bc
T6	27.3333c
T7	19.6000b
T8	34.2000e
T9	0.000a
T10	0.000a
T11	0.000a
T12	0.000a



Figure 4.6. Visual comparison of 12 treatments in Lobelia.

4.3.5 *Paulownia fortunei*

Results in table 4.6 show highest number of mean in Treatment T10 which is 21.6000 and Treatment T1, T3, T4, T5, T7, T9, T12 shows almost 0 mean number of shoots and it can also be observed in figure 4.6 which shows that there callus formation in treatments T5, T7, T9, T12 but no shoots were observed but in treatment T1, T3 and T, there is not even callus formation. There is no significant difference between treatment T8 and T11. Treatments T2, T6 and T12 are significantly different from each other.

Table 4.6. Comparison of mean number of shoots for Poulownia

Treatments	Mean number of shoots
T1	0.000a
T2	9.0000b
T3	0.000a
T4	0.000a
T5	0.000a
T6	16.2667d
T7	0.000a
T8	4.4000b
T9	0.000a
T10	21.6000e
T11	5.2667b
T12	1.0667a



Figure 4.7. Visual comparison of 12 treatments in Poulownia.

Overall, the results show that all these plant species react differently to these new in silico designed cytokinin oxidase inhibitors and there was significant difference among treatments, and it varies from plant to plant. Although cytokinin oxidase inhibitors play vital role in plant tissue culture but still some species did not react to these new compounds. Another observation was these new in silico designed oxidase performed well in combination with 2iP and BA. Very less or almost no shoots were observed when these compounds were applied alone except CPPU which is an artificial cytokinin.

4.4 Results of the GFP expression by leaf infiltration

After two days of leaf infiltration, the leaves were observed under UV light. Very little GFP expression was observed. However, this provided an evidence that spinach responds to the foreign DNA and the expression can be dependent on the virulence of the bacteria, the efficiency of the vector transfer, the sage of the plant while injecting. Figure 4.8 shows the side by side comparison of the injected leaf under normal light and under UV light and GFP expression can be observed.

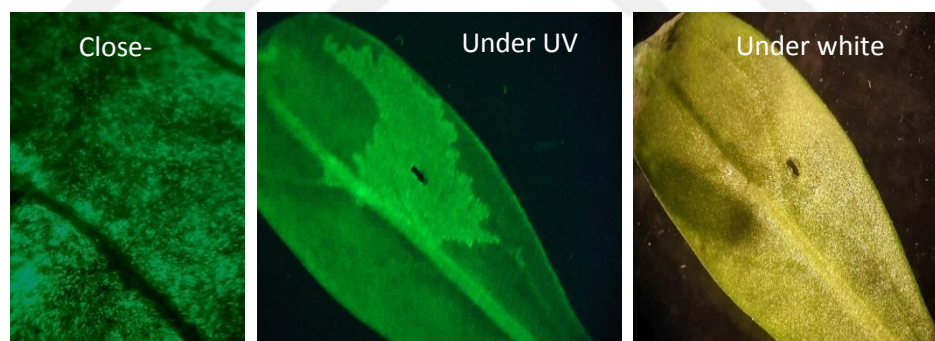


Figure 4.8. Results of the GFP expression in spinach leaf.

4.5 Results of the CRISPR-Cas9 workflow

As the whole project was divided in two parts, tissue culture and molecular part, the results of the molecular part are discussed in this section. The literature study of the genes responsible for the flower induction or suppression provided an evidence that no genes have been cited in Spinach which play role in flower control. In fact, no literature was found for the close relatives of spinach i.e. chard, sugar beet and quinoa. So, it was decided to first identify the genes which could possibly be involved in flower control in Spinach. Several articles suggested the role of FT-like genes. These genes, as discussed before, play varying role in flower induction in different plants. So, the presence of these genes was checked by BLAST tool of NCBI and Spinachbase.org. The results showed more than 50% homology for all the genes and so these genes were chosen to conduct the further experimentation.

4.5.1 PCR and gel electrophoresis results of 1st and 2nd set of primer pairs

All the primer pairs and the mixtures of primer were checked by PCR and results were carefully analyzed for further experimentation. Due to several mistakes, the experiment was a little bit delayed however, at the end confident results were obtained.

