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# OPTIMIZATION OF IN-VITRO REGENERATION AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF LACTUCA SATIVA

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United Arab Emirates University

**MASTER THESIS NO. 2024: 22**

**College of Science**

**Department of Biology**

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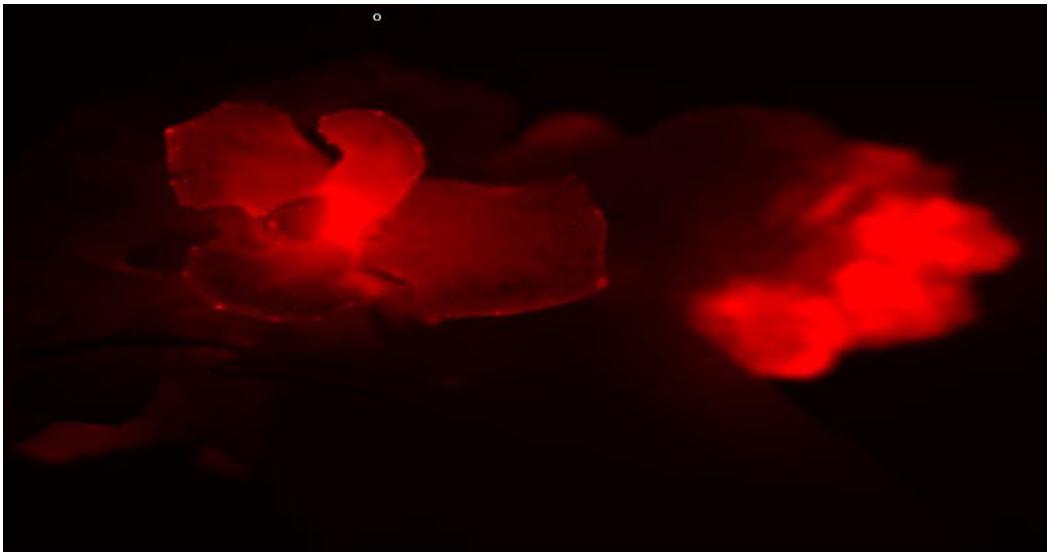

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Mohamed Omer Elbashir ElSiddig

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Molecular Biology and Biotechnology

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Cover: Transgenic lettuce emitting red florescence under the florescence microscope
(Photo: By Mohamed Omer Elbashir Elsiddig)


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## Declaration of Original Work

I, Mohamed Omer Elbashir Elsiddig, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Optimization of In-Vitro Regeneration and Agrobacterium-Mediated Transformation of Lactuca Sativa"* hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Khaled Amiri UAEU. This work has not previously formed the basis for awarding any academic degree, diploma, or similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been appropriately cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation, and publication of this thesis.

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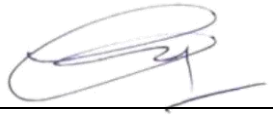
Signature 

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# Abstract

Lettuce (*Lactuca sativa* L.) is a leafy vegetable consumed worldwide, mainly as a green salad. It is an herbaceous, self-pollinated annual plant of the family Asteraceae. Lettuce is low in calories, fat, and sodium. It is a good source of fiber, iron, folate, and vitamin C. Improving existing lettuce varieties for water use efficiency, photosynthesis efficiency, salt tolerance, increased nutritional content, etc., is the need of the hour, considering that lettuce is a crop ideal for vertical farming in the UAE.

This study aimed to develop a highly efficient *in vitro* regeneration and Agrobacterium-mediated transformation protocol for a popular lettuce variety, 'Paris Island cos' belonging to the romaine type. The study analyzed 11 different tissue culture media-hormone combinations for shoot regeneration efficiency from lettuce cotyledon explants. Murashige and Skoog media supplemented with vitamins, 3% sucrose, 0.1 mg/L BAP, and 0.1 mg/L NAA had the highest shoot regeneration percentage in this study and were used in the Agrobacterium transformations.

The study analyzed the effect of several transformation parameters, such as 'explant preculture,' 'optical density of Agrobacterium culture,' 'infection method,' and 'time of co-cultivation,' etc., to optimize the transformation. The highest transformation efficiency of 14% was observed when shallow wounded cotyledon explants from 7-day-old seedlings were transformed under the following conditions: preculture for 24 hours, Agrobacterium optical density of 0.5 at 600 nm, infection time of 10 minutes, and a co-cultivation period of 3 days under 22°C under dark conditions. After optimizing the transformation conditions using a vector harboring the reporter gene RFP, the study further transformed the gene *SbSI-1*, reported to function in salt and drought tolerance in the halophyte (*Salicornia brachiata*), and obtained transgenic plants. The expression levels of the transgene were analyzed by quantitative real-time PCR. The study successfully developed an efficient in vitro regeneration and Agrobacterium-mediated transformation protocol for the lettuce variety 'Paris Island cos'.



**Keywords:** Lettuce, *in vitro* regeneration, Agrobacterium-mediated transformation, Transgenic plants.

# Title and Abstract (in Arabic)

## تحسين عملية تكون الخلايا النباتية وعملية التعديل الوراثي بواسطة البكتيريا داخل المختبر لنبات الخس ( *Lactuca sativa*)


***الملخص***

يعتبر الخس (*Lactuca Sativa*) من الخضراوات الورقية والتي يتم استهلاكها في جميع أنحاء العالم وبشكل أساسي من ضمن مكونات السلطة الخضراء. وهو نبات عشبي سنوي ذاتي التلقيح ويتبع لفصيلة الستيرايسيات (Asteraceae). ويحتوي الخس على سعرات حرارية قليلة جداً بالإضافة إلى نسبة قليلة من الدهون والصوديوم. كما أنه يعتبر مصدر جيد للألياف والحديد وفيتامين C. إن تحسين أصناف الخس الموجودة حالياً من حيث تقليل استخدام المياه، وزيادة كفاءة التمثيل الضوئي، وتحمل الملوحة، بالإضافة إلى زيادة المحتوى الغذائي صارت ضرورة ملحة مع الأخذ في الاعتبار أن الخس هو محصول مثالي للزراعة العمودية في دولة الإمارات العربية المتحدة.

تهدف هذه الدراسة إلى تطوير بروتوكول عالي الكفاءة في المختبر لعملية تكوين الخلايا النباتية بالإضافة إلى التعديل الوراثي المطلوب باستخدام البكتيريا (*Agrobacterium tumefaciens*) على صنف الخس "Paris Island cos " والذي ينتمي إلى نوع رومين(Romaine) . قامت الدراسة أيضا بتحليل 11 تركيبة مختلفة من الأوساط الغذائية الزراعية تحتوي على هرمونات تساعد على نمو الأنسجة من أجل تحسين كفاءة الخس على تكوين الخلايا النباتية ونمو أوراق نبات الخس من الورقة الفلقية. الوسط الغذائي والذي يتكون من Murashige and Skoog والفيتامينات، 3% من السكروز، 0.1 ملغ/لتر BAP و0.1 ملغ/لتر NAA أثبت أن لديه أعلى نسبة نمو لأوراق نبات الخس في هذه الدراسة وبالمثل تم استخدم هذا الوسط الغذائي في عملية التعديل الوراثي بواسطة البكتيريا (*Agrobacterium tumefaciens*).

بالإضافة الى ما سبق فقد قامت الدراسة ايضا بتحليل تأثير عدد من الدلالات والطرق التي تؤثر على التعديل الوراثي مثل: الزراعة المسبقة للأوراق وتعداد البكتريا وقت الزراعة المشتركة بالإضافة الي طريقة حقن الجين وما إلى ذلك من طرق لتحسين التعديل الوراثي. وقد لوحظ أن أعلى كفاءة تحول للخس بنسبة 14% عندما تم تحويل ورقة الفلقة المجروحة بجروح سطحية على شتلات عمرها 7 أيام تحت الشروط التالية: الزراعة المسبقة لمدة 24 ساعة، تعداد البكتيريا الزراعية (OD) يجب أن تكون 0.5 عند 600 نانومتر، ووقت الإصابة بالعدوى 10 دقائق، وفترة الزراعة المشتركة لمدة 3 أيام على درجة حرارة 22 درجة مئوية في ظل ظروف مظلمة. بعد تحسين ظروف التعديل الوراثي باستخدام ناقل (Vector) والذي يحتوي على الجين المراسلRFP ، قامت الدراسة أيضاً بتحويل جين مستخدم مسبقاً يعمل على تحمل الملوحة والجفاف من نبات الساليكورنيا براكياتا (*Salicornia brachiata*) والنباتات المعدلة وراثياً باستخدام نفس الجين. تم تحليل مستويات التعبير الجيني في النباتات المعدلة وراثيا بواسطة استخدام Real-time PCR.

نجحت الدراسة في تطوير بروتوكول فعال يساعد على تكون الخلايا النباتية ونجاح عملية التعديل الوراثي داخل المختبر بوساطة (*Agrobacterium tumefaciens*) لصنف الخس'Paris Island cos' .

**مفاهيم البحث الرئيسية:** الخس، تكون الخلايا النباتية في المختبر، التعديل الوراثي باستخدام البكتيريا، النباتات المعدلة وراثيا.

# Acknowledgments

I am immensely grateful to everyone who contributed to making this thesis a reality. I also owe a debt of gratitude to my committee, with a special acknowledgment to my advisors, Prof Khaled M. Amiri and Dr. Suja George, for their unwavering support, invaluable guidance, and continuous encouragement throughout this journey.

I extend my heartfelt thanks to my work colleagues, whose assistance and guidance were instrumental in shaping this thesis. Their contributions have been invaluable, and I deeply appreciate their efforts.

This thesis would not have been possible without the support and encouragement of my family. Their unwavering belief in me and constant encouragement motivated me during challenging times.

Lastly, I express my sincere appreciation to all the individuals and resources that have contributed to this work in any way. Your support has been invaluable, and I am truly grateful for the opportunity to embark on this academic endeavor.

# Dedication

*Words can hardly describe my thanks and appreciation to you. You have been my source of inspiration, support, and guidance. You have taught me to be unique and determined, to believe in myself, and always to persevere. I am truly thankful and honored to have you as my parents and sister*

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# List of Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-Cichlorophenoxyacetic Acid |
| ABA | Abscisic Acid |
| BAP | 6-benzylaminopurine |
| CDD | Conserved Domain Database |
| CDS | Coding Regions |
| CKs | Cytokinin's |
| Ct | Cycle Threshold |
| GA | Gibberellic Acid |
| GOI | Gene Of Interest |
| IAA | Indole-3-Acetic Acid |
| IBA | Indole-3-Butyric-Acid |
| MCS | Multiple Cloning Sites |
| MIC | Minimum Inhibitory Concentration |
| MS | Murashige and Skoog |
| MSA | Multiple Sequence Analysis |
| NOS | Nopaline Synthesis |
| ORF | Open Reading Frame |
| PCR | Polymerase Chain Reaction |
| RFP | Red Fluorescent Protein |
| T-DNA | Transfer- DNA |
| Ti-Plasmid | Tumor Inducing Plasmid |

# Chapter 1: Introduction

## 1.1 Overview

Crop improvement strategies, including genetic transformation methods and non-transgenic approaches like micropropagation, rely on the plant's ability to regenerate whole crop plants efficiently through tissue culture. A key advantage in practical applications is using a multiple-shoot regeneration system that bypasses a slow intermediate callus stage. The success of an *in vitro* regeneration system is influenced by various factors, including the species and variety of the plant, the type of explant used, and the composition of hormones and nutrients in the culture media. The efficiency of regeneration protocols can vary significantly between different genotypes within the same species, indicating a strong genotype bias. Therefore, tailoring and optimizing *in vitro* regeneration protocols for the genotype under consideration is crucial. This customization ensures the highest possible efficiency and reliability in producing regenerated plants, whether for genetic transformation purposes or large-scale micropropagation efforts aimed at crop improvement. Crop improvement programs utilize breeding or transgenic technologies to improve desired traits in the species of choice. The development of transgenic plants modified with a gene of interest from another species with the desired trait is an ideal method for crop improvement as it transcends the barriers of sexual reproduction, is precise, and less time-consuming compared to breeding. To develop a transgenic plant, however, it is essential to standardize Agrobacterium-mediated transformation protocols for the species in question.

Lettuce (*Lactuca sativa* L.) is a leafy vegetable consumed worldwide, mainly as a green salad. It is an herbaceous, self-pollinated annual plant of the family Asteraceae. Lettuce is low in calories and sodium. It is a good source of fiber, iron, folate, and vitamin C. Improving existing lettuce varieties for water use efficiency, photosynthesis efficiency, salt tolerance, increased nutritional content, etc., is the need of the hour, considering that lettuce is a crop ideal for vertical farming in the UAE.

This study aimed to develop a highly efficient *in vitro* regeneration and Agrobacterium-mediated transformation protocol for a popular lettuce variety, 'Paris Island cos, ' belonging to the romaine type. Additionally, after optimizing the transformation conditions, the study further transformed a gene previously reported functioning in salt and drought tolerance in the halophyte *Salicornia brachiata* and obtained transgenic plants.

### 1.2 Statement of the Problem

The lettuce variety, "Paris Island cos", is a popularly consumed nutritious leafy vegetable. The species is ideal for vertical farming due to its suitability for growth in hydroponic systems and short life cycles. Improving existing lettuce varieties for water use efficiency, photosynthesis efficiency, salt tolerance, increased nutritional content, etc., will further enhance the status of the crop as an ideal system for vertical farming. For crop improvement programs in 'Paris Island cos,' it is essential to develop high-efficiency *in vitro* regeneration and Agrobacterium-mediated transformation protocols in this variety. A research survey revealed that no such efficient protocols exist for this variety. Hence, this study developed high-efficiency *in vitro* regeneration and Agrobacterium-mediated transformation protocols in this variety. The study also aimed to demonstrate the reliability of the developed protocols by transforming a gene previously reported functioning in salt and drought tolerance in the halophyte *Salicornia brachiate* into the chosen lettuce variety.

### 1.3 Research Objectives

- To develop a highly efficient *in vitro* regeneration protocol for the chosen lettuce variety, 'Paris Island cos'.
- To develop a highly efficient Agrobacterium-mediated transformation protocol for the chosen lettuce variety, 'Paris Island cos'.
- To demonstrate the suitability of the developed protocols by transforming a foreign gene into the selected variety and to assess the transgene expression levels.

## 1.4 Relevant Literature

### *1.4.1 Optimization of In Vitro Regeneration in Selected Plant Variety*

Unlike animal cells, plant cells have a remarkable ability to regenerate tissues and organs from differentiated cells, which involves converting one cell type to another. This plasticity, known as totipotency, allows the regeneration of whole organs and plants via the dedifferentiation of cells and reprogramming of cell fates. This feature of plant cells is extremely useful in plant tissue culture, wherein multiple plants are regenerated from a small amount of starting plant material *in vitro*. *In vitro* regeneration can happen through various modes that require intricate molecular, regulatory, and signaling pathways.

The three main modes of regeneration are (1) Tissue regeneration, (2) *De novo* organogenesis, and (3) somatic embryogenesis (Bull & Michelmore, 2022).

Tissue regeneration, which produces new meristems, is often observed in lower plants such as bryophytes (mosses, liverworts, hornworts, etc.) (La Farge et al., 2013). However, vascular plants (higher plants with xylem and phloem for distributing resources through the plant) follow *De novo* organogenesis or somatic embryogenesis for regeneration. During *De novo* organogenesis, whole organs are regenerated from explants. *De novo* organogenesis can be direct regeneration (development of organs directly from explants) or indirect regeneration (development of organs from explants through an intermediate undifferentiated callus phase). Unlike *De novo* organogenesis, somatic embryogenesis involves the regeneration of embryo or embryo-like structures from somatic cells, which can develop into a whole plant. Similar to *De novo* organogenesis, somatic embryogenesis can be direct (development of embryo or embryo-like structures directly from explants) or indirect (development of embryos from explants through an intermediate undifferentiated callus phase) (Figure 1).

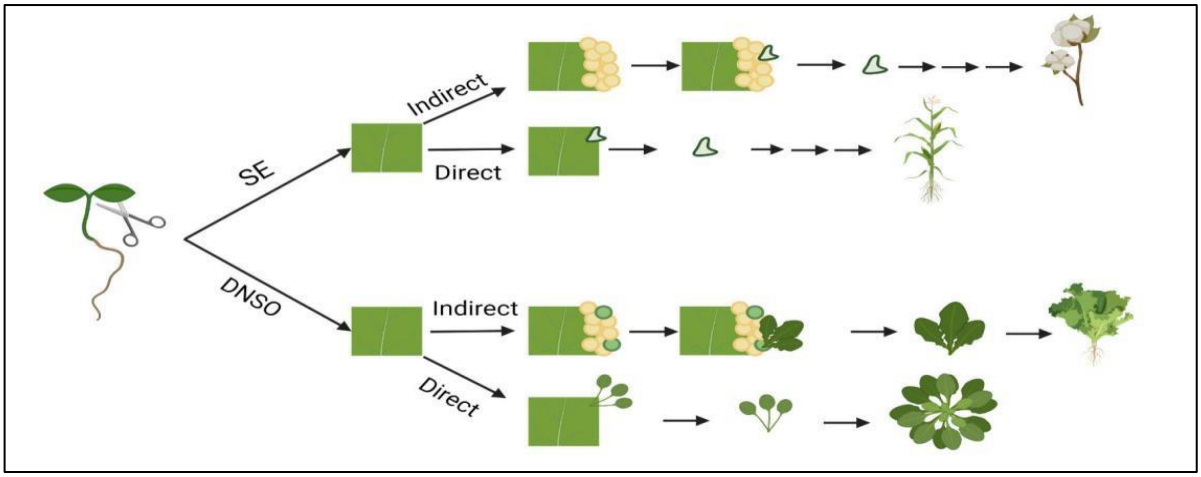


Figure 1: Pathways of *in vitro* regeneration of vascular plants. Somatic embryogenesis (SE) and *De novo* shoot organogenesis (DNSO) can occur directly on the explant or indirectly with the formation of pluripotent callus as an intermediate step reproduced from (Bull & Michelmore, 2022)

The *in vitro* regeneration process is complicated and depends on species, plant genotype, source plant material (known as explant), nutrients and Plant Growth Regulators (PGRs), culture conditions, etc.