The results of 1st set of primer pairs: The genes were found in Arabidopsis and the sequences were used to check the homology in Spinach. NCBI BLAST tool showed positive results and four genes FtIG1, FtIG2, FtIG3 and FtIG4 were chosen. However, a mistake happened while making the 1st set of primers. The sequences which were obtained from NCBI were mRNA sequences which means, these sequences did not have the sequences of introns or untranslated regions of the genes. Due to this mistake, the PCR results were rather unsatisfying. Figure 4.9 shows the PCR results of the PCR of FtIG1, FtIG2, FtIG3 and FtIG4 genes. FtIG1 amplified a larger fragment of >1500bp while the expected size was 576bp. As a whole, all four genes showed highly nonspecific amplifications.

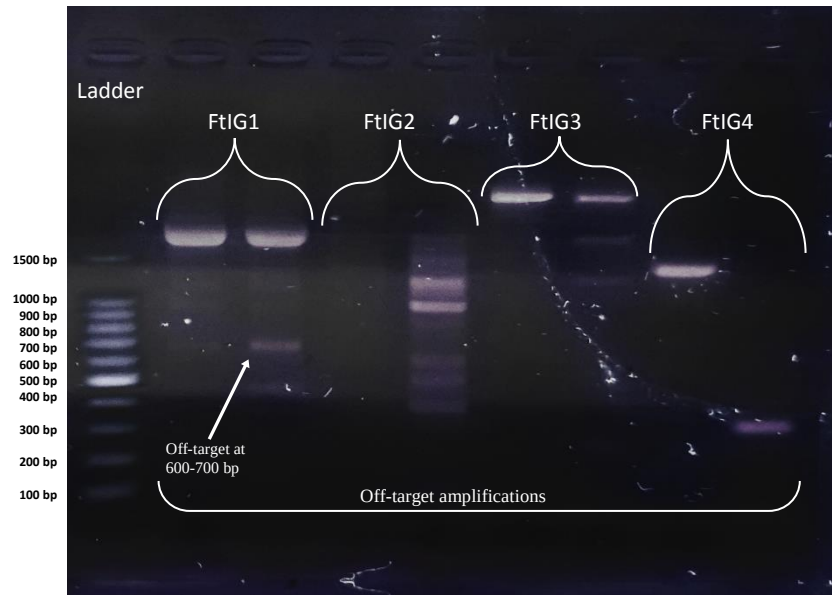


Figure 4.9. 1st PCR result of 1st set of primer pairs.

These results were totally unexpected. However, a very light band was observed in one of the amplifications of FtIG1 which was close to 600-700bp. This band was thought to be the expected band and it was decided to continue the further experimentation on FtIG1's amplification of $\approx 600-700$ bp.

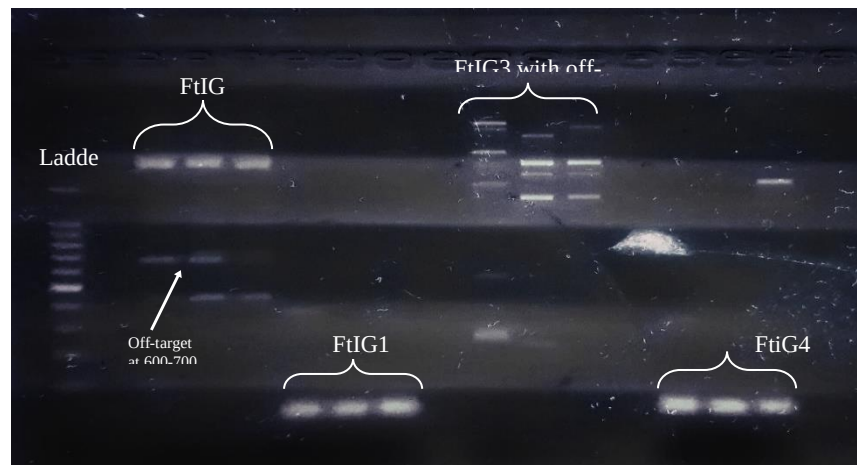


Figure 4.10. 2nd PCR result of 1st set of primer pairs.

Another PCR was run on the same primers with further optimized PCR composition and PCR protocol. This PCR resulted the same as the previous one. The amplifications of FtIG2 and FtIG4 did not make any amplifications and probably the primers were seen in the gel and FtIG3 showed so many non-specific

amplifications. However, the results were clearer, and All the primer pairs produced non-specific amplifications. Figure 4.10 shows the electrophoresis results of this PCR. From this PCR as FtIG1 showed the fragment size of ≈ 600 -700bp, this band was cut from the gel, DNA was excised and was sent for sequencing.

The results of 2nd set of primer pairs: As the mistake of making primers from mRNA sequences was realized, the complete sequences of all the four genes FtIG1, FtIG2, FtIG3 and FtIG4 as well as FTIP (which was discovered later in literature study) were obtained from the spinachbase.org. These sequences included the introns, exons, and untranslated regions. The new set of primers were ordered. This time, each forward primer was run on the PCR with its designed reverse primer, but also different primer mixtures were tried. The reason of trying these primer mixtures was to amplify even larger sequences which could be used for sequencing of the gene and the reason of trying all the possible mixtures was to be sure to get the successful primer mixtures as some primer could make primer-dimers. As 38 samples were run (34 test samples and 4 control) on different gels, Figure 4.11 shows the results of all the amplifications. Primer no. 1, 2 and 3 did not amplify any fragment not even any off-target amplification. Primer no. 7 and 9 showed expected band sizes along with slight non-specific amplification. One repetition of the primer no. 14 did not show results probably due to human error. While all the remaining samples showed very good results and all the bands were according to expected sizes. It was an interesting observation that the mistake of making primers from mRNA region of the gene did not provide any good results while the markers from the complete sequence of the gene gave all good results. Even the mixture of the primers gave very good results.

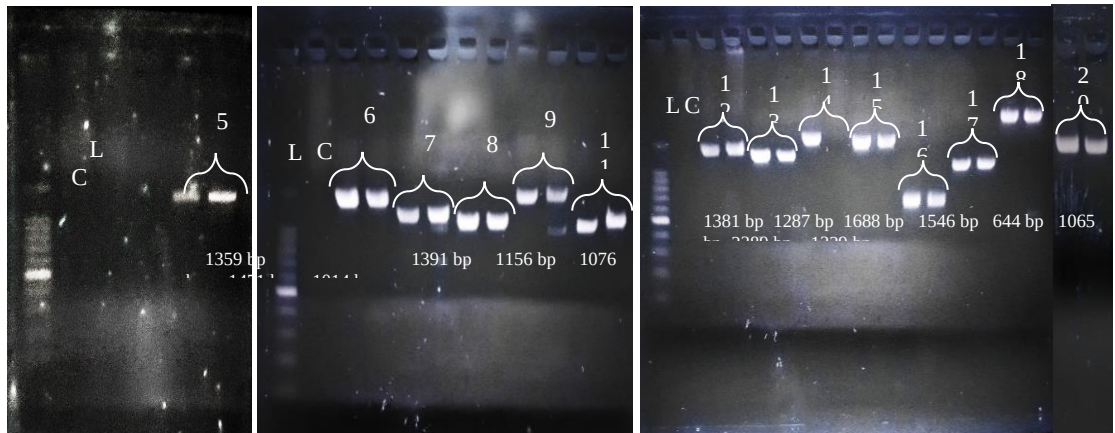


Figure 4.11. PCR results of primer no. 5 to 20.

Based on the results of this PCR, primer no. 1, 2, and 3 were repeated to be sure that these did not have any specific or non-specific amplification. Primer no. 5, 6, 9, 11, 12, 13, 14, 15, and 20 were chosen for amplification to prepare samples for sequencing. The reason of choosing only these was that all these amplify a specific part of the gene where the target exon of the gene will be almost in the center of the sequence. For FTIP, several samples were chosen so that gene could be sequenced from several parts and the target sequence of interest could be obtained. Figure 4.12 shows the results of PCR amplifications of primer no. 1, 2 and 3. So, it was concluded that the sequence of FtIG1 downloaded from spinachbase.org was not present in the spinach under experiment. While Figure 4.13. shows the results of the primer no. 5, 6, 9, 11, 12, 13, 14, 15 and 20. for the PCR amplifications of the remaining selected primer pairs and primer mixtures were very good. These bands were then cut very carefully, DNA was excised from the gel and was sent for sequencing to LGC company.