A suitable regeneration protocol that works for one genotype would often require considerable modifications, especially in the case of nutrient and hormone combinations, for different genotypes of the same species(Altpeter et al., 2016). Thus, standardizing a suitable regeneration protocol for your species and genotype of choice is often the most crucial and limiting step in crop improvement programs employing plant transformation.

Optimization of the *in vitro* shoot regeneration media involves identifying the right combinations of micro and macronutrients, vitamins, carbon sources, and hormones. Even the choice of media gelling agent can make considerable differences in shoot regeneration percentages. Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) is a widely used plant tissue culture media comprising micro and macronutrients required for plant growth. Different versions of this media are commercially available, including those with vitamins. Other than MS, different basal salt mixtures are also commercially available, including Anderson basal salt mixture, Blaydes basal medium, Chu's N6 basal medium, etc, tailored to improve tissue culture efficiency in different species.

Plant Growth Regulators (PGRs) are hormones and chemicals that affect plant development and growth, including root and shoot growth, stem elongation, flowering, aging, and many other conditions. Minimal concentrations of these substances can produce significant growth changes in plants. PGRs can be naturally occurring compounds or synthetically produced. To date, auxins, gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), ethylene, brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), florigen, and strigolactones (SLs) have been reported as plant hormones. Each group contains naturally occurring hormones in plants and synthetic counterparts (Asami & Nakagawa, 2018).

Auxins and cytokinins are commonly used in plant tissue culture media for inducing shoot and root regeneration. Auxin causes several plant responses, including phototropism (bending towards light), geotropism (downward root growth in response to gravity), promotion of apical dominance, flower and fruit formation, formation of adventitious roots, etc. Commonly used auxins include naturally occurring ones such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA), and phenylacetic acid (PAA) as well as synthetic auxin-like substances such as naphthalene-1-acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and picloram (George et al., 2023). Cytokinins promote cell division in plant shoots and roots, affecting apical dominance, axillary bud growth, and leaf senescence.

There are two types of cytokinins: adenine (kinetin, zeatin, and 6-benzylaminopurine (BAP)) and phenylurea cytokinins (diphenylurea and thidiazuron)(Asami & Nakagawa, 2018). The ratio of auxins to cytokinins in the culture media affects the cell's fate. In general, increasing the ratio of cytokinin to auxin results in a shift from root to shoot organogenesis. However, identifying the right hormones and their ratios is crucial for *in vitro* shoot regeneration.

### *1.4.2 Lettuce (Lactuca sativa L.)*

Lettuce is a leafy vegetable consumed worldwide, mainly as a green salad. It is an herbaceous, self-pollinated annual plant of the family Asteraceae. Lettuce varieties differ in their colors, sizes, and shapes (Figure 2). Lettuce varieties have been grouped into six main types based on leaf shape, size, texture, head formation, and stem type. They are

crisphead lettuce, butterhead lettuce, romaine or cos lettuce, leaf or cutting lettuce, stem or stalk (Asparagus), and Latin lettuce(Kim et al., 2016). China, as the largest producer of lettuce globally, primarily cultivates stem lettuce varieties. Lettuce is the 3rd most consumed vegetable in the US, contributing to about 22% of the total lettuce production worldwide. Romaine lettuce accounted for 30.1% of lettuce production in the US in 2014.

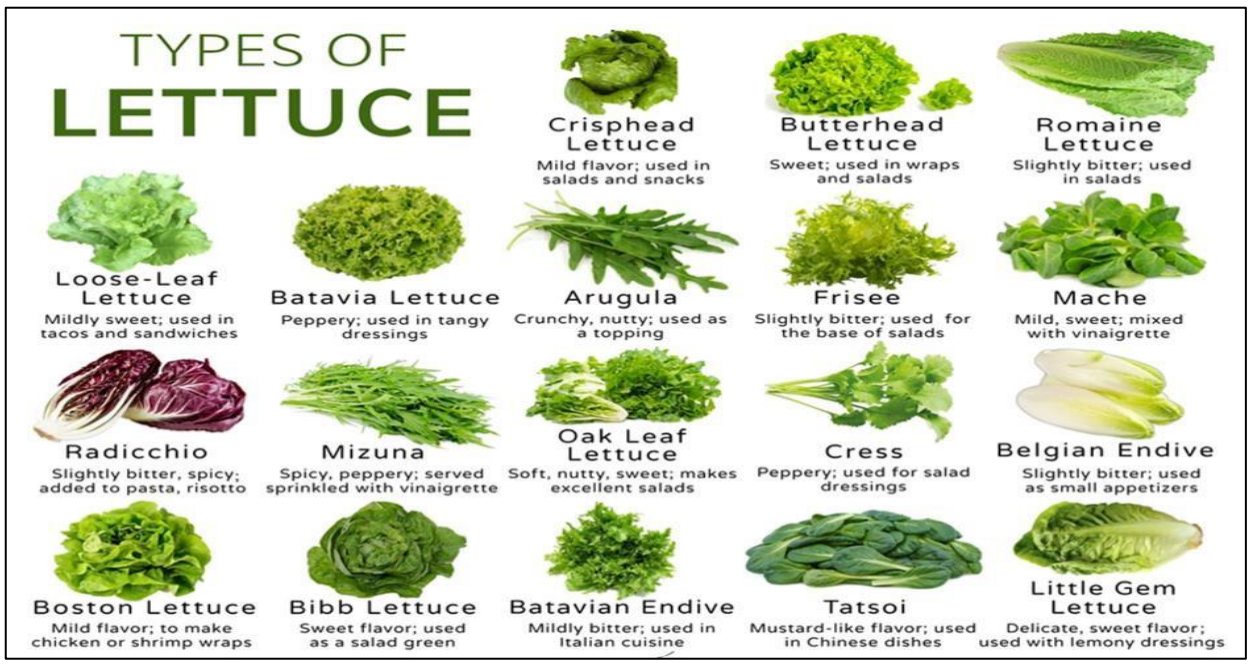


Figure 2: Morphology of some common Lactuca species and their relatives (Hassan et al., 2021)

Lettuce contributes significantly to the nutritional content of diets as it is popularly consumed in salad mixes. Lettuce is generally eaten raw, and this helps retain more nutrients compared to other vegetables, such as potatoes, which are cooked or processed. Lettuce is low in calories, fat, and sodium and is a good source of fiber, iron, folate, and vitamin C. It is also a good source of various other health-beneficial bioactive compounds. Various bioactive compounds in lettuce possess anti-inflammatory, cholesterol-lowering, and anti-diabetic activities. Crisphead lettuce, though widely popular, is comparatively low in minerals, vitamins, and bioactive compounds. However, leaf-type and romaine lettuce are more nutritious with folate content than other rich leafy vegetable sources (Kim et al., 2016).

Hydroponics, or growing plants without soil, is gaining popularity among farmers in the United Arab Emirates, especially in growing local fruits and vegetables with minimal water resources. Vertical farming aids in controlling the plant growth conditions and maximizes crop production with minimum energy, nutritional, labor, and space inputs. Vertical farming systems are ideal for the UAE as this mode of farming counters the challenges presented by the country's limited arable land and water. Supported by the country's beneficial policies, the number of hydroponic farms in the UAE increased from 50 in 2009 to 1,000 in 2016. Lettuce is one of the crops that is amenable to growth in hydroponic media. Taking advantage of this, several initiatives to grow lettuce in vertical farms have started in the UAE.

Improving existing lettuce varieties for water use efficiency, photosynthesis efficiency, salt tolerance, increased nutritional content, etc., is the need of the hour, considering that lettuce is a crop ideal for vertical forming in the UAE. Crops improvement programs involving large-scale production of clonally propagated plants from a minimum number of starting materials (micropropagation) or genetic engineering for enhancing the traits mentioned above require an efficient *in vitro* regeneration system. This study aims to develop a highly efficient *in vitro* regeneration and Agrobacterium-mediated transformation protocol for a popular lettuce variety, 'Paris Island cos, ' belonging to the romaine type.

Previous studies have reported standardization of *in vitro* regeneration and Agrobacterium-mediated transformation protocols for different lettuce varieties(Armas et al., 2017; Curtis et al., 1994). However, this study has not encountered such protocols for 'Paris Island cos.' Developing these protocols in this nutritious variety will pave the foundation for future trait improvement programs in this variety.

### *1.4.3 Agrobacterium-Mediated Transformation of Plants*

*Agrobacterium tumefaciens*, a Gram-negative soil bacterium with a rod-shaped morphology, is recognized as a phytopathogen due to its ability to infect plants naturally, mainly through wound sites, leading to the development of crown gall disease (Figure 3).

Upon infection by Agrobacterium, affected plants form rough, woody, tumor-like galls on their roots, trunks, and occasionally branches. These galls harm plant health, disrupting the normal flow of nutrients and water, thus impeding plant growth and vigor. The mechanism underlying Agrobacterium-mediated infection involves transferring and integrating a specific region known as transfer-DNA (T-DNA) from its tumor-inducing plasmid (Ti) into the plant genome. This process is initiated when Agrobacterium senses the presence of phenolic molecules released from actively growing cells in a plant wound. The integration of T-DNA into the plant genome represents a pivotal step in the pathogenic process of Agrobacterium, ultimately leading to the formation of crown gall disease in infected plants. Once the T-DNA is integrated into the plant genome, the genes encoded within them are expressed. The T-DNA region contains genes that direct the production of phytohormones such as auxin and cytokinin, which can lead to uncontrolled cell proliferation, producing the crown gall tumor. Other genes in the T-DNA region direct the production of novel compounds called opines, which serve as nutrients for the colonizing bacteria (Figure 3) (Flores-Mireles et al., 2012).

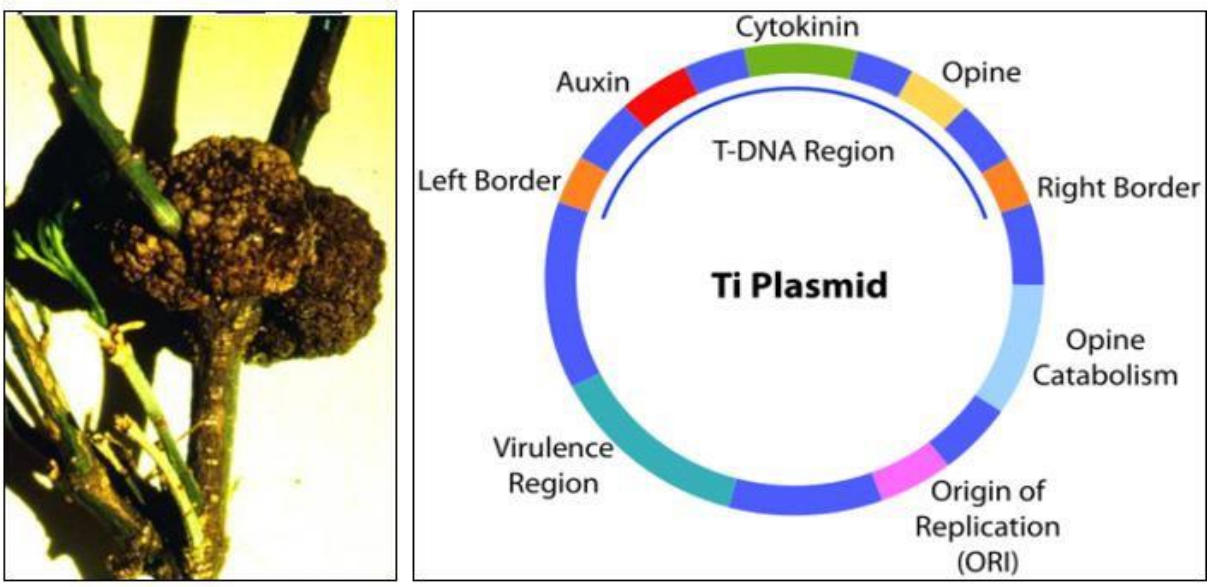


Figure 3: Formation of crown gall on a plant, structure of Ti plasmid (www.wikipedia.org)

Agrobacterium-mediated transfer of T-DNA is a Complex process essential for the genetic modification of plants. The desired genes for this transfer are harbored within the virulence (Vir) region of the Ti plasmid, spanning approximately 30 kilobases. Chemical signaling molecules, such as phenolics and sugars, emanating from wounded plant cells trigger the initiation of T-DNA transfer. These signals are detected by the sensory protein VirA, which initiates a phosphorylation of VirG protein. Upon phosphorylation, VirG orchestrates the expression of various Vir operons, including virB, virC, virD, virE, and virF.

Critical to T-DNA processing are the conserved 25-base pair imperfect repeats at the ends of the T-region, known as border sequences. The virD2 protein, an endonuclease specific to T-DNA borders, cleaves the T-DNA between bases 3 and 4 of the 25 bp T-DNA border and attaches itself to the 5’ end of the T-strand. This enzymatic activity is facilitated by VirD1, which exhibits a topoisomerase-like function.

Subsequently, the single-stranded DNA-binding protein VirE2 binds to the nicked T-DNA, forming a T-complex nucleoprotein complex.

A transmembrane pore composed of proteins encoded by the virB operon facilitates the T-complex's translocation into the plant cell. Upon entry into the plant cell, nuclear localization signals present in VirD2 and VirE2 guide the T-complex to the nucleus, utilizing the plant's nuclear import machinery. VirF, characterized by an F-box motif, potentially destabilizes the T-complex, facilitating targeted proteolysis of VirD2 and VirE2 before T-DNA integration(Figure 4).In addition, several bacterial chromosomal and plant genes play indispensable roles in various stages of the pathogenesis process, including bacterial attachment, signal transduction, nuclear import of T-DNA, targeted proteolysis, and T-DNA integration.

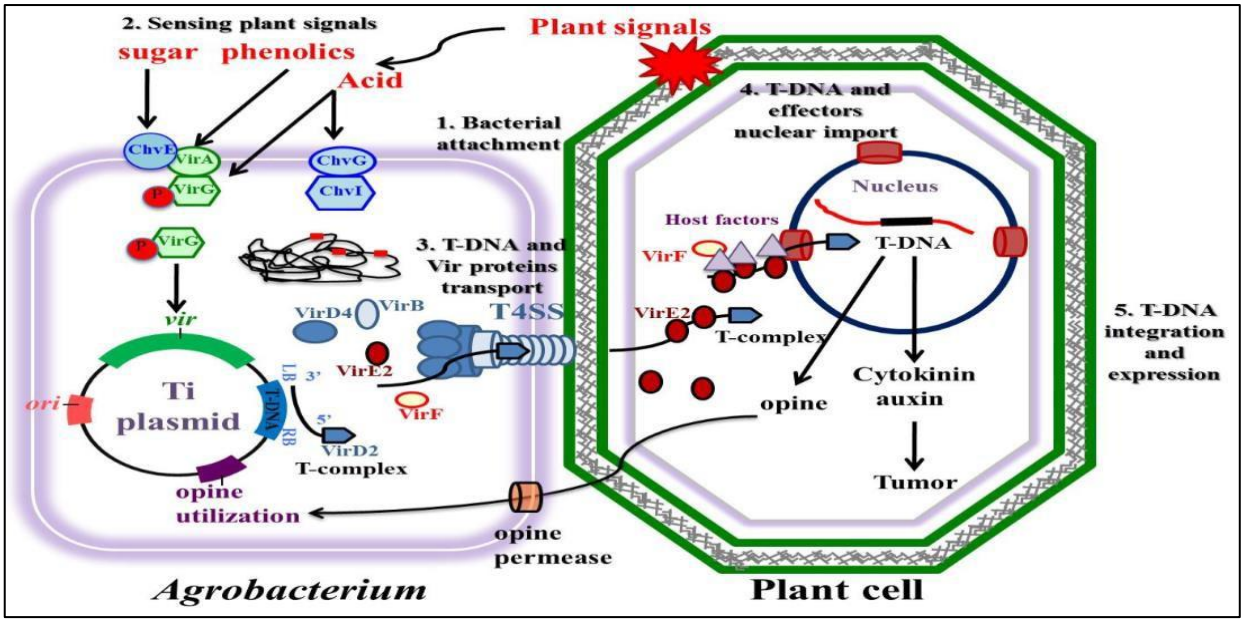


Figure 4: Agrobacterium infection, T-DNA transfer, and integration into the plant genome (1) Attachment of A. tumefaciens to the plant cells. (2) Sensing plant signals by A. tumefaciens and regulating virulence genes in bacteria following transduction of the sensed signal. (3) Generation and transport T-DNA and virulence proteins from the bacterial cells into plant cells. (4) Nuclear import of T-DNA and effector proteins in the plant cells. (5) T-DNA integration and expression in the plant genome (Hwang et al., 2017)

Using Agrobacterium's horizontal gene transfer capability has revolutionized plant genetic modification, facilitating the introduction and integration of genes of interest (GOIs) into plant genomes. The strategy revolves around incorporating the GOI within the T-DNA region of the Ti plasmid, flanked by border sequences, leveraging Agrobacterium-mediated transfer to achieve plant genome integration. However, the native Ti plasmid contains oncogenes responsible for auxin and cytokinin production and opine synthesis genes, which can induce uncontrolled proliferation of recipient plant cells.