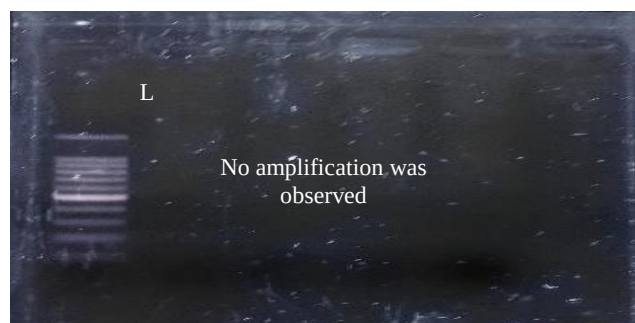


Figure 4.12. PCR results of primer no. 1, 2 and 3.

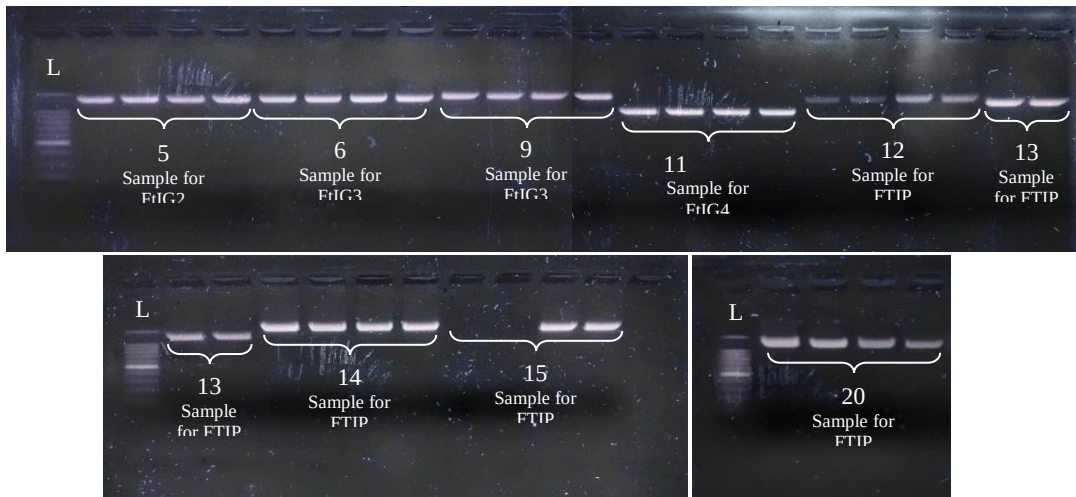


Figure 4.13. PCR results of primer no. 5 to 20 for DNA excision.

4.5.2 Results of sequencing of the fragments

As the LGC company had very specific criteria for sample preparation, it was important to be careful about the concentration of DNA and primers. The DNA was excised from the gel using the DNA excision kit from Promega.

Sequencing of the 1st set of primer pairs: The amplification of 600-700bp of FtIG1 gene was used. According to the DNA excision kit the final volume of nuclease-free water to elute the DNA from SV minicolumn was 50µl. This protocol was followed the same way, but the DNA obtained at the end was less concentrated. To make this DNA more concentrated, it was decided to freeze-dry the DNA and then dilute it with less water to get concentrated product. However, after freeze-drying and resuspension of the DNA, when the DNA samples were run on the gel to check whether there was any DNA damage or not, the DNA band was not of expected size. It was thought that the DNA might have ligated making a larger fragment than expected. To overcome this problem, the DNA was incubated at 95°C for 4 minutes and was cooled down at room temperature. This incubation was followed to denature the DNA strands and then allowing it to anneal again. This incubation did not also work, and the DNA band remained larger than expected. Figure 4.14 shows the results of the freeze-dried DNA sample. Anyhow, the sample for sequencing from this DNA sample was prepared and was sent for sequencing. The LGC representative explained that the sample

was not good and no sequence amplification could be performed. So, no sequence was received for this gene.

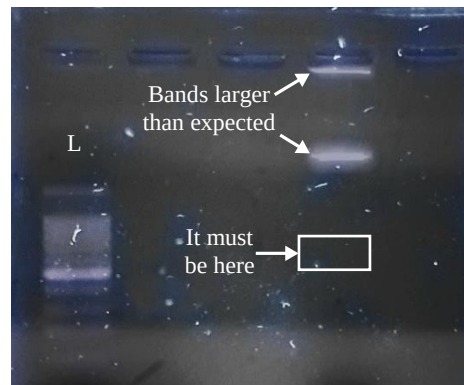


Figure 4.14. DNA damage of the PCR product of FtIG1 after freeze-drying.

Sequencing of the 2nd set of primer pairs: The 2nd set of primer pairs provided good amplifications which according to the expected fragment sizes. This time the DNA excision from gel was eluted in 35µl nuclease-free water to have a concentrated DNA sample. The samples were prepared very carefully considering the concentration and quantity of the DNA and primers. The samples of primer no. 5, 6, 9, 11, 12, 13, 14, and 15 were sent for sequencing with forward as well as reverse primers. Table 4.7 shows the results of the sequencing for each sample. The homology of the sequences was checked by ClustalW homology at ClustalOmega. The homology and the chromatogram analysis revealed that the genes had SNPs at many points and all the five genes had heterozygous alleles. Figure 4.15 shows a segment of the chromatogram of primer no. 14 and heterozygous base can be observed. Since the sequenced results were not enough, no further conclusions could be made based in this data.

Table 4.7. Sequencing results of primer no. 5, 6, 9, 11, 12, 13,14 and 15

Primer no.	Sequence
5 with forward primer	TATAGTTCAGACTTAATTCACATACAGAGAAAT
5 with reverse primer	No sequence recieved
6 with forward primer	ATGGCAAAGGAGCGTCAGCTTGAGATTTCCTCAGAATCCAACAATACC TTGAAGAAATCTGTCTCATCTCCTTTTCATGAACACTGCCTCGAAGATGT ATTCAATCTCTGAAGTTAGAAAACACAACACTGCTGACTCTGCATGGA TCATCGTCCACGGTCACATCTATGACTGTACACGTTTCTTGAAAGATCA TCCAGGAGGCACAGATTCTATCCTGATAAACGCATGTACAGATTGCAC CGAAGAGTTTGAAGCCATTCACTCTGACAAAGCCAAGAAGCTTTTGG AGATTACCGTATCAGTGAACATCATCA
6 with reverse primer	No sequence recieved
9 with forward primer	ATGCCCATAGGTCATTTATTGCTATTGGGCCTTGGGCCTTAATGGATGG CTGACTAGCCATAGTGGTAGGTTTGATGCTGTTGTTTAGAGCCATTGG GCTTTGGACTTAGTGGCCCAATTGAATAT
11 with forward primer	No sequence recieved
11 with reverse primer	ATATCAAATAATACTACTTCGTAAGAGATTATTTTGA
12 with forward primer	ATCCAACAATACCTTGAAGAAATCTGTCTCATCTCCTTTTCATGATCACT GCCTCAAAGATGTATTCAATCTCTGAAGTTAGAAAACCAACACTGCT GACTCT
12 with reverse primer	CATACTTGTCTCTGCAAGACACCGGCCACAAAGACATATGTCAATTAG TTAAGCTTGATATATACAATGATGTTCCCTCTCGTTGAGGAATGAGGAA GAGTGAGAGAGAG
13 with forward primer	TAGACATTTCTTAAGATTACTGGGACTCGTTTACATTTCTGAGAGATGA AAAGAAAGAGGCTAGCTTTTTGAAAAATCTTCTTATCATTTAGCTACT GATGGTCAAATCCAAAAG
13 with reverse primer	AAGACATATGTTAATTTGTTAAGCTTGATATATACAATATGTTCCTTTC ATTGAGGAATGAGGACGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA GAGAGAGAGAGAG
14 with forward primer	ATCCGCTCACAATTCACACAACATACGAGCCGGAAGCATAAAGTGTA AAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC GCTCACTGCCCCTTTCCAGTCGGGAAACCTGTCGTGCCAG
14 with reverse primer	CTTCTTTCATCTTCATCATCATCTTTCTGTTGTTAGCCTTCTCATACT CTGCTAGTCTGCTTCACCTTGAGTCTCCAATTTCTTTTCTGCAGATTTA ATACATAGGTATTCTAGTTTGTGAGAATAGTGAACATTTTTCACATT TTTAATCAAACACAATTACAACTAGCATACCTATGTATTAATCTGC AGGAAAAAGAAATTGGAGACTC
15 with forward primer	TTCTTTTGTGCCGAGGAGAAAGTAATGCAGGTTGTA
15 with reverse primer	ATTTAGTCTTGCATATTGCTTGATATTCCGTAACAAATAGTACTACGT CTATAACAACCTCAGATGCTAAAGAGATCCAAACGGAGCTTTAGTTGA TGGTTCGTTTGTGCATCTCTTTTGAACGGGTGGC