A disarmed Ti plasmid lacking oncogenes is employed. While this deletion abolishes tumor-forming capabilities, it preserves T-DNA transfer and integration functionalities. The binary vector system, consisting of two plasmids, serves as a fundamental tool in the process of Agrobacterium-mediated transformation of higher plants. The cloning vector harbors T-DNA borders, multiple cloning sites (MCS), replication functions for *Escherichia coli* and *Agrobacterium tumefaciens*, selectable marker genes, reporter

genes, and supplementary elements crucial for enhancing transformation efficiency. This vector is employed alongside a helper plasmid housed within the selected Agrobacterium strain. They help plant the genome. In plant transformation protocols, the gene of interest (GOI) is typically inserted into the cloning vector's multiple cloning site (MCS), which is flanked by desired promoter and terminator sequences. This recombinant vector is subsequently introduced into Agrobacterium cells that carry a helper plasmid. The cloning vector contains selection markers that allow for the identification of transformed bacteria during the selection process. Subsequently, these bacteria infect the chosen explant. Following co-cultivation, the explant is transferred to a selection medium conducive to the growth of plant cells containing the integrated T-DNA region, carrying both plant selection markers and the GOI (Figure 5).

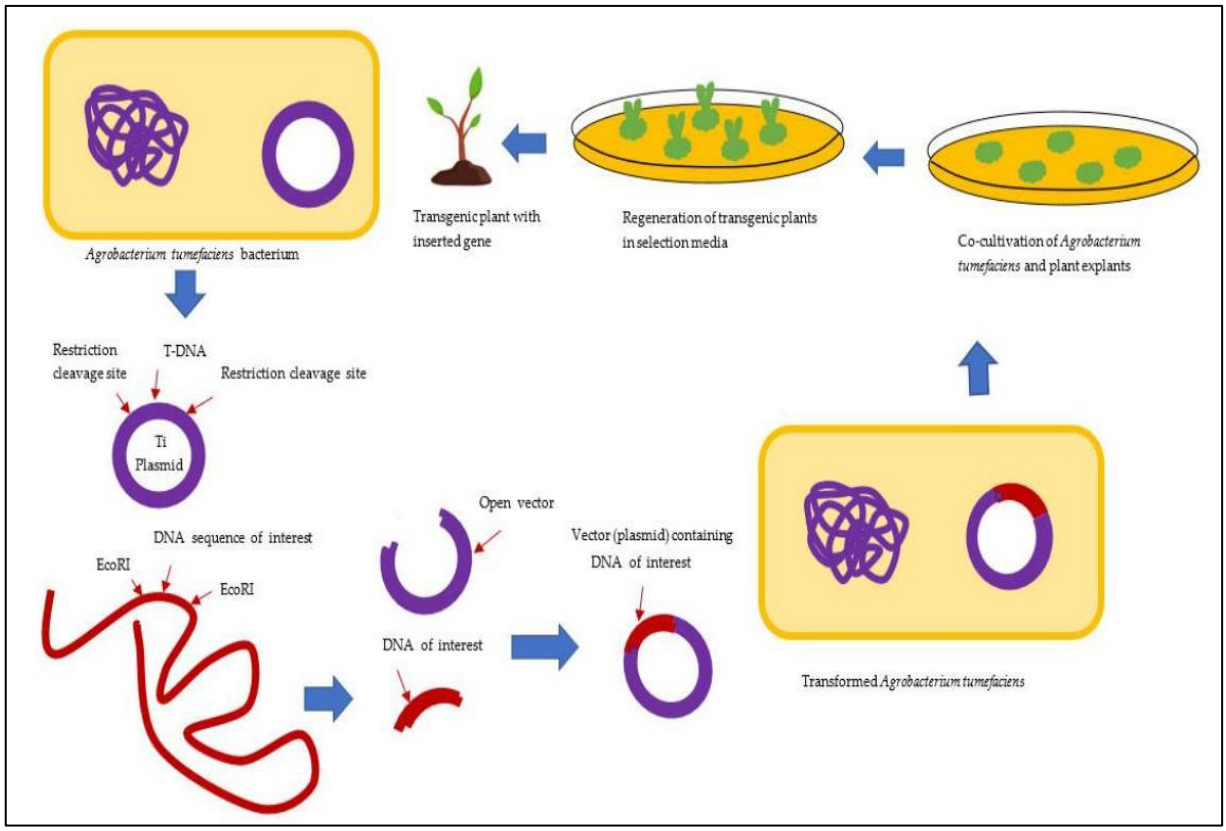


Figure 5: Schematic representation of steps involved in Agrobacterium-mediated genetic transformation of plants (Ghimire et al., 2023)

### *1.4.4 Developing Abiotic Stress-Tolerant Crops*

Extreme environmental conditions such as drought, soil salinity, high variations in temperature, flooding, etc., are detrimental to plant growth and yield and are collectively termed abiotic stresses. Several studies have reported the extent of yield loss in various plant species due to abiotic stresses. It has been estimated that most cultivated crops suffer more than 50% yield losses under various abiotic stresses during their life cycle (Boyer, 1982).

Various abiotic stresses can affect plants alone or together. Under the changing climatic conditions, the incidents of abiotic stresses will be predicted to increase, thus increasing the yield loss (Godoy et al., 2021).

Hence, it is the need of the hour to develop crop plants that can survive under these stress conditions better to sustain their productivity in the future. Plants, being sessile organisms, are unable to avoid abiotic stresses physically. Hence, they have developed morphological, physiological, biochemical, and molecular mechanisms that help them cope with the stresses. At the molecular level, the adverse effects of abiotic stress fall into three broad categories: homeostatic imbalance, disruption of growth and metabolic activities during stress, and generation of Reactive Oxygen Species (ROS). When plants experience changes in their environment, they activate a complex series of processes that begin with stress perception and culminate in activating specific genes. These genes, known as stress-responsive genes, are responsible for the plant's physical, chemical, and biological adaptations that ultimately determine whether the plant will thrive or succumb to the stress (George et al., 2007).

Several stress-responsive genes have been reported previously from model organisms and highly stress-tolerant species. Genetic transformation of sensitive crop plants using these genes was reported to increase their tolerance to stress.

For instance, transgenic tomato plants that overexpress an Arabidopsis C repeat/dehydration-responsive element binding factor 1 (*CBF1*) were reported to be more resistant to drought stress than wild-type tomatoes (Hsieh et al., 2002). Genetic

engineering of apples by over-expressing a cold-inducible *Osmyb 4* gene from rice improved its tolerance to cold and drought stress (Pasquali et al., 2008).

The study conducted by Jin et al. (2009) demonstrated that the overexpression of the stress-responsive transcription factor *DREB1b* increased the tolerance of cold and drought stress in transgenic grapevine.

Their findings underscored the pivotal role of *DREB1b* in enhancing the plant's ability to withstand adverse environmental conditions, thereby offering potential strategies for improving stress resilience in crops.

Similarly, research conducted by Khare et al. (2010) focused on introducing a mannitol-1-phosphate dehydrogenase (*mtlD*) gene from bacteria into tomato plants. The study revealed that the transformed tomato plants exhibited enhanced tolerance to abiotic stresses, presumably attributed to the role of *mold* in modulating plant responses to environmental challenges. This investigation shed light on the potential utility of genetic engineering approaches in improving crop stress tolerance mechanisms.

*Salicornia brachiata,* recognized for its high salt tolerance, thrives in saline environments, demonstrating a dependence on salt for optimal growth. Previous investigations have highlighted its significance as a model plant for explaining the salt adaptation and tolerance mechanisms characteristic of halophytes. Studies conducted by (Jha et al., 2009; Kumari et al., 2017) have mainly utilized *Salicornia brachiata* to delve into these mechanisms.*SbSI-1*, identified as a novel Salt Inducible nuclear protein gene derived from *Salicornia brachiata*, stands out as a noteworthy subject of study. This gene is particularly interesting due to its potential implications in understanding the molecular pathways underlying salt stress response and adaptation mechanisms within halophytes.

The study findings indicate that transgenic tobacco plants, engineered to overexpress the *SbSI-1* gene, demonstrated notable improvements in various growth parameters, including enhanced growth performance, seed germination rates, cell viability, pigment contents, and starch accumulation. Moreover, these transgenic tobacco plants exhibited higher tolerance to drought and salt stress conditions, as evidenced by higher tolerance

indices than non-transgenic counterparts. However, it is essential to note that there is currently no available literature reporting the transformation of the *SbSI-1* gene in the lettuce cultivar Paris Island cos.

# Chapter 2: Material and Methods

## 2.1 Materials and Reagents

All materials and reagents used in this study are mentioned in Appendices (Appendix A-D).

Table 1: List of primers name and sequences used in this study

| **Primer name** | **Sequence 5′ - 3′** | **No. of bp** |
|---|---|---|
| RFP_FRAG_F | TCACTGTTGATACATATGGCCTCCTCCGAGAAC | 33 |
| RFP_FRAG_R | TTCAGAATTGTCGACCTACAGGAACAGGTGGTGG | 34 |
| *SbSI*_FRAG_F | TCACTGTTGATACATATGATGCCTAATAAACATATCATGGAACAACC | 47 |
| *SbSI*_FRAG_R | TTCAGAATTGTCGACTTAACGCTTCCCTTGTT | 32 |
| pRI201-AN_VEC_F | AATTCTGAATCAACAACTCTCCTGGC | 26 |
| pRI201-AN_VEC_R | TATCAACAGTGAAGAACTTGCTTTTGATCTTT | 32 |
| GENE1_SEQ_F | CACGGGGGACTCTAGATACATCAC | 24 |
| GENE1_SEQ_R | CTTAAGCACACAAGCTAGCTTTTTATTTGACAC | 33 |
| M13F | GTAAAACGACGGCCAGT | 17 |
| M13R | CAGGAAACAGCTATGAC | 17 |
| LS_TUB_RT_F | TAGGCGTGTGAGTGAGCAGT | 20 |
| LS_TUB_RT_R | AACCCTCGTACTCTGCCTCTT | 21 |
| *SbSI*_ RT_F | ACAACAATTTTTCACAAACCTCCA | 24 |
| *SbSI*_ RT_R | GAACGAAGAAGGGGAATGGTG | 21 |

## 2.2 Polymerase Chain Reaction (PCR)

All standard PCR reactions in this study were conducted according to the following protocol: PCR reaction mix was prepared and distributed into individual PCR tubes, with the final concentrations of components set as follows: Taq DNA Polymerase Buffer at 1X concentration, dNTPs at 0.25 mM each, specific forward and reverse primers at 50 ng each, Taq DNA Polymerase at 0.1 µl, and a DNA template concentration between 50-100 ng in 20 µl reaction. PCR reaction conditions were as follows: an initial denaturation step at 94°C for 5 minutes to facilitate pre-amplification denaturation, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at a temperature (X°C) specific to the primer pair for 30 seconds (where X°C varies with the annealing temperature of the primers), extension at 72°C for a duration determined by the length of the amplicon,

and a final extension step at 72°C for 5 minutes. After amplification, PCR products were visualized by electrophoresis on an agarose gel, DNA size markers, negative controls and positive controls. This approach ensured the accurate assessment of PCR amplification products and the validation of experimental results.

### 2.3 Agarose Gel Electrophoresis

Agarose powder was weighed per the desired gel percentage and dissolved using 1X TBE buffer. The solution was heated to boiling and cooled to warm temperatures using an ice bath. 5 µl of the DNA staining solution HydraGreen was added tandpoured into gel loading trays with the combs in place. The gel was aet, and electrophoresis was carried out in 1X TBE buffer at a voltage ranging from 60 -120 V for 15-30 minutes, depending on specific purposes.

### 2.4 Isolation and Purification of Plasmid DNA

Plasmid DNA extraction is a fundamental molecular biology procedure crucial for various downstream applications. This study employed the QIAprep Spin Miniprep Kit from Qiagen for efficient plasmid isolation , the isolation prortocl was provided by the manufacturer. Bacterial cells were harvested and resuspended in Buffer P1, followed by the addition of Buffer P2 to denature proteins and release plasmid DNA. Buffer N3 was then added to precipitate proteins and genomic DNA, with the resulting supernatant containing plasmid DNA transferred to a spin column. After washing to remove impurities, purified DNA was eluted using Buffer EB. The eluate, containing plasmid DNA, was suitable for downstream applications. Analysis for quality and quantity was performed using agarose gel electrophoresis and spectrophotometric estimation. This method offers a simple and efficient approach for isolating purified plasmid DNA, facilitating various molecular biology experiments.

### 2.5 Purification of DNA Fragments from Agarose Gels

The QIAquick Gel Extraction Kit was employed to purify DNA fragments from agarose gels. Initially, the desired DNA fragment was carefully excised from the gel and dissolved in Buffer QG at 50°C. After vortexing and dissolution, isopropanol was added

to the sample, which was then transferred to a QIAquick spin column and centrifuged. The flow-through was discarded, and the column was washed with Buffer QG and Buffer PE to remove any impurities. Finally, the purified DNA was eluted using Buffer EB and analyzed for quantity and purity.

This method efficiently purifies DNA fragments from agarose gels, providing high-quality DNA suitable for downstream applications.

### 2.6 Spectrophotometric Estimation of Nucleic Acids

The concentration and purity of nucleic acids in the solution were determined using a Shimadzu UV-visible spectrophotometer (UV1601), and absorbance was measured at 260 nm and 280 nm wavelengths. An optical density (OD) of 1 at 260 nm signifies a concentration of 50 µg of double-stranded DNA or 40 µg of RNA. We also assessed the purity of the nucleic acids by calculating the A260/A280 ratio, which provides information on the level of protein contamination in the nucleic acid sample.

### 2.7 Preparation of *E. coli* Competent Cells and Transformation

In the protocol outlined by Chung et al. (1989), *Escherichia coli* (E. coli) cells are cultured in LB broth until reaching an optical density at 600 nm (A600) of 0.5-0.6, indicating exponential growth. After centrifugation and resuspension in ice-cold TSS solution, competent cells are frozen in liquid nitrogen for long-term storage at -80°C. Thawed cells are immediately used for transformation experiments.For transformation, competent cells are mixed with a ligation mix or intact plasmid and incubated on ice for 30 minutes. A brief heat shock at 42.5°C for 45 seconds is applied, followed by adding LB medium and further incubation at 37°C with shaking for 1 hour to allow recovery and plasmid expression. Transformed cells are then centrifuged, and the medium is partially removed before carefully resuspending the cells.Transformed cells are plated onto LB plates with appropriate antibiotics for colony selection and incubated overnight at 37°C to promote colony formation. Subsequent colony PCR analysis identifies transformed cells containing recombinant plasmids. This concise protocol offers a streamlined approach for preparing and transforming competent *E.coli* cells, facilitating efficient molecular biology experiments.

## 2.8 Colony PCR for Selection of Transformants

Recombinant colonies were identified utilizing the colony PCR method. Initially, surviving bacterial colonies were individually picked using sterile toothpicks and streaked onto LB medium supplemented with the appropriate antibiotic selection for subsequent growth. Following streaking, a sterile toothpick carrying bacterial cells from the selected colonies was placed into a 0.2 ml PCR tube containing sterile water.A PCR mix was prepared according to established protocols and aliquoted into individual PCR tubes.

The PCR setup included samples from the bacterial colonies, a DNA ladder for size reference, negative controls containing all PCR components except bacterial cells, and positive controls containing the plasmid.Subsequently, the PCR products were subjected to electrophoresis on an agarose gel to visualize the amplification results. This process allowed for identifying and verifying recombinant colonies based on the presence or absence of the expected DNA bands, compared against the controls and DNA ladder.

## 2.9 Cloning of RFP Visual Marker Gene and *SbSI-1* Gene from *Salicornia brachiata* into Plant Transformation Vector pRI201-AN

The coding region (CDS) of the Red Fluorescent Protein (RFP) gene was obtained as part of a plasmid from addgene (Plasmid #68220, www.addgene.org). The coding region of the *SbSI-1* gene from *Salicornia brachiata* was commercially synthesized. CDS were amplified from respective plasmids using forward and reverse primers designed for in-fusion cloning (Table 1).

RFP CDS was amplified using RFP_FRAG_F and RFP_FRAG_R. SbSI-1 CDS was amplified using primers SbSI_FRAG_F and SbSI_FRAG_R. the processe of in-fusion cloning is shown in figure 6. The amplification PCR was carried out using the high-fidelity Taq polymerase, CloneAmp™ HiFi PCR polymerase (Takara Biosciences, Japan). The 25 l PCR mix contained 12.5 l CloneAmp HiFi PCR Premix, 1.25 l each of the forward and reverse primers, and 10 ng plasmid; the final volume was made up to 25 µl with nuclease-free water. The reaction conditions were as follows: pre-amplification denaturation at 98°C for 30 sec, 20 PCR cycles at 98°C for 10 sec, 60°C for 20 sec, 72°C

for X sec* (*—varies with the length of the amplicon, 5 s/kb), and final extension at 72°C for 5 min.

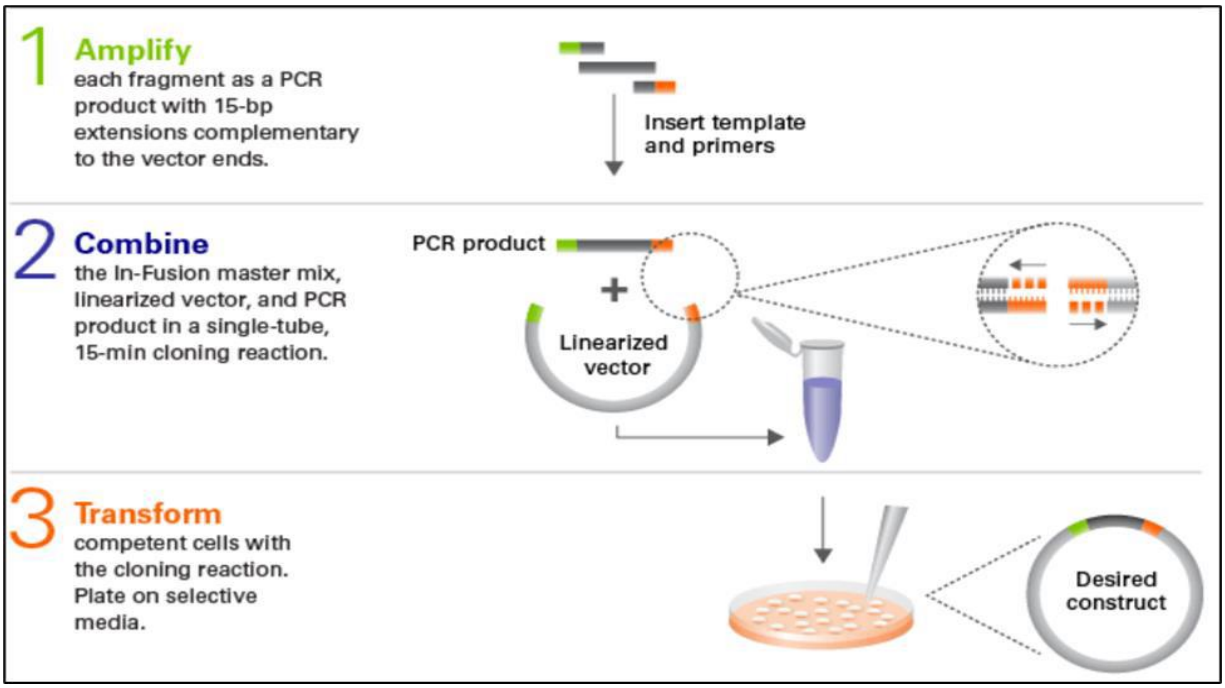


Figure 6: Overview of In-Fusion Cloning (www.takarabio.com)

pRI201-AN vector was linearized using primers pRI201-AN_VEC_F and pRI201-AN_VEC_R (Table 1) using HiFi PCR polymerase. The linearization allows the candidate genes to be cloned in Multiple Cloning Site (MCS) 1 of pRI201-AN between the NdeI and SalI restriction enzyme sites, keeping both sites intact after cloning. The PCR extension time was modified to allow amplification of the 10 kb vector. The PCR products were electrophoresed in an agarose gel.

Cloning of coding regions of the RFP gene and *SbSI-1* gene individually to MCS1 of the pRI201-AN vector was carried out by In-Fusion seamless cloning (Takara Biosciences, Japan). To clone, an in-fusion cloning mixture was prepared to contain 100 ng linearized vector, 50 ng fragment (RFP/ SbSI-1), and 2 l of 5X In-Fusion HD Enzyme Premix; the volume was made up to 10 µl with sterile water. The mix was incubated at 50°C for 15 minutes and chilled on ice for 5 minutes. The in-fusion mix was used for the transformation of *E. coli* cells. After transformation, the cells were plated on LB agar media containing 50 µg/ml kanamycin. *E.coli* cells surviving anti-antibiotic-containing

tens were surveyed for the presence of recombinant plasmid by colony PCR using primers GENE1_SEQ_F and GENE1_SEQ_R .Plasmid was isolated from PCR-positive single bacterial colonies.

### 2.10 Sequencing of Recombinant Plasmids

The plasmids were sequenced at an external facility (Macrogen, Korea) using primers GENE1_SEQ_F and GENE1_SEQ_R. The sequence of the insert was confirmed using Snapgene software (www.snapgene.com).

### 2.11 *In Silico* Analysis

The sequence of the *SbSI-1* gene from *Salicornia brachiata* was obtained from prior studies (Kumari et al., 2017). To identify the Open Reading Frames (ORFs) within *SbSI-1*, the 'Translate tool' available on EXPASY (www.expasy.org) was utilized, and the putative coding region was determined based on the longest ORF exhibiting predicted start and stop codons. Subsequently, protein subcellular localization prediction was performed using the WoLF PSORT tool (https://wolfpsort.hgc.jp/). Predictions regarding molecular mass were conducted using the ProtParam tool on EXPASY (www.expasy.org), while putative transmembrane predictions were generated using CCTOP (https://cctop.ttk.hu/).To assess sequence similarity, the BLASTP program was used to search against the non-redundant protein sequences (nr) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### 2.12 Plant Material and Sterilization

The lettuce cultivar Paris Island cos seeds were acquired from commercial vendors; seeds were prepared for experimentation. First, we soaked the seeds in water overnight and then subjected them to a 10% sodium hypochlorite (NaOCl) solution, specifically Clorox, with two drops of a surfactant called Tween 20. After 10 minutes of gentle agitation, the seeds were rinsed with sterilized distilled water to eliminate any residual traces of bleach. Then, they dried the seeds on a sterile filter paper before placing them onto germination media within Petri dishes . The Petri dishes containing the seeds were kept in dark conditions at approximately 25°C until the radicle emerged, which typically

occurred within 3-4 days. The dishes were transferred to a light environment characterized by a 16-hour light cycle and 8-hour dark cycle, with a daytime temperature of 26°C and a nighttime temperature of 24°C.

The humidity was maintained at approximately 70%. Throughout the experiments, 7-day-old seedling lettuce cotyledons were used as explants.

### 2.13 Standardization of *In Vitro* Shoot Segeneration of Lettuce

Murashige and Skoog (MS) media (see appendix A) , served as the foundational medium for this study, comprising macro-elements, micro-elements, vitamins, and 3% sucrose. The powder was dissolved in water following manufacturer instructions, and the pH was adjusted to 5.8 using either HCl or NaOH based on pH meter readings. Subsequently, phyto agar was incorporated to achieve a concentration of 0.8%. The media underwent sterilization via autoclaving at 121°C and 15 psi for 20 minutes. Stock solutions of various Plant Growth Regulators (PGRs) were prepared at a concentration of 1 mg/L in appropriate solvents. For *in vitro* regeneration experiments, the media was melted and allowed to cool before desired PGRs were added (Table 2), ensuring aseptic conditions. The media was then poured into Petri dishes to solidify completely. Explants from 7-day-old lettuce seedlings, specifically cotyledons, were utilized for transformation studies. The base of lettuce cotyledons was excised, and the explants were transferred to the prepared culture medium. Plates were maintained under a light cycle of 16 hours of light and 8 hours of dark, with a temperature of 26°C during the day and 24°C at night, alongside a humidity level of approximately 70%. Subsequent subculturing of the explants into the same medium occurred after two weeks.

Data about the percentage of explants forming callus, shoots, roots, and the number of shoots per explant, etc., were collected after 28 days in culture. All experiments were conducted with three replicates comprising 12 explants per plate.

### 2.14 Rooting and Acclimatization

After 28 days of growth, healthy shoots exceeding 1 cm in length were selected and carefully moved to 3% MS media without hormones to trigger the rooting process. These

plantlets were kept in the rooting media for three weeks for proper root development. Once the plantlets produced strong roots, they were gently removed from their bottles and rinsed thoroughly to remove any remaining medium. The plantlets were then planted in pots filled with a soil and compost mixture in a 2:1 ratio.

All pots were covered with perforated polythene covers to facilitate acclimatization for two weeks. After a two-week acclimatization period in the growth room, the covers were removed, and the plantlets were moved to larger pots to support further growth and development. This process ensured a smooth transition for the plantlets from *in vitro* culture to ex-vitro conditions.

Table 2: Plant Growth Regulator combinations used for standardizing *in vitro* shoot regeneration of lettuce from cotyledons

| **Media** | **NAA (mg/L)** | **IAA (mg/L)** | **Kinetin (mg/L)** | **BAP (mg/L)** | **Zeatin (mg/L)** | **Activated charcoal (mg/L)** |
|---|---|---|---|---|---|---|
| LM1 | 0.025 | 0.025 | 1.0 | | | |
| LM2 | 0.05 | 0.05 | 0.5 | | | |
| LM3 | 0.05 | 0.05 | 2.0 | | | |
| LM4 | 0.5 | 0.5 | 2.0 | | | |
| LM5 | 1.0 | 1.0 | 2.0 | | | |
| LM6 | 0.1 | | | 0.1 | - | - |
| LM7 | 0.1 | | | 0.5 | - | - |
| LM8 | 0.05 | | | 1.0 | - | - |
| LM9 | 0.1 | | | - | 2.0 | - |
| LM10 | 0.25 | | | 5 | - | 200 |
| LM11 | 0.5 | | | 10 | - | 200 |

## 2.15 Analysis of the Inhibitory Effect of Kanamycin on *in vitro* Shoot Regeneration of Lettuce

Media combination LM6, which was found to be ideal for shoot regeneration from lettuce explants, was used to analyze the inhibitory effect of kanamycin on shoot regeneration. Cotyledon explants prepared as described above were placed on LM6 media (3% MS, 0.1 mg/L NAA, 0.1 mg/L BAP) supplemented with various

concentrations of the antibiotic kanamycin (10, 20, 30, 50 mg/L). LM6 media without any antibiotic was used as a control in this experiment.

All media combinations also contained 300 mg/L of timentin, which was used to stop Agrobacterium's overgrowth after transformation. The explants were subcultured after 2 weeks into the same media. Explant viability and growth were observed weekly, and data on the percentage of explant viability and shoot growth were observed after four weeks. The experiment involved three replicates of 12 explants each.

### 2.16 Preparation of Agrobacterium Competent Cells and Transformation

Agrobacterium-competent cells were prepared following the method outlined by Kámán-Tóth et al. (2018). Initially, a single colony of Agrobacterium LBA4404 was inoculated into 25 ml of LBA media supplemented with 10 mg/L rifampicin and cultured overnight at 28°C with agitation at 230 rpm. The bacterial cells were then spread onto LB agar plates containing 10 mg/L rifampicin and incubated overnight at 28°C to ensure the formation of a bacterial layer covering the plate surface.

After collection from multiple plates to obtain sufficient volume, the bacterial cells were washed and resuspended in using 4ml ice-cold 10% (v/v) sterile glycerol. The suspension was divided into microcentrifuge tubes, centrifuged, and resuspended successively in ice-cold 10% (v/v) sterile glycerol to yield a concentrated competent cell suspension. Aliquots of this suspension were frozen in liquid nitrogen and stored at -80°C until further use.

For electroporation, a mixture of plasmid DNA and Agrobacterium cell suspension was prepared and transferred into a cold electroporation cuvette. Electroporation was performed using specific parameters 2.5 kV voltage, 25 μF capacitance, and 400 Ohm resistance. Following the pulse, LBA medium was added to the cuvette, and the bacterial suspension was transferred to a centrifuge tube. After incubation at 28°C for 2 hours, the transformed cells were spread onto selective agar plates and incubated for 2 days at 28°C. Positive Agrobacterium colonies were selected based on antibiotic resistance and further verified using colony PCR with specific primers GENE1_SEQ_F and GENE1_SEQ_R. This method of competent cell preparation and electroporation enables

efficient transformation and offers a robust approach for various molecular biology applications in Agrobacterium-mediated genetic engineering.

### 2.17 Agrobacterium-Mediated Transformation of Lettuce

Lettuce seeds were germinated as described above; for agrobacterium-mediated transformation, the cotyledons of 7-day-old seedlings were used. The base of the cotyledons was cut off, and 2-3 shallow wounds were made on the adaxial side of cotyledon explants with a sharp, sterile blade. The explants were placed on LS-Preculture media for 24 hours under light for pre-culture.

pRI201-AN vectors cloned with RFP/*SbSI-1* coding regions (1RED-AN, 1SbSI-AN) were transformed to Agrobacterium strain LBA4404 competent cells through electroporation as described above. Cells from a single positive bacterial colony harboring the desired vector were grown in a liquid LB medium with 50 mg/L kanamycin and 10 mg/L rifampicin to OD 0.5 at 600 nm. The bacterial cells were resuspended in an equal volume of liquid LS-Infection medium.

The precultured explants were infected using the infection bacterial solution for 10 minutes with mild shaking. The explants were then briefly dried and co-cultivated in solid LS-CC media at 22°C in the dark for three days. The explants were then transferred to selection media; LS-PSM (See appendix C), supplied with 50 mg/L kanamycin and 300 mg/L timentin and maintained at a light cycle of 16 light and 8 hours dark, temperature of 26 a day and 24°C night and humidity of 70%. After two weeks, the explants were sub-cultured to the same selection media (LS-PSM). After one month of growth in the selection media, the well-formed regenerated shoot explants were moved to rooting media, LS-rooting, in bottles and allowed to grow for three weeks. The rooted plants were hardened, as described in the above sections.

### 2.18 Fluorescence Analysis and Microscopy

In the initial transformation experiments, the efficiency of the transformation protocol was evaluated using the recombinant vector 1RED-AN. The presence of red fluorescence in explants and surviving shoots grown on selection media was assessed using fluorescence microscopy. This involved utilizing a Zeiss Discovery V12

microscope equipped with a Zeiss Rhodamine cube KSC 295–815D for excitation at 540/25 and emission at 605/55 wavelengths.

Images were captured using an Optronics digital camera (Nikon D60) with manual exposure settings. Additionally, images under bright fields were recorded using consistent exposure times for all samples.

### 2.19 Isolation of Plant Genomic DNA

Genomic DNA isolation from plant tissues followed a modified protocol used by Murray and Thompson (1980). Initially, 3-5 grams of plant tissue were ground using liquid nitrogen to maintain tissue integrity. The powdered tissue was then mixed with 10 ml of CTAB buffer and incubated at 65°C for 30 minutes to facilitate cell lysis and release of DNA. After centrifugation at 10,000 RPM for 10 min, the upper aqueous phase containing the DNA was carefully transferred to a separate tube.

Isopropanol was added to the aqueous phase to precipitate the DNA, which was subsequently stored at -20°C for 30 minutes to enhance precipitation.Following centrifugation, the DNA pellet was washed with 70% ethanol to remove contaminants, air-dried, and dissolved in TE buffer containing 10 µg/ml RNase A. The mixture was incubated at 37°C for 1 hour to remove RNA contaminants. Further purification of the DNA was achieved through phenol-chloroform extraction, with the aqueous phase transferred to a fresh tube after each extraction step. To precipitate the DNA again, 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the aqueous phase. After centrifugation, the DNA pellet was washed with 70% ethanol, air-dried, and dissolved in TE buffer for downstream applications.This method ensures the isolation of high-quality genomic DNA suitable for various molecular biology analyses, including PCR, restriction digestion, and sequencing.

### 2.20 PCR Analysis of Transgenic Plants

Total genomic DNA was isolated from a regenerated lettuce plant in LS-PSM media as described in section 2.19. The incorporation of the T-DNA region containing RFP/ *SbSI-*

*1* coding regions was confirmed using PCR with primers M13 forward and M13 reverse to amplify the 35S promoter–coding region-terminator segment.

### 2.21 Isolation of Total RNA from Plant Tissue

Total RNA extraction from plant tissues followed the protocol established by Chomczynski and Sacchi in 1987 with minor modifications. Plant tissue (3-10 grams) was ground into a fine powder using liquid nitrogen, followed by mixing with 18 ml of RNA extraction solution and allowing it to thaw. The sample was then transferred to a sterilized tube containing 1.8 ml of 2 M sodium acetate (pH 4.0). After adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), vortexing, and ice incubation, the lysate was centrifuged at 13,000 rpm for 30 minutes at 4°C.

The upper aqueous phase containing RNA was collected and mixed with an equal volume of isopropanol for overnight precipitation at -20°C. Following centrifugation, the RNA pellet was washed with chilled 70% ethanol, air-dried, and resuspended in an RNA extraction solution. Further RNA purification involved another round of isopropanol precipitation, centrifugation, ethanol washing, and pellet resuspension in nuclease-free water. The quantity and quality of the RNA sample were assessed using agarose gel electrophoresis and a nanodrop test. This protocol ensures the extraction of high-quality total RNA suitable for downstream applications such as reverse transcription, PCR, and gene expression analysis.