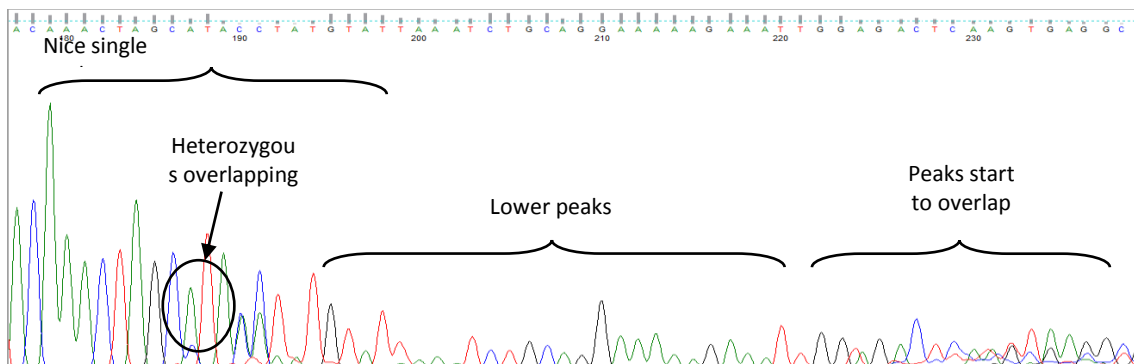


Figure 4.15. Chromatogram of sequencing of primer no. 14.

As a next step, PCR cloning was followed to separately amplify each allele of the FtIG3 gene and then send it for sequencing. Only one gene was chosen at this time to first see the results of PCR cloning and then same procedure was decided to be followed for amplifications of the remaining genes.

4.5.3 Results of PCR cloning

The transformed bacteria were transformed by the ligated vector (the vector provided in the kit ligated with the PCR product of FtIG3). Two plates (1 time and 10 times concentrated) were incubated for each ligation to observe the efficiency of the competent agrobacterium. 1X ligation plates had 10 times less bacteria compared to the 10X plates. This method was followed to be sure to obtain results in case in 1X plate no colony grows, at some colonies could be observed in 10X plate. However, all the plates showed that the bacteria were very efficient, and many colonies were observed.

The pJet cloning vector comes with a resistance gene to ampicillin and has a suicidal gene in it as well. This suicidal gene is silenced if the ligation reaction is successful. So, if the ligation reaction was not successful, the suicidal gene in the vector kills the bacteria after transformation. All the colonies which are visible in the Figure 4.16 were transformed as no other bacteria could grow with unsuccessful ligation. Further, as the bacteria have ampicillin resistance, they survive in the LB media and any other contaminant dies because of the antibiotic. So, it had been a two-way control system. The ampicillin kills any contaminant which does not has resistance to it and the suicidal gene kills all the non-transformed bacteria. So, the growth in the plates only had transformed bacteria. However, some possibilities might occur i.e. there can be a frameshift at the insertion point of the vector. This frameshift will disrupt the sequence of the suicidal gene and the bacteria can still grow on LB plate without any ligated gene.

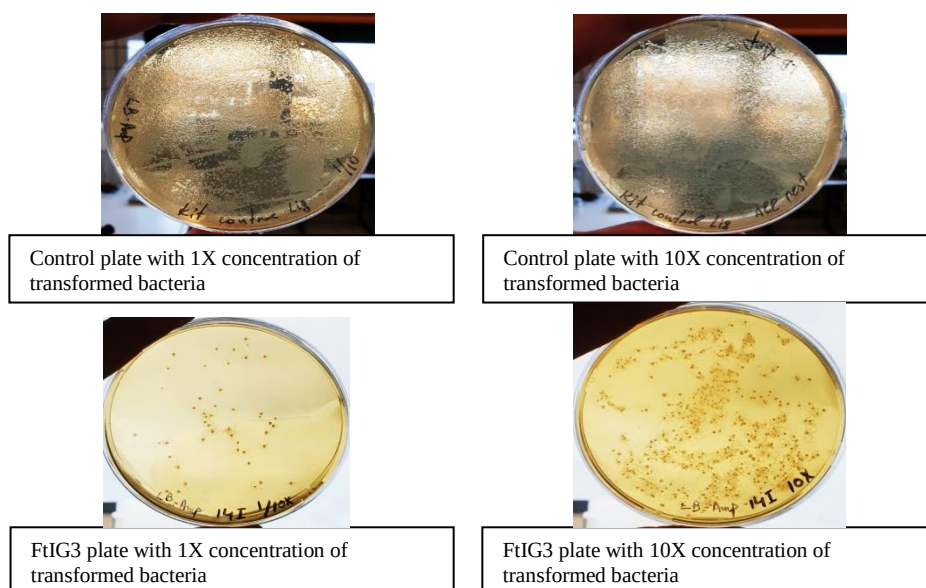


Figure 4.16. Overnight growth of transformed bacteria containing the amplified fragment of FtIG3 and the Control PCR provided in the kit.

From these plates, 12 single cell bacterial colonies were taken under sterile condition and were carefully transferred on another LB plate as shown in Figure 4.17 All these single cell colonies grew very well and were used for plasmid isolation and sequencing.



Figure 4.17. Growth of 12 transformed colonies.

5. DISCUSSIONS

More research needs to be conducted on the flowering of spinach, its tissue culture establishment and flowering gene knockout procedure improve its quality and solve this problem. Plant tissue culture is not only beneficial in multiplying plants but also helps in study of important processes for example cell metabolism, signalling pathways of genes in plants and in production of pathogen free cultures. Cell cultures are playing their role in examine many biological processes in the field of agriculture and biochemistry (Thorpe, 2007). In plant tissue culture, plant hormones auxins, gibberellins, cytokinin etc plays important role in growth and development of plants. In most cases, these hormones work by interacting with each other (Gaspar et al., 1996b). In our studies, we have focused on role of newly designed cytokinin oxidase inhibitors to enhance the activity of cytokinin for the growth and development of 14 different plants including spinach as these inhibitors stops the activity of cytokinin oxidase which degrades cytokinin and hinders the growth (Zalewski et al., 2010). It was observed that these compounds showed activity in 5 important crops but in combination with 2iP and BA. As these newly designed hormones alone have no or very less cytokinin-like activity, they possibly target cytokinin degradation by inhibiting cytokinin oxidase/dehydrogenase. They can be potential compounds to enhance efficiency of in vitro protocols and can enhance the regeneration process for economical important crops.

Spinach is a difficult crop to grow in vitro laboratory. It showed very slow germination in in-vitro culture. The results reveals that there is a dire need to develop new protocols for tissue culture of spinach as it an important crop and successful cell culture of spinach can lead to not only study of flowering genes and Crisper/Cas studies but it will open doors in study of biological processes in spinach. With our protocols spinach showed less than 50% of growth overall and it took very long even to develop that 50% of spinach culture.

The GFP expression in the spinach leaf shows that the plant is easy to genetically modify and it responds to the foreign DNA. However, it is highly dependent on the type of foreign DNA, the state of the plant, the type of vector

and agrobacterium and the method used to transform the plant. (Ahuja, 1988; and Binns, 1990). Further research on this plant following the aim to knock-out flower induction genes will reveal the outcomes and how the plant responds to such genetic changes.

Working on plant genetics is an interesting part of the molecular research. In our research the target was to suppress flowering in Spinach to avoid bolting problem (Chitwood et al., 2016) by knocking-out flower induction gene(s). This research focused the growers of Spinach as bolting is a problematic factor and if there is no bolting, growers can earn more profit as after bolting the leaves of spinach lose taste (Molly, 2018). Several other cultural practices are used to delay/suppress bolting (Bonny, 2019), but genetic basis of bolting has not been properly studied yet. However, before reaching that end goal of suppressing bolting, the primary goal has been to establish a working CRISP-Cas9 protocol which targets only the intended part of the gene and rest of the genome stays intact (Zhang et al., 2015), the outcomes of such gene knock-out could have positive or negative results on overall growth of plant. The choice of genes to target had been a crucial factor as no documented data could be found and the experiment was conducted on hypothetical basis. The construct of CRISPR-Cas9 had already been a limiting factor and the selected genes were an extra limiting factor. Even though the Cas9 protocol could have been successfully established, the knock-out of the selected genes was still a question whether this knock-out will suppress bolting or not.