### 2.22 Reverse Transcription and cDNA Synthesis

The QuantiTect® Reverse Transcription Kit from Qiagen, USA, facilitated the generation of complementary DNA (cDNA) from total RNA. Initially, total RNA stored at -80°C was thawed on ice. In contrast, gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water were thawed at room temperature. To eliminate genomic DNA, 1 µg of total RNA was mixed with 2 µl of 7X gDNA Wipeout Buffer, and RNAse-free water was added to make up a final volume of 14 µl. This mixture was incubated for 2 minutes at 42°C and then placed on ice.Subsequently, a reverse transcription reaction mix was prepared using the 14 µl genomic DNA wiped-out mixture sample. Quantiscript Reverse Transcriptase (1 µl), 5X

Quantiscript RT Buffer (4 µl), and RT Primer Mix (1 µl) were added, bringing the total reaction volume to 20 µl.

The mix was incubated for 15 minutes at 42°C, followed by 3 minutes at 95°C to inactivate the Quantiscript Reverse Transcriptase. This process efficiently converted total RNA into cDNA, facilitating downstream molecular biology applications.

### 2.23 Quantitative Real-Time PCR (qRT-PCR)

Quantitative Real-time PCR (qRT-PCR) was employed to evaluate transgene expression levels in transgenic plants. The QuantiTect Reverse Transcription Kit from Qiagen was utilized for RNA reverse transcription, following the protocol in section 2.22. Gene-specific primers were designed using Primer 3.0 software (Table 1). The primer design criteria included: an amplicon length within the 70-150 bp range, a GC content between 20 to 80%, avoidance of runs of identical nucleotides, a primer melting temperature (Tm) of 58 to 60°C, and no more than two G and/or C bases within the five nucleotides at the 3′ end. Amplicon sites were selected to ideally span one or more introns to prevent genomic DNA amplification, and primer pairs were designed to be specific to the target gene, avoiding amplification of pseudogenes or related genes.

PowerUp™ SYBR™ Green Master Mix was used to prepare the the qRT-PCR reaction mixture, and profiling was conducted using the StepOnePlus™ Real-Time PCR System. Thermal cycling conditions comprised an initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

Each qRT-PCR analysis included three biological and three technical replicates to ensure result robustness and reproducibility. The lettuce tubulin gene was selected as the internal reference gene for normalization. Relative gene expression levels were evaluated using the 2^(-ΔΔCT) method outlined by Livak and Schmittgen (2001).

Ct values were recorded in an Excel spreadsheet, representing the cycle threshold where fluorescent intensity surpasses background levels. ΔCt was calculated by subtracting the Ct value of the reference gene from that of the target gene. Subsequently, ΔΔCt was determined by subtracting the ΔCt of the control sample from that of the treated sample.

The Negative value (-ΔΔCT) was utilized as the exponent of 2 in the equation to determine the fold change of the treated target gene relative to the control.

### 2.24 Data Collection and Statistical Analysis

The data were statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All data were subjected to a one-way analysis of variance (ANOVA), and means were compared using Duncan's multiple range test (DMRT). Differences in means ± SD (standard deviation) were analyzed using DMRT at "Duncan's multiple range test" at $P \leq 0.05$.

# Chapter 3: Results and Discussion

## 3.1 Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

This study employed HotStarTaq DNA Polymerase from Qiagen for general PCR reactions. This polymerase utilizes a chemically mediated hot start mechanism, ensuring complete inactivation until the initial heat activation step at the onset of PCR. Supplied with QIAGEN PCR Buffer, HotStarTaq DNA Polymerase effectively minimizes nonspecific amplification products and primer dimers, enhancing the specificity of PCR amplification. The polymerase exhibits an extension rate of 2–4 kb/min at 72°C, with a half-life of 10 minutes at 97°C and 60 minutes at 94°C. In addition, it demonstrates an amplification efficiency of ≥105 fold compared to conventional Taq polymerase. The PCR conditions detailed in section 2.2 were successfully employed to amplify desired fragments from plasmids, plant genomic DNA, and colony PCRs. For most PCRs in this study, 30 cycles were sufficient to amplify the desired fragments. However, in rare instances where the amplified fragment appeared faint, the number of cycles was increased to 35.

Remarkably, less than 20 ng of template DNA proved adequate for successfully amplifying fragments, underscoring the efficiency and sensitivity of DNA Polymerase in PCR applications. from the plasmid DNA, while amplification from genomic DNA required template concentrations between 50 to 100 ng. The percentage of agarose in agarose gel electrophoresis was decided based on the expected amplicon size. Lower concentrations were used for large fragments; higher concentrations were used for smaller ones. An agarose concentration range of 0.8% to 1.5% was sufficient for this study.

## 3.2 Isolation and Purification of Plasmid DNA

This study employed the QIAprep Spin Miniprep Kit from Qiagen to isolate Plasmid DNA. This kit offers a streamlined procedure by eliminating the need for Phenol extraction and ethanol precipitation, thus simplifying DNA purification. The methodology is based on the modified alkaline lysis method described by (Birnboim & Doly, 1979).

Initially, bacterial cells were lysed under alkaline conditions, facilitating the release of plasmid DNA. Subsequently, the lysate was neutralized and adjusted to high-salt–binding conditions to optimize DNA binding to the silica membrane. Upon centrifugation, the cleared lysate was ready for purification, with the membrane selectively retaining DNA while allowing passage of RNA, cellular proteins, and metabolites. A brief wash step with Buffer PE efficiently removed salts, ensuring the purity of the isolated plasmid DNA.

The purified DNA was eluted from the column using Buffer EB. During this study, significant quantities of plasmid DNA ranging from 4 µg to 10 µg were typically isolated. Notably, the quantity of isolated plasmid correlated with its size, with smaller plasmids yielding larger quantities and vice versa. Importantly, plasmids isolated using this method exhibited minimal RNA and protein contamination, making them suitable for direct utilization in downstream studies without the need for further purification steps.

### 3.3 Purification of DNA Fragments from Agarose Gels

This study used the QIAquick Gel Extraction Kit to elute DNA fragments from agarose gels. The PCR products containing the desired fragments were run on agarose gels. During electrophoresis and subsequent steps, special care was taken to avoid fragment degradation and contamination. Fresh 1X TBE buffer was used for electrophoresis, the voltage was set to less than 70V, and care was taken not to exceed 20 minutes of run time. The fragment was excised with a little excess gel around the band. The gel slice was excised as quickly as possible, as exposure to UV light damages DNA.

The results showed that the quantity of eluted DNA was higher for smaller fragments (0.5 – 1 µg), while for larger fragments like the linearized vector, less quantity was obtained (0.1 – 0.5 µg). incubating the sample at 37°C for 10 min increased the quantity of the DNA elution. Additionally, a second elution was performed to increase the net amount of DNA whenever necessary.

### 3.4 Spectrophotometric Estimation of Nucleic Acids

In this study, we assessed the purity of nucleic acids using spectrophotometric analysis, which measures absorbance at specific wavelengths (260 nm and 280 nm) using a spectrophotometer. The purity of DNA and RNA samples was determined by the A260/A280 ratio, with the ideal range being 1.8 to 2.0 for pure DNA and RNA, respectively. A lower A260/A280 ratio suggests the presence of contaminants like proteins, while strong absorbance at 270 nm and 275 nm indicates possible phenol contamination. The study's nucleic acid samples isolated using commercially available kits consistently met quality and quantity standards. However, some samples isolated using laboratory-made buffers and protocols initially fell below expectations. In such cases, we repeated the experiment to ensure high-quality nucleic acids.

### 3.5 Preparation of *E. coli* Competent Cells and Transformation

In this study, we used the *E.coli* strain DH5-Alpha to transform the ligation mix and increase the quantity of plasmids. DH5-Alpha is a strain engineered explicitly for laboratory cloning, boasting multiple mutations that enhance its transformation efficiency.

The mutations present in the DH5-Alpha strain include dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1) (Durfee et al., 2008). These mutations confer various advantages, such as enabling blue-white screening for recombinant cells (due to the lacZ Delta M15 mutation), reducing endonuclease degradation (endA1 mutation), and minimizing homologous recombination (recA1 mutation) for stable inserts. Furthermore, DH5-Alpha is not resistant to common antibiotics, making it suitable for transforming and selecting plasmids containing antibiotics like kanamycin and ampicillin. To prepare competent DH5-Alpha cells, we utilized a one-step protocol described by Chung et al. (1989). Previous experiments conducted at KCGEB have demonstrated the efficiency and simplicity of this method compared to the conventional $CaCl_2$ method. This study protocol proved highly efficient, consistently yielding recombinant bacteria carrying the desired plasmid on the first attempt.

### 3.6 Colony PCR for Selection of Transformants

Colony PCR is a technique utilized to Determine the presence or absence of transformed plasmids within bacterial colonies. Single colonies surviving on specific antibiotic-containing media are selected and placed into the PCR reaction solution, where they are lysed during the Initial heating step. This lysing process enables the release of plasmid DNA from the bacterial cells, which serves as a template for PCR amplification.

Primers designed to target the plasmid DNA specifically are used to detect the presence of the desired plasmid within a bacterial colony. This method eliminates the need to isolate plasmids from numerous colonies for screening purposes, making it particularly suitable for rapidly screening many colonies. It is essential to avoid adding a large number of bacterial cells to the PCR reaction mix, as this can lead to inefficient PCR due to an excess of bacterial debris and elevate the likelihood of nonspecific amplification. Moreover, colonies from fresh bacterial plates yield better results compared to those from older plates. It is crucial to incorporate positive and negative controls into colony PCR experiments.

### 3.7 Cloning of RFP Visual Marker Gene and *SbSI-1* Gene from *Salicornia brachiata* into the Plant Transformation Vector pRI201-AN and Sequencing

The pRI201-AN binary vector facilitates efficient transgene expression in dicotyledonous plant cells. It incorporates the ColE1 origin of replication from *E. coli*, enabling the plasmid to replicate in multiple copies within *E.coli*, facilitating the isolation of recombinant plasmids in large quantities post-transformation. Additionally, it contains a mutant-type replication origin (Ri ori) from the *Rhizobium rhizogenes* Ri plasmid, ensuring the maintenance of the plasmid in a low copy number within Agrobacterium (Nishiguchi et al., 1987).

This characteristic reduces the likelihood of multiple T-DNA copies integrating into the plant genome post-agrobacterium-mediated transformation, thereby facilitating the generation of single-copy T-DNA-integrated transgenic plants. The vector also includes a bacterial selection marker gene, the aminoglycoside phosphotransferase gene (NPT III) from *Streptococcus faecalis*, providing resistance to the kanamycin antibiotic.

The T-DNA region of the plasmid is delimited by the left and right border sequences (LB and RB, respectively).

Adjacent to the ColE1 origin, the vector features the first Multiple Cloning Site (MCS) positioned next to the RB, flanked by a constitutively expressing 35S promoter from the Cauliflower Mosaic Virus (CaMV) on the 5' end and a transcription termination sequence from the heat shock protein 18.2 gene from Arabidopsis thaliana on the 3' end (Nagaya et al., 2010).

Furthermore, the vector incorporates a 58 bp sequence corresponding to the translational enhancer from the 5'-UTR sequence of the Arabidopsis thaliana Alcohol dehydrogenase gene (Sugio et al., 2008), enhancing transgene expression levels in transgenic plants. A second MCS is included after the heat shock protein terminator to integrate a second transgene if required. The marker gene for selecting transgenic plants is a plant codon-optimized aminoglycoside phosphotransferase gene (NPT II), driven by a Nopaline Synthase (NOS) promoter and flanked by a NOS terminator and polyA signal adjacent to the LB (Figure 7).

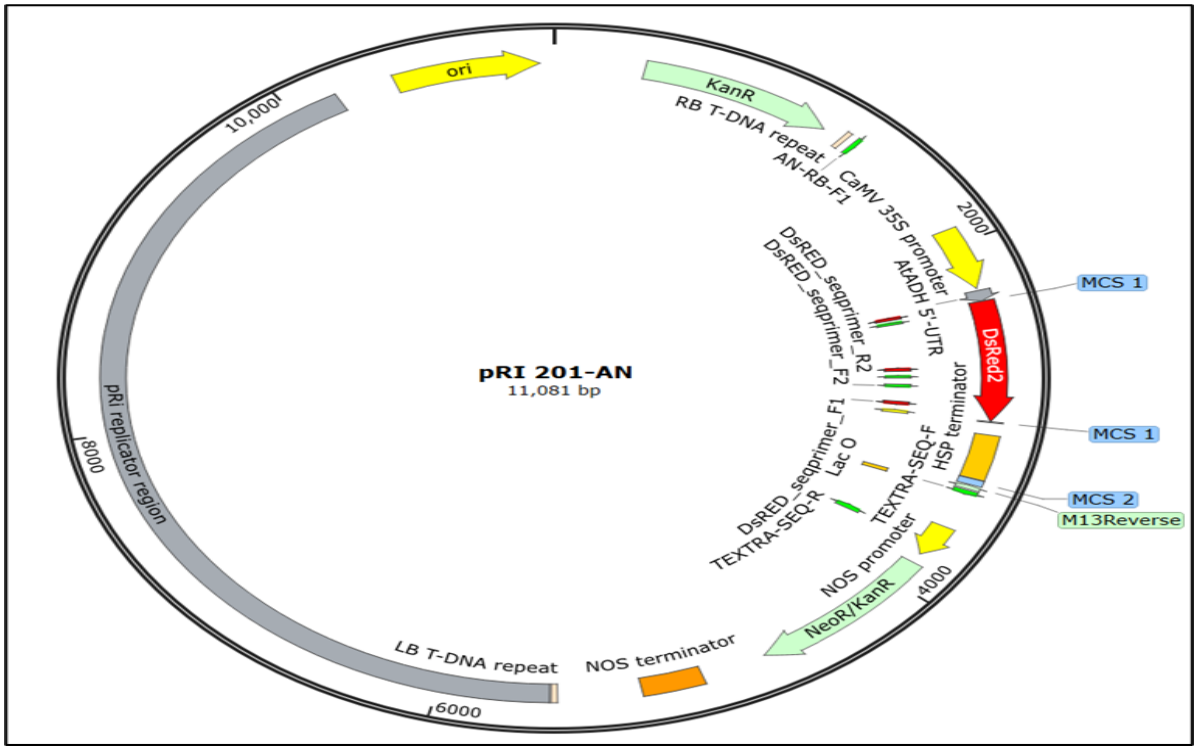

Figure 7: Vector map of 1RED-AN. The figure shows the *DsRED* gene cloned in the MCS1 of vector pRI201-AN vector under the CaMV 35S promoter. The vector carries selection marker genes for antibiotic kanamycin resistance

*DsRed* is a red fluorescent protein (RFP) derived from the *Discosoma* coral. RFPs serve as effective visual marker genes for transformation vectors and have been utilized in various organisms, including animals, fungi, and plants, to identify transformed cells (Rodrigues et al., 2001; Terahara et al., 2012; Zhang et al., 2015).This study obtained *DsRed* from addgene (www.addgene.org). The Plasmid repository was used as a visual marker to evaluate the transformation protocol's efficiency (Figure 7).

The *sbSI-1* gene from *Salicornia brachiata*, an extreme halophyte of the family Chenopodiaceae known for its high salt tolerance and suggested as a model for studying salt adaptation mechanisms, was cloned into the binary vector pRI201-AN to assess its potential in enhancing abiotic stress tolerance in transgenic lettuce. (Lv et al., 2012; Rathore et al., 2015) Previous studies have established an expressed sequence tag (EST) database for *S.brachiata* in response to salt stress, containing numerous novel and hypothetical genes potentially involved in salt tolerance mechanisms.

The *SbSI-1* gene, identified from this database, has improved drought and salinity tolerance in *E. coli* and transgenic tobacco. (Jha et al., 2009).

The In-Fusion seamless cloning method provided by Takara Biosciences, Japan, facilitated the cloning of *SbSI-1* into the pRI201-AN vector for lettuce transformation. This method allows for rapid and directional cloning of DNA fragments into plant transformation vectors. By recognizing 15-bp overlaps at the ends of DNA fragments, the In-Fusion Enzyme efficiently and precisely fuses PCR-generated inserts with linearized vectors, enabling seamless integration of the desired sequence. This study used the 'snapgene' software to design primers with 15-bp overlaps at their ends to amplify the fragments and linearize the vector (Table 1). The cloning protocol was highly efficient in joining the fragment to the linearized vector, and colonies carrying the recombinant plasmids were obtained easily (Figure 8).

Unlike the regular PCRs conducted in this study, the amplification of fragments and linearization of the vector were conducted with a high-fidelity Taq polymerase to minimize base incorporation errors during polymerization. For this purpose, the CloneAmp HiFi PCR Premix used in this study has a taq polymerase with an exceptionally low error rate of 12 mismatched bases per 542,580 total bases.

The polymerase is fast, with an extension time of 5 sec/kb, and has a hot-start antibody that prevents nonspecific amplification at room temperatures.