In this study, among the several limiting factors, the choice of mRNA sequences for making molecular markers had clearly delayed the experiment. The mRNA sequence does not contain the sequence of introns, untranslated regions, or peptide sequences of the gene, instead it only contains the sequence of the coding region as known as CDC region. So, designing molecular markers for mRNA is a limiting factor as the sequence of the marker may be covering two exons where the intron sequence had been erased during translation (Gupta et Rustgi, 2004). Moreover, another considerable point is that finding the whole sequence of a gene is very important in such studies. The whole gene sequences in this study had been obtained from Spinachbase.org. The new molecular markers had been

designed very carefully (Khan, 2015) and the PCR results had been positive for all the markers except for the markers of FtIG1 gene. The optimization of PCR is rather simple method; however, minor mistakes can produce negative results (Williams, 1989).

The DNA isolation and gene sequencing had been an interesting case study in this experiment, as an interesting behavior of DNA was observed after freeze-drying it. Freeze-drying the DNA either damages the fragment or it makes the fragments to make ligations with each other making a much larger fragment (Iuso et al., 2013) which could not be reverted either. The sequencing results of this DNA were also not positive. Due to limitation of time, freeze-drying was avoided, and DNA was eluted in less quantity of nuclease-free water to have concentrated DNA. The sequencing results of these DNA samples and the analysis of the chromatograms provided an evidence that all the genes in our spinach plant were indeed heterozygous and that had been the reason of unsuccessful sequencing (« SBTengine », 2020).

Lastly, to acquire the sequences of heterozygous genes, it was important to separate the two alleles and then send each allele separately for sequencing. PCR cloning had been another fascinating piece of technology where the alleles were ligated with the vector and the vector were taken by the bacteria (Gopal, 2014). The two-way control i.e. the suicidal gene and antibiotic, provided an extra security that only the transformed bacteria which have our gene of interest, grow and there is no other contaminant growing on the LB plates. This whole study provides a solid evidence that such experimentation seems quite easy and simple in theory however, practically, there might be little obstacles that delay the overall experiment and it also provides and evidence of how research is taken forward through many possible solution to overcome any such obstacle.

6. CONCLUSION

Spinacia oleracea is a commercially important leafy vegetable. In spring flowering occurs (known as bolting) as soon as spring days are longer than 14 hours. Bolting makes it impossible to mechanically harvest spinach. This implies that the crop does not reach its potential yield as required by growers. In our experiment CRISPR/Cas gene knockout method has been utilized to solve this problem. The results of in-vitro germination by seed and regeneration of spinach revealed that it is very difficult as overall germination and regeneration percentage was less than 50% in all types of growth medias, concentrations, and growing protocols. Therefore, further research must be conducted on tissue culture development of spinach as successful tissue culture production is required for growth of genetically modified spinach after Crisper/Cas transformation. In our experiment, the new in silico compounds have produced potentially good results in tissue culture regeneration of several other crops i.e *Nicotiana tabacum*, *Drosera rotundifolia*, *Coleus esculentum*, *Lobelia erinus*, *Paulownia fortune*. However, the success of the tissue culture widely depends on the genotype. There have been several studies where different genotypes of same plant exhibited significantly different tissue culture growth under same media and growth conditions. On the other hand, as regeneration of plant species is highly dependent on genotype, the cytokinin inhibitors can be further optimized for better regeneration. The sequencing of the targeted genes had been a difficult part of the experimentation. The analysis of the sequencing results showed that both Ft and FTIP1 genes are heterozygous and show SNPs. The heterozygosity of the targeted genes resulted in taking another approach to sequence heterozygous genes i.e. PCR cloning. The results of PCR cloning were confident and further experimentation is required to complete the project. The entire project was highly effected due to COVID-19 and many activities were delayed by several weeks. Overall, our studies and results can further help to proceed the research on flowering gene knockout and tissue culture establishment in spinach.

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16/09/2020

A handwritten signature in dark ink, appearing to read 'Irum Saadia' with a stylized flourish at the end.

Irum Saadia KHAN

CURRICULUM VITAE

Irum Saadia Khan was born in Pakpattan, Pakistan on 5th January 1997. She went to Bright Grammar High School Lahore for her secondary education and Government Degree College for women, Lahore Pakistan for intermediate studies. She did her bachelor's in agriculture sciences from University of the Punjab, Pakistan and graduated in 2018. For her master's studies, she got selected for a prestigious scholarship for Europe from 2018-2020 and she studied Plant Breeding under Erasmus Mundus Joint master's degree Program (emPLANT). Her first year of masters was in institute polytechnic UniLaSalle, France and her second year was in Ege University, Turkey. She did her master thesis from Ghent University Belgium.