In this study, using the CloneAmp HiFi PCR Premix, the 10 kb pRI201-AN was successfully linearized and was used for cloning. 2 recombinant plasmids each/construct (1RED-AN, 1SBSI-AN) were sequenced, and no sequence errors were found within the inserted coding regions for both the genes (Figure 8).

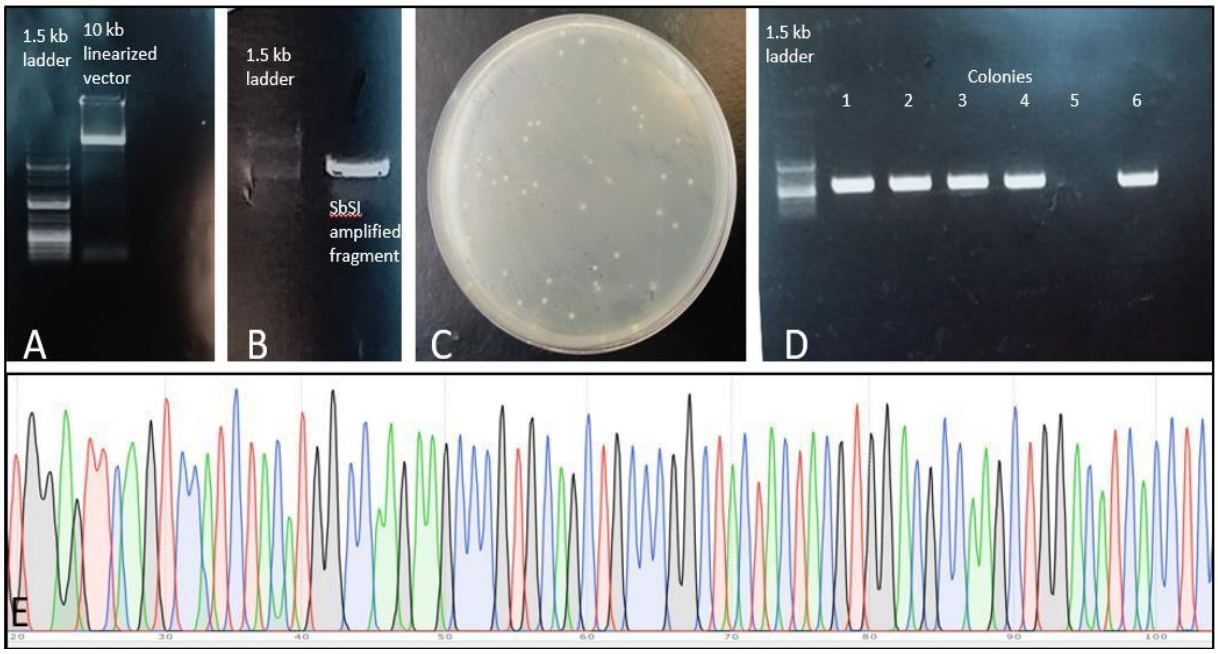


Figure 8: Stages of In-Fusion cloning of SbSI-1 gene into linearized pRI201-AN vector. A) vector amplification using high fidelity Taq polymerase, B) fragment amplification, C) bacterial colonies surviving on antibiotic media after transformation with

### 3.8 *In Silico* Analysis of *SbSI-1* Gene

The mRNA sequence of the *SbSI-1* gene, sourced from previous publications (Yadav et al., 2012), spans a total length of 917 base pairs (bp), featuring an open reading frame (ORF) of 477 bp responsible for encoding a protein consisting of 159 amino acids (Figure 9). The *SbSI-1* protein possesses a molecular weight of 18389.65 and a theoretical isoelectric point (pI) of 8.51. Upon amino acid composition analysis, histidine was the most abundant amino acid, constituting 16.4% of the protein, followed by proline at 11.9%. Notably, the protein exhibits a grand average hydropathicity (GRAVY) value of -1.084. Proteins characterized by negative GRAVY values are considered hydrophilic, while positive values suggest hydrophobic properties.

Further analysis of the *SbSI-1* protein sequence involved a comparison against the non-redundant protein sequences (nr) database at NCBI utilizing the BLAST algorithm. BLAST, an acronym for Basic Local Alignment Search Tool, facilitates the identification of regions displaying local similarity between sequences. This computational tool compares nucleotide or protein sequences against sequence databases, providing statistical assessments of matches. Upon conducting a BLASTP analysis, it was revealed that the protein exhibiting the highest degree of homology to *SbSI-1* corresponds to a polycomb group protein Pc-like protein derived from Chenopodium quinoa, with a query cover of 66% and an identity of 54.55%.


Figure 9: ORF and predicted amino acid sequence of SbSI-1. The figures were generated using the site www.expasy.com

The results indicated that *SbSI-1* is unlike any previously characterized proteins in the database (Figure 10). A Multiple Sequence Analysis (MSA) of the *SbSI-1* protein with the first five BLASTP hits showed that all the proteins were rich in histidine residues in the N-terminal. The proteins showed more sequence similarity towards the C-terminal. The phylogenetic analysis showed *SbSI-1* clustering with hypothetical proteins from *Nyssa sinensis* and *Beta vulgaris*. Protein subcellular localization predictions indicated that *SbSI-1* had a high chance of being located in the chloroplast, followed by the nucleus. Previous studies have indicated that *SbSI*-1 is localized to the nucleus (Kumari et al., 2017).

*SbSI-1* has been reported as highly upregulated in *Salicornia brachiate* plants subjected to salt and drought stresses. Transgenic tobacco plants that overexpressed the *SbSI-1* gene demonstrated enhanced physiological traits, including improved seed germination, higher pigment contents, enhanced growth performance, increased cell viability, elevated starch accumulation, and elevated tolerance index under conditions of drought and salt stress, when compared to wild-type tobacco plants. Notably, the overexpression of *SbSI-1* resulted in a reduction in Reactive Oxygen Species (ROS) content, thereby reducing ROS-induced damage during stress episodes. Moreover, the transgenic plants exhibited heightened levels of antioxidant enzymes, an increased net photosynthetic rate, and enhanced osmotic and redox homeostasis under stress conditions. Furthermore, the upregulation of tobacco transcription factors *NtDREB2* and *NtAP2* in transgenic plants provides additional insights into the role of *SbSI-1* in augmenting tolerance to abiotic stress (Kumari et al., 2017). However, despite these findings, the precise functional role of *SbSI-1* remains unknown. The search for conserved domains within the *SbSI-1* protein did not reveal the presence of any conserved domains, which may suggest a potential function of this protein. Additionally, the protein lacked transmembrane domains, indicative of its non-membrane localization. Moreover, there was no discernible signal peptide directing the localization of the protein to organelles or membranes.

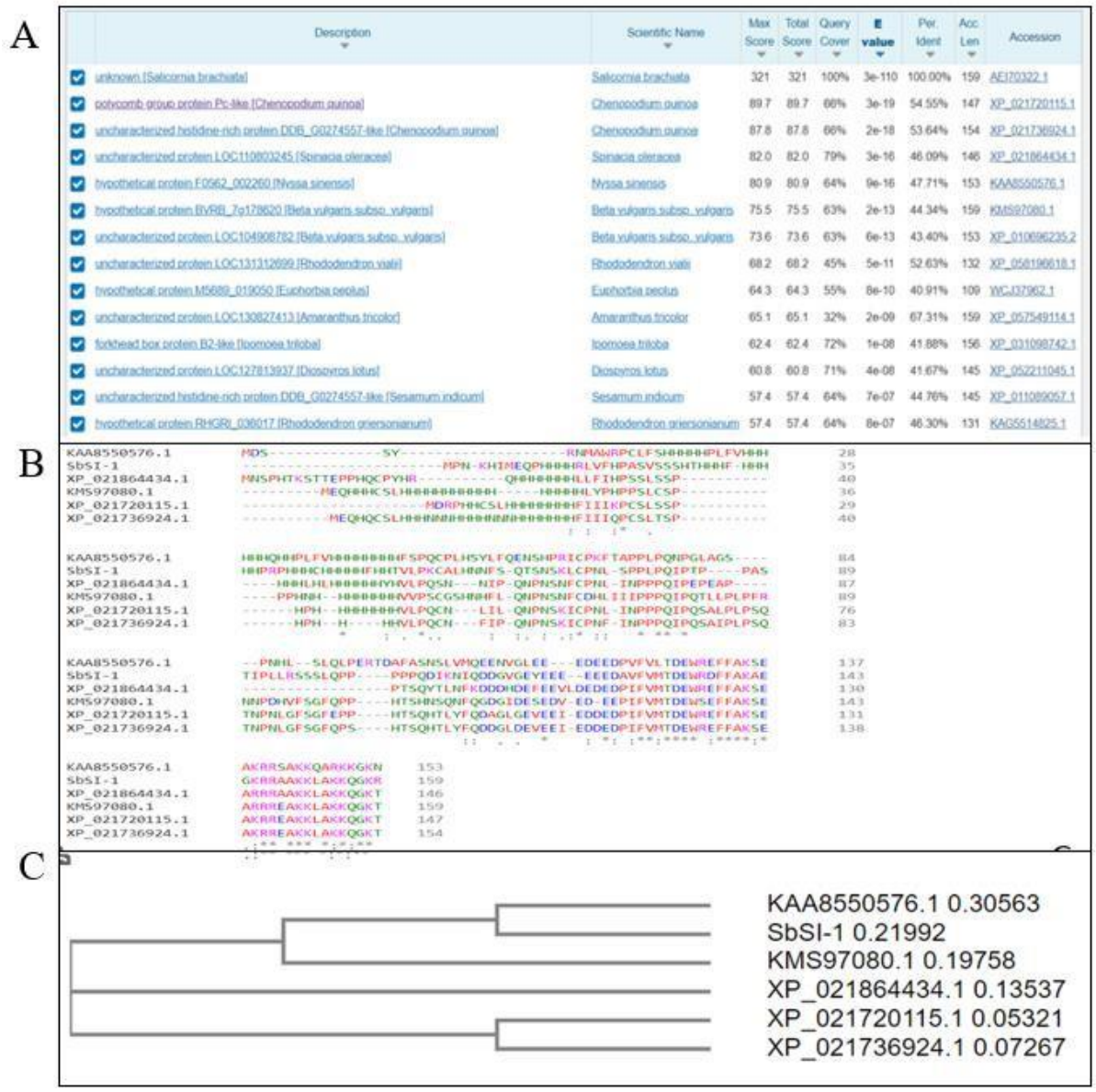


Figure 10: Bioinformatic Analysis of *SbSI-1* protein A) Results of BLAST search, B) Multiple Sequence Analysis result , and C) phylogenetic analysis of *SbSI-1* protein

### 3.9 Plant Material and Sterilization

The sterilization regime used was successful in eliminating any contamination during seed germination of lettuce. Nearly 90% of seeds showed radicle emergence within 3-4 days of sowing, and after transferring to light, cotyledons emerged. Within 7 days of sowing, the cotyledons were grown enough for downstream experiments. Very few seedlings had emerging true leaves at this point. The study observed that the seed germination percentage drastically reduced with age of the seeds. Less than 20% germination was often observed when an old batch of seeds was used.

The germination percentage of older seeds could be increased with short-term treatment (1 day) at lower temperatures (4°C). A beneficial effect of cold treatment on seed germination has been previously reported (Ilyas et al., 2022). Fungal contamination was observed in a few plates, and seedlings from those plates were not used for any experiments.

### 3.10 Standardization of *In Vitro* Shoot Regeneration of Lettuce

Crop improvement efforts through genetic transformation approaches and some non-transgenic approaches, such as micropropagation of species for obtaining large amounts of plantlets from small starting material, ultimately rely on regenerating whole plants reliably and efficiently through tissue culture. For most practical applications, a multiple-shoot regeneration system without a slow intermediate callus stage is advantageous. The efficiency of an *in vitro* regeneration system heavily depends on the species, variety, explant type, and hormonal and nutrient composition of the culture media. Very often, a regeneration protocol that works well for one variety will not be as efficient in another species, indicating a solid genotype bias. Thus, it is essential to optimize *in vitro* regeneration protocols for the genotype in question.

Several previous studies have reported *in vitro* shoot regeneration of different lettuce varieties. The regeneration responses were highly variable across experiments and heavily dependent on genotype, with many cultivars showing strong recalcitrance. Multiple shoot regeneration of 90.8 ± 7.9% from cotyledon explants was observed in lettuce variety 'RSL NFR' when MS medium supplemented with 200 mg/l of activated charcoal, 3% sucrose, 10 mg/l BAP and 0.5 mg/l NAA was used. However, the same media showed varying shoot regeneration efficiencies in other cultivars (Armas et al., 2017). In the lettuce cultivar Romaine, a combination of 0.1 mg/L NAA and 0.4 mg/L BAP produced maximum shoot regeneration (Gómez-Montes et al., 2015). An older study involving twenty-two lettuce genotypes highlighted the genotype differences in *in-vitro* responses in lettuce (Ampomah-Dwamena et al., 1997). A detailed literature survey found no established *in vitro* shoot regeneration protocols for the variety, 'Paris Island cos', chosen in this study. In this study, lettuce explants showed signs of organogenesis within 14 days of culture in the 11 media combinations.

Shoot regeneration was observed in LM1, LM2, LM3, LM6, and LM7 media, while callus regeneration was observed in LM4, LM5, LM8, LM9, and LM10 media. Roots were not found to be regenerating in any of the media combinations by day 14.

However, by day 28 in culture, some of the explants in LM4, LM5, and LM9 had displayed root regeneration. All the media combinations except LM11 showed some level of shoot regeneration, although the percentage of shoot regeneration varied widely (Figure 11). Additionally, considerable variation was observed in the length of the regenerated shoots (Table 3). The percentage of callus regeneration was highest in LM8 and lowest in LM2. The percentage of shoot regeneration was highest in LM6 (92.5%) and lowest in LM11(0%).

The average number of shoots/explants was highest in LM6 (2.29) and lowest in LM8 (1.00). The average shoot length was highest in LM10 (18.75 mm) and lowest in LM9 (4.00 mm). Based on the data collected and morphological observations, LM6 was selected as a media combination for future experiments.

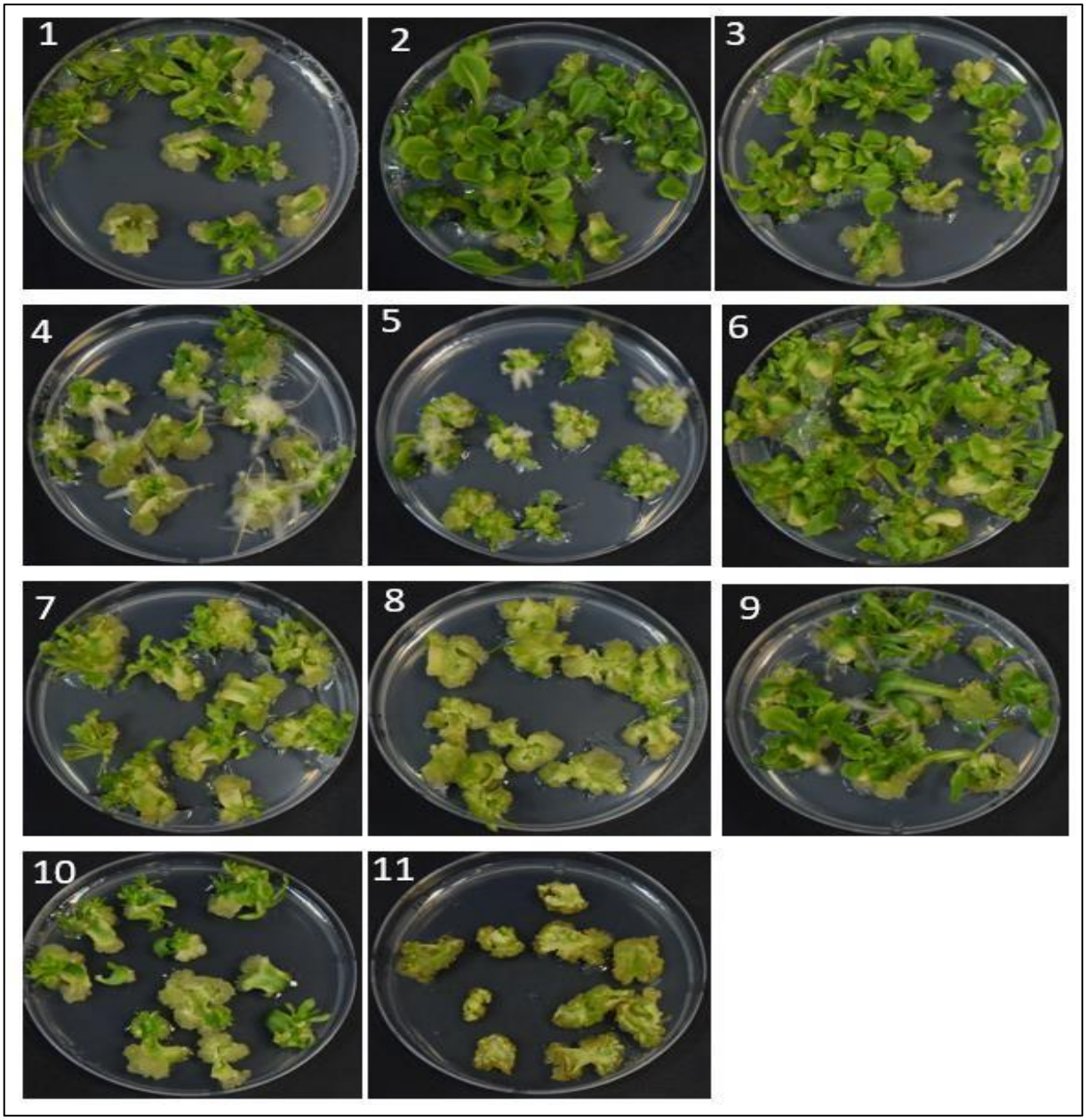


Figure 11: Explant growth in selected hormone combinations after 28 days. Cotyledon explants from 7-day-old lettuce seedlings were cultured on 3% MS media with different hormone combinations (LM1- LM11) and subcultured to the same media after two weeks. The pictures were taken on day 28 of culture. The data on the percentage of shoot regeneration, average number of shoots/explant, and average shoot length were recorded. All experiments were conducted with three replicates comprising 12 explants per plate

Table 3: Effect of different hormone combinations on shoot regeneration from cotyledon explant

| Media | Shoot Regeneration % | Average No. of shoots/explant | Shoot average length (mm) |
|---|---|---|---|
| LM1 | 50.00 bcd | 1.50 bc | 16.87 a |
| LM2 | 85.00 ab | 1.85 ab | 6.75 b |
| LM3 | 60.00 abcd | 1.45 bc | 17.50 a |
| LM4 | 37.50 cde | 1.33 bc | 14.37 a |
| LM5 | 32.50 de | 1.15 c | 7.75 b |
| LM6 | 92.50 a | 2.29 a | 5.50 b |
| LM7 | 67.50 abcd | 1.33 bc | 16.25 a |
| LM8 | 35.00 cde | 1.00 c | 8.25 b |
| LM9 | 75.00 abc | 1.36 bc | 4.00 bc |
| LM10 | 60.00 abcd | 1.25 bc | 18.75 a |
| LM11 | 0 | 0 | 0 |

The table shows the percentage of shoot regeneration and mean length of shoots per explant. The data is collected on day 28. Data are the means of four replications. The data were statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All data were subjected to a one-way analysis of variance (ANOVA), and means were compared using Duncan's multiple range test (DMRT). Differences in means ± SD (standard deviation) were analyzed using DMRT at "Duncan's multiple range test" at $P \leq 0.05$.

### 3.11 Lettuce *In Vitro* Rooting and Acclimatization

The lettuce shoots transplanted to 3% MS media developed roots within three weeks. The acclimatization and hardening protocols used were ideal for plant survival, and no loss of plants was observed at this stage. The pot plants flowered within 3-4 months, and seeds were collected within 4-5 months of planting (Figure 12).

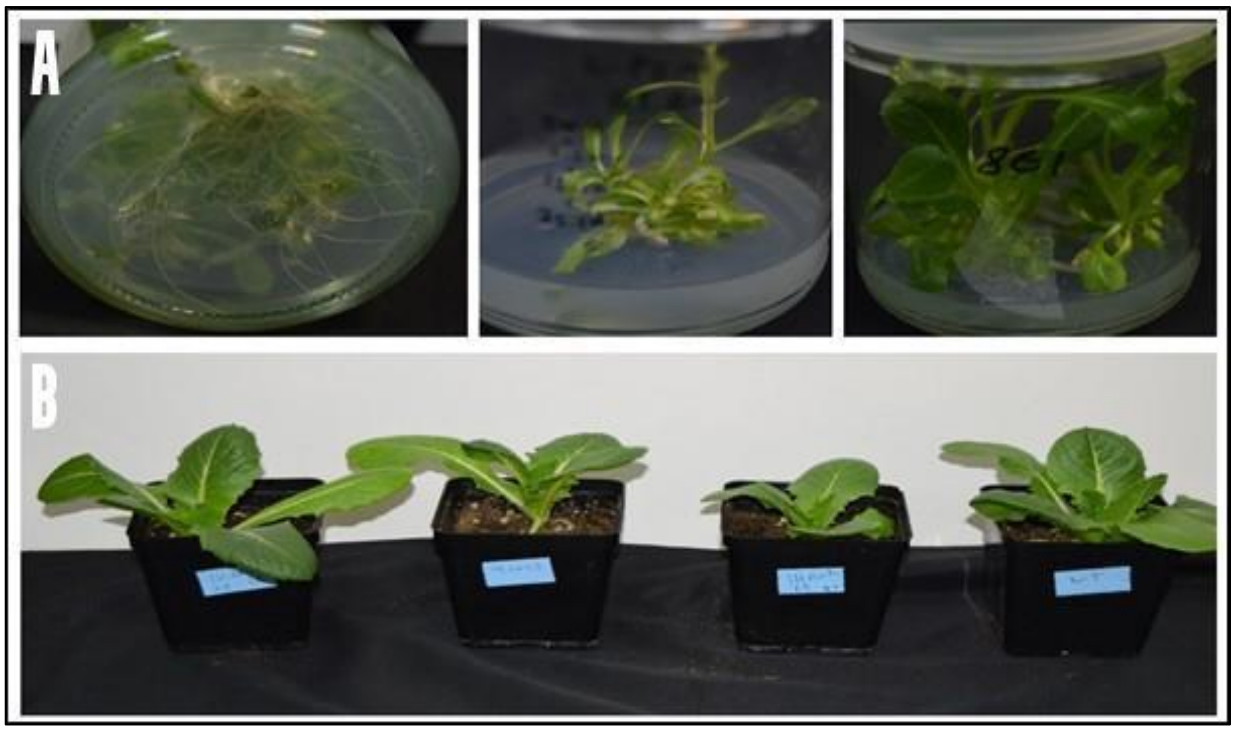


Figure 12: Rooting, hardening, and growth of transgenic lettuce in soil. A) Lettuce in root induction media MS.3% after 2 weeks gorwth in soot regenration media LM6**.** B) Lettuce in soil after two week of accmulization (representative figures shown)

### 3.12 Analysis of the Inhibitory Effect of Kanamycin on *In vitro* Shoot Regeneration of Lettuce

Using genes conferring antibiotic tolerance is a standard method of selecting transgenic plants over non-transgenic plants after Agrobacterium-mediated transformation. Commonly used selectable marker genes involve those conferring resistance to antibiotics such as kanamycin and hygromycin. Including such genes allows the transgenic cells to survive on a medium containing the antibiotic. The pRI201-AN vector used in this study carries the gene for aminoglycoside phosphotransferase (neomycin phosphotransferase), which can inactivate kanamycin (Figure 7).

Placing the transformed explants in a medium containing kanamycin ensures that only those cells that have integrated the T-DNA carrying the selection marker gene can survive and regenerate in the medium. Depending upon species, genotype, and explant type, plant cells can survive and regenerate on different concentrations of selected antibiotics.

In order to ensure that only transgenic cells survive on the antibiotic-containing media, it is necessary to identify the minimum concentration of the antibiotic in which no growth and regeneration happens.

This study conducted a Minimum Inhibitory Concentration (MIC) test for kanamycin using the selected explant type, cotyledons. At a concentration of 10 mg/L kanamycin, shoot regeneration was observed from nearly 50% of the explants after 28 days of culture in LM6 media. 20 mg/L kanamycin severely inhibited shoot regeneration. Still, smaller shoots were observed in less than 10% of the explants. The explant also showed callus growth under this concentration. 30 mg/L kanamycin completed inhibited shoot regeneration. However, minor callus growth was observed in many explants. 50 mg/L completely inhibited growth in the explants, which progressively showed signs of necrosis as the days in the culture progressed (Figure 13). Based on these results, a kanamycin concentration of 50 mg/L was selected as the MIC for kanamycin.

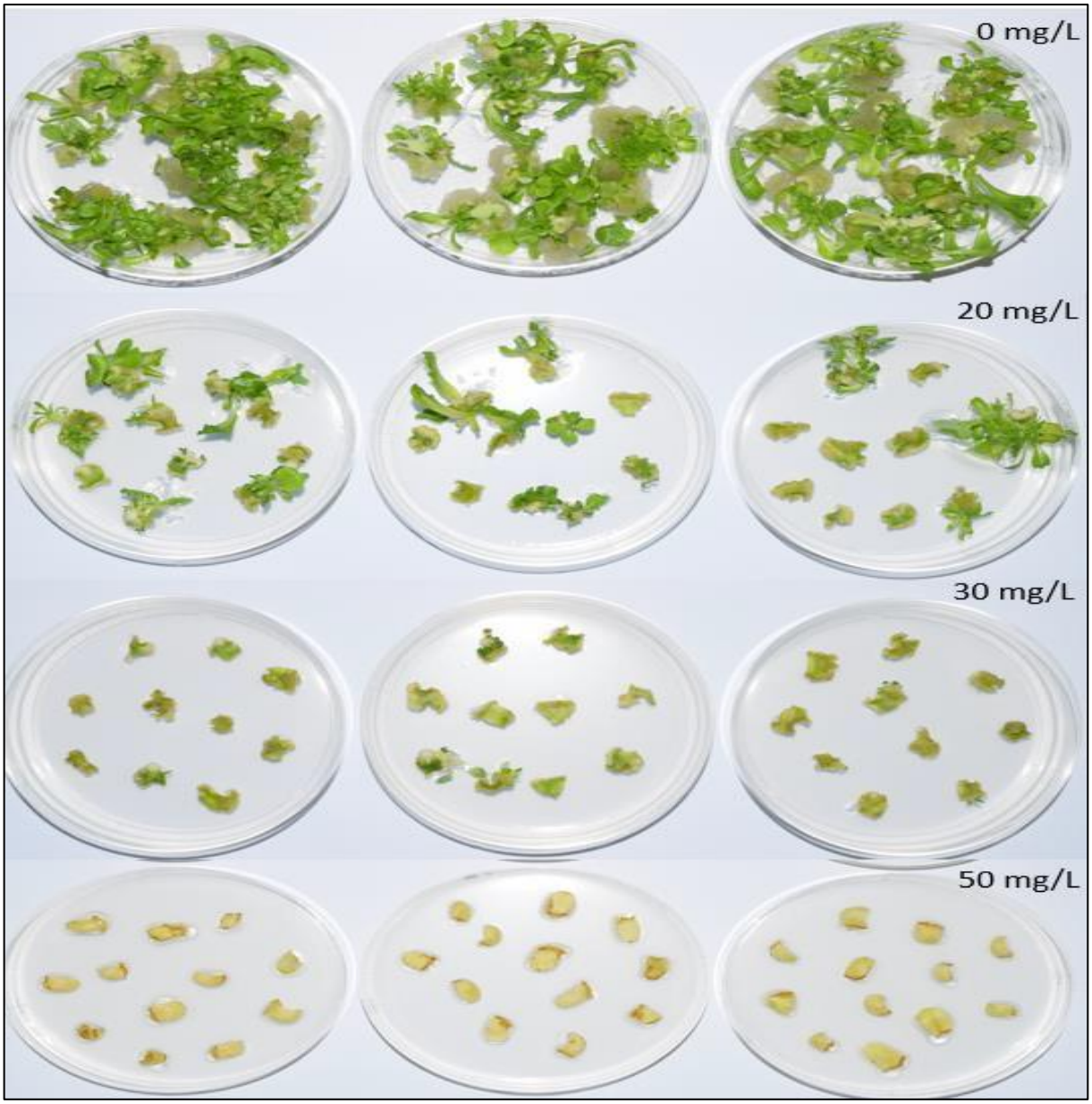


Figure 13: Effect of different concentrations of kanamycin on the growth of lettuce explants in shoot regeneration media. Lettuce explants were grown in LM6 media supplemented with different concentrations of kanamycin and growth pattern was observed for 28 days. The concentration of kanamycin that completely limited regeneration from explants was selected for future experiments

### 3.13 Preparation of Agrobacterium Competent Cells and Transformation

Various Agrobacterium strains are available for the transformation of different plant species. The different strains differ in their chromosomal background, the bacterial selection marker gene, Ti plasmid, plasmid selection marker gene, etc. (Table 4) (Hellens et al., 2000).Commonly used strains include LBA4404, EHA101, EHA105, GV3101, AGL-1 etc. Due to the differences in chromosomal background and Ti plasmid, these strains differ in their virulence and the range of plants susceptible to T-DNA transfer via their vir genes.

Table 4: Disarmed Agrobacterium tumefaciens strains defined by the Agrobacterium chromosomal background and the Ti plasmid they harbor (Hellens et al., 2000)

| Agrobacterium Strain | Chromosomal Background | Marker Gene | Ti PLasmid | Marker Gene | Opine |
|---|---|---|---|---|---|
| LBA4404 | TiAch5 | rif | pAL4404 | spec and strep | Octopine |
| GV2260 | C58 | rif | pGV2260 (pTiB6S3DT-DNA) | carb | Octopine |
| C58C1 | C58 | – | Cured | – | Nopaline |
| GV3100 | C58 | – | Cured | – | Nopaline |
| A136 | C58 | rif and nal | Cured | – | Nopaline |
| GV3101 | C58 | rif | Cured | – | Nopaline |
| GV3850 | C58 | rif | pGV3850 (pTiC58Donc.genes) | carb | Nopaline |
| GV3101::pMP90 | C58 | rif | pMP90 (pTiC58DT-DNA) | gent | Nopaline |
| GV3101::pMP90RK | C58 | rif | pMP90RK (pTiC58DT-DNA) | gent and kan | Nopaline |
| EHA101 | C58 | rif | pEHA101 (pTiBo542DT-DNA) | kan | Nopaline |
| EHA105 | C58 | rif | pEHA105 (pTiBo542DT-DNA) | – | Succinamopine |
| AGL-1 | C58, RecA | rif, carb | pTiBo542DT-DNA | – | Succinamopine |

This study used the LBA4404 strain for lettuce transformations. LBA4404 carries an octopine-type disarmed Ti plasmid pAL4404 without self-transport function in the TiAch5 chromosomal background. Its chromosomal DNA contains a rifampicin resistance gene, and the plasmid contains a streptomycin resistance gene. This strain has successfully transformed various species, including tomatoes, tobacco, and other plants (Van Eck et al., 2019).

This study's competent cell preparation protocol was less tedious and time-consuming than other commonly used protocols. However, the transformation efficiency was less than the other protocols (based on observations in the lab, not statistically analyzed).

No escapes were observed after transformation, and any bacterial colony surviving on antibiotic selection media invariably contained the transformed plasmid. Thus, recombinant bacteria were recovered in more than 95% of the transformation experiments.

### 3.14 Agrobacterium-Mediated Transformation of Lettuce

Several factors intricately influence the efficiency of Agrobacterium-mediated transformation in plants, encompassing the bacterial strain employed, the type of explant, the duration of explant preculture, the optical density of the bacterial culture for infection, the concentration of Acetosynringone, infection duration and method, co-cultivation period, temperature during co-cultivation, as well as the type and concentration of antibiotics employed to deter Agrobacterium overgrowth during selection. Standardizing these parameters is essential to develop a transformation protocol to optimize transformation efficiency.

This study assessed two bacterial strains, namely EHA105 and LBA4404, for their impact on transformation efficacy. Initial trials with EHA105, recognized as a supervirulent strain, frequently resulted in bacterial overgrowth during co-cultivation, necessitating supplementary explant washing in antibiotic solutions before transfer to selection media. Despite these precautions, bacterial overgrowth in selection media was commonly observed, ultimately leading to explant death despite including antibiotics in the media to prevent Agrobacterium overgrowth. Consequently, EHA105 was excluded from subsequent experiments. Conversely, strain LBA4404 did not result in bacterial overgrowth during co-cultivation or selection, except in isolated instances.

The selection of an appropriate explant significantly influences transformation efficiency, with leaf, hypocotyl, roots, cotyledons, immature embryos, anthers, somatic embryos, etc., commonly utilized as explants. An explant with a well-established and highly efficient shoot regeneration protocol is preferred.

Cotyledons were initially selected as explants for this study, given the prior literature demonstrating their effectiveness in transforming various lettuce varieties (Curtis et al., 1994; Marveeva et al., 2009). Initial transformations employing the 1RED-AN vector exhibited robust red fluorescence in transformed explants, affirming their suitability for further experimentation (see Figure 14). the initial assessment of transformation efficiency, the recombinant vector 1RED-AN was employed, and the presence of red fluorescence was examined in explants and surviving shoots cultivated on selection media.

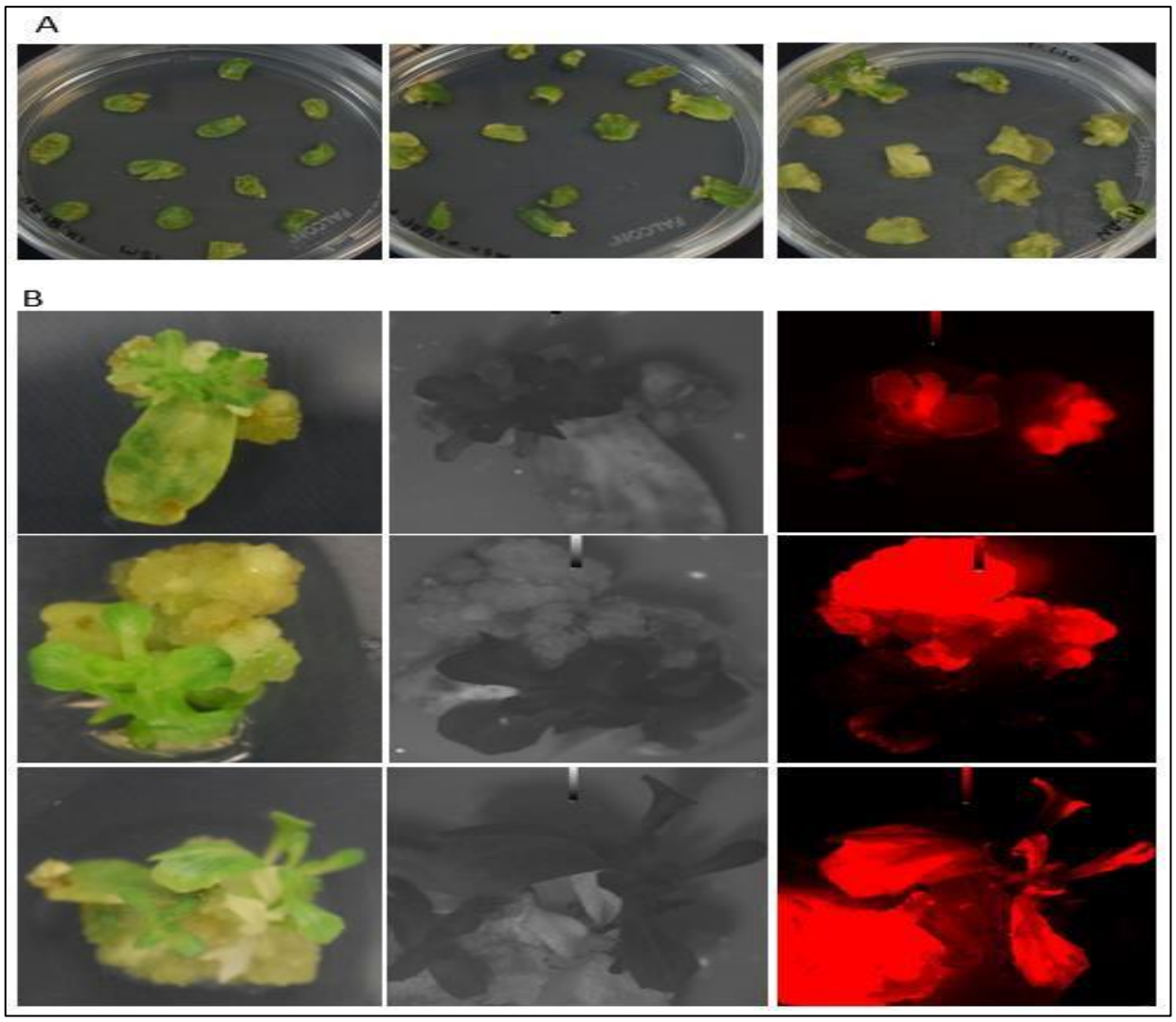


Figure 14: Agrobacterium-mediated transformation and shoot regeneration of lettuce. A) shoot regeneration from 1RED-AN transformed explants in selection media, B) RFP fluorescence observed in regenerated shoots using fluorescence microscopy

This evaluation utilized fluorescence microscopy with a Zeiss Discovery V12 microscope equipped with a Zeiss Rhodamine cube KSC 295–815D, employing excitation at 540/25 and emission at 605/55 wavelengths.

Imaging was performed using an Optronics digital camera (Nikon D60) with manual exposure settings. Additionally, bright-field images were captured with consistent exposure times across all samples.

This study briefly analyzed various factors influencing the efficiency of Agrobacterium-mediated transformation in plants. Notably, the impact of explant preculture on transformation efficiency was briefly assessed, revealing a significant decline in explant survival rates when freshly wounded explants were directly subjected to transformation without any preculture. However, a one-day preculture period in preculture media improved explant survival rates; hence, the transformation experiments were conducted after one day of preculture.

Optical density (OD) measurement at 600 nm became a pivotal method for gauging bacterial culture growth stage and density. Optimal OD600 values were crucial for maximizing transformation efficiency while minimizing Agrobacterium overgrowth during co-cultivation. Accordingly, experiments were conducted within the OD600 range of 0.5-0.8, as values exceeding 0.8 resulted in excessive Agrobacterium overgrowth post-transformation, leading to explant mortality, while lower values reduced transformation efficiency and transgenic plant recovery.

A standardized protocol utilizing 100 μM Acetosynringone (AS) and a 10-minute infection period with mild shaking was adopted, as these parameters were optimal for transforming the selected lettuce variety. Additionally, a three-day co-cultivation period was employed across all experiments. Two infection methods were analyzed, with method 1 involving slight wounding of explants and method 2 utilizing sonication followed by vacuum infiltration. Although both methods yielded surviving explants expressing high levels of red fluorescent protein (RFP) post-transformation, shoot regeneration was notably higher in wounded explants (14%) compared to sonicated and vacuum-infiltrated explants (3%), rendering the former as the preferred explant infection method.

Furthermore, the effect of co-cultivation temperatures on Agrobacterium overgrowth and transformation efficiency was investigated, with temperatures of 22°C and 25°C analyzed.

Co-cultivation at 25°C for three days resulted in Agrobacterium overgrowth and explant mortality, prompting the selection of 22°C for co-cultivation. Timentin was utilized as the antibiotic to prevent Agrobacterium overgrowth during selection, with a concentration of 300 mg/L effectively curbing bacterial proliferation. This concentration was maintained during the rooting phase of plants. Initial transformation standardization experiments were conducted using the construct 1RED-AN, facilitating the assessment of transformation efficiency through visual observation of red fluorescence in surviving explants. After two weeks of transformation, transient transformation efficiency approached nearly 100% when analyzing RFP expression in explants. However, only approximately 14% of explants regenerated red fluorescent shoots under ideal conditions. Subsequent transformation with 1SbSI-AN was undertaken following the standardization of the transformation protocol using 1RED-AN.

### 3.15 Analysis of Transgenic Plants

Genomic DNA was extracted from a confirmed positive 1SbSI-AN transgenic plant cultivated in soil. Subsequent PCR analysis using primers designed to amplify the insert revealed the presence of the T-DNA region, harboring the insert, in all examined plants. Additionally, total RNA was isolated from selected transgenic lettuce plants, followed by qRT-PCR analysis to assess the expression levels of the *SbSI-1* gene. The findings revealed considerable variation in gene expression levels across different transgenic lines, with line 8 exhibiting the highest expression level (as depicted in Figure 15).

These results provide evidence of the successful integration of the *SbSI-1* gene into the genome of the transgenic plants, followed by transcription into the corresponding mRNA molecules.

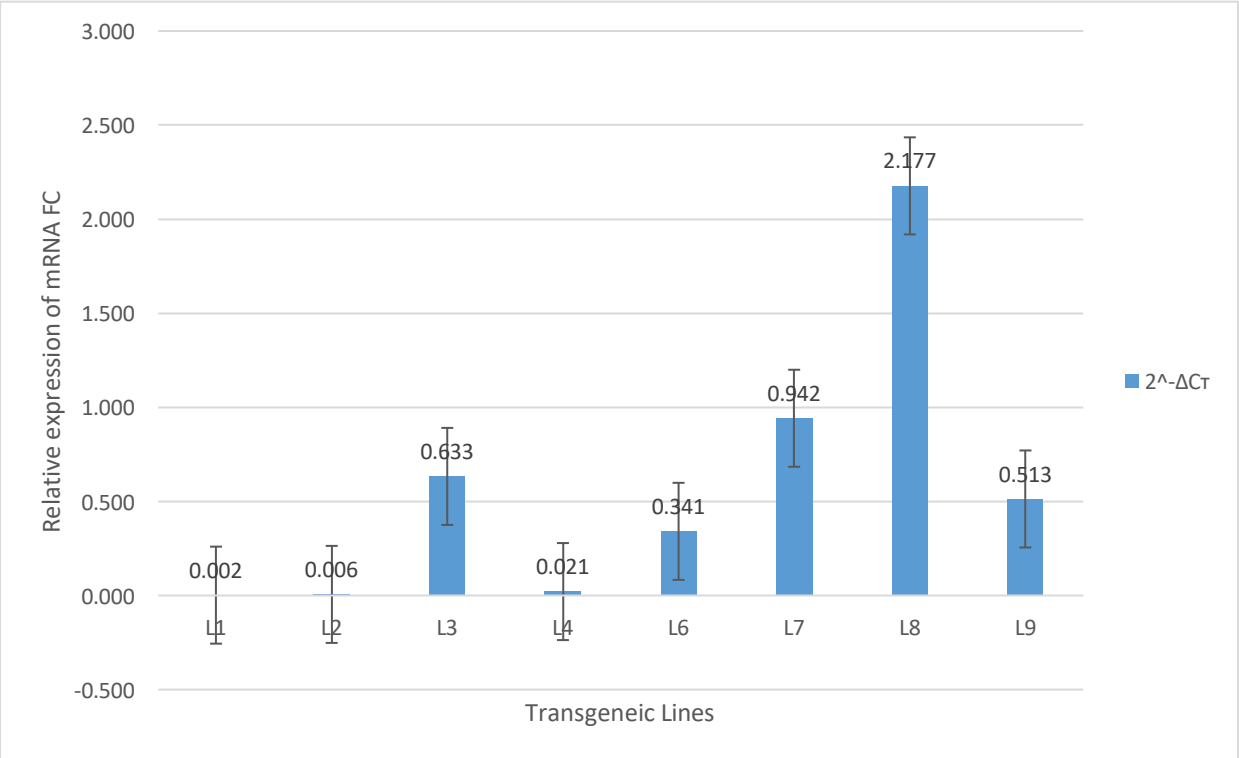


Figure 15: qRT-PCR analysis of transgenic lettuce. The Y-axis shows the relative expression of *SbSI-1* mRNA compared to control wild-type plants. X-axis shows the transgenic lines analyzed. Each qRT-PCR analysis included three biological and three technical replicates. The lettuce tubulin gene was used as the internal reference gene for normalization. The standard deviation was calculated using Excel

# Chapter 4: Conclusion

The study's primary objective was establishing highly efficient *in vitro* regeneration and agrobacterium-mediated transformation protocols tailored explicitly for the lettuce variety 'Paris Island cos.' A pivotal aspect of this study was the development of a shoot regeneration medium that exhibited an exceptional efficiency of over 95% in generating shoots from lettuce explants.

The study successfully achieved its purpose by implementing a standardized Agrobacterium-mediated transformation protocol, which achieved a stable transformation efficiency of 14%. These accomplishments underscore the efficacy and reliability of the protocols in facilitating the incorporation of foreign genes into the Paris Island cos lettuce variety.

Furthermore, our investigation encompassed a validation process wherein the successful integration of the transgene was tested via Polymerase Chain Reaction (PCR) analysis. Subsequently, the transcription expression level of the transgene lines was confirmed through quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

Overall, establishing highly efficient *in vitro* regeneration and transformation methodologies tailored for "The Paris Island Cos". Lettuce variety enhances our understanding of plant biotechnology and paves the way for expedited advancements in lettuce genetic improvement and trait modification endeavors.

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# Appendices

## Appendix A

List of the materials and chemicals used in the present study and their sources

| Material | Description | Source |
|---|---|---|
| Plant growth media | Murashige and Skoog basal medium with vitamins | PhytoTechnology Laboratories, USA |
| Plant growth regulator | Indole-3-acetic acid (IAA) | PhytoTechnology Laboratories, USA |
| | Indole-3-butyric acid (IBA) | |
| | Naphthaleneacetic Acid (NAA) | |
| | 2,4-Dichlorophenoxyacetic Acid (2,4 D) | |
| | 6-benzylaminopurine (BAP) | |
| | Kinetin | |
| | Zeatin | |
| | Activated charcoal | |
| Carbon source | Sucrose | Sigma, MO, USA |
| General chemicals | LB media, agarose, and others | Sigma, MO, USA |
| *E.coli* strain | DH5-Alpha | Available at KCGEB |
| Agrobacterium strains | EHA105, GV3101, LBA4404 | |
| Plasmids | pRI201-AN | Takara Bio Inc, Japan |
| Antibiotics | Ampicillin, kanamycin, rifampicin, timentin, hygromycin | PhytoTechnology Laboratories, USA |
| Molecular weight Markers | Invitrogen DNA 1kb Plus Ladder | Invitrogen, USA |
| DNA Loading dye | DNA Gel Loading Dye (6X) | Thermo Scientific, USA |
| RNA loading dye | RNA Gel Loading Dye (2X) | Thermo Scientific™,USA |
| Cloning kit | In-Fusion® HD Cloning Kit | Takara Bio Inc, Japan |
| Plasmid isolation kit | QIAGEN Plasmid Mini Kit | Qiagen, Germany |
| Gel elution kit | QIAquick Gel Extraction Kit | Qiagen, Germany |
| Taq DNA Polymerase | HotStarTaq DNA Polymerase | Qiagen, Germany |
| Primers | - | Microgen, Korea |
| Reverse transcripttion kit | QuantiTect® Reverse Transcription Kit | Qiagen, Germany |
| qRT-PCR kit | PowerUp™ SYBR™ Green Master Mix | Applied Biosystems, USA |

## Appendix B

List of buffers and solution used in this study

| | |
|---|---|
| 10 X TBE buffer | 1L<br><br>108 g Tris<br>55 g Boric acid<br>40 ml of 0.5 M EDTA (pH 8.0) |
| Transformation and storage solution (TSS) | 85%( vol/vol) LB broth.<br>10% (wt/vol) PEG 8000.<br>5% (vol/vol) DMSO.<br>50 mM $MgCl_2$.<br>The final pH was adjusted to 6.5, autoclaved, and stored at 4°C |
| Plant genomic DNA extraction buffer | 2% Cetyl Trimethyl Ammonium Bromide (CTAB).<br>50 mM EDTA (pH 8.0).<br>100 mM Tris-HCl (pH 8.0).<br>1.4 M NaCl.<br>Autoclaved and 1% β-Mercaptoethanol was added before use. |
| Phenol: Chloroform | Equal quantities of Tris saturated phenol (pH 7.6) and chloroform were mixed and stored in amber-colored bottles. |
| 3 M Sodium acetate (pH 5.2) | 40.8 g of sodium acetate.3H20 was initially added to 80 ml water. The pH was adjusted to a pH of 5.2 by adding glacial acetic acid. The solution was made up to 100 ml and autoclaved. |
| RNA Extraction buffer | 4 M Guanidinium isothiocyanate<br><br>25 mM Sodium acetate (pH 7.0)<br><br>0.5% Sarcosyl<br><br>0.1% β-Mercaptoethanol |

## Appendix C

Composition of various culture media used in this study

| Purpose | Media | Composition |
|---|---|---|
| *E. coli* growth | Luria Bertani Broth | Tryptone 10 g/L<br>Yeast extract 5 g/L<br>Sodium chloride 10 g/L<br>pH was adjusted to 7.0 and autoclaved<br>For solid LB agar medium, 15 g /L agar was added before sterilization |
| Agrobacterium growth | Luria Bertani Broth for *A.tumefaciens* | Tryptone 10 g/L<br>Yeast extract 5 g/L<br>Sodium chloride 5 g/L<br>pH was adjusted to 7.0 and autoclaved.<br>For solid LB agar medium, 15 g /L agar was added before sterilization. |
| Plant growth | Murashige and Skoog (MS) basal medium with vitamins, pH 5.8 | **Macro elements** **mg/l**<br>$NH_4NO_3$ 1650<br>$KNO_3$ 1900<br>$CaCl_2.2H_2O$ 440<br>$MgSO_4.7H_2O$ 370<br>$KH_2PO_4.H_2O$ 170<br>**Microelements** **mg/l**<br>$H_3BO_3$ 6.2<br>$MnSO_4$ 22.3<br>$ZnSO_4.7H20$ 8.6<br>KI 0.83<br>$Na_2MoO_4.4H_20$ 0.25<br>$CuSO_4.5H_20$ 0.25<br>$FeSO_4.7H_20$ 27.85<br>$Na_2EDTA$ 37.25<br>**Vitamins** **mg/l**<br>Myo-inositol 100<br>Nicotinic acid 0.5<br>Pyridoxine HCl 0.5<br>Thiamine HCl 0.1<br>**Amino acid** **mg/l**<br>Glycine 2<br>**Carbohydrate** **g/l**<br>Sucrose 30 |
| Seed germination | LS-SGM | 2.35 g/L MS salts and vitamins<br>20 g/L sucrose<br>Set pH to 5.8.<br>8 g/L agar<br>Sterilize by autoclaving |

| | | |
|---|---|---|
| Explant preculture | LS-Preculture | 4.71 g/L MS salts and vitamins<br>30 g/L sucrose<br>Set pH to 5.8.<br>8 g/L agar<br>Sterilize by autoclaving and add;<br>0.1 mg/L NAA<br>0.1 mg/L BAP |
| Agrobacterium infection | LS-Infection | 4.71 g/L MS salts and vitamins<br>30 g/L sucrose<br>Set pH to 5.8.<br>Sterilize by autoclaving and add;<br>100 μM Acetosynringone |
| Co-cultivation | LS-CC | 4.71 g/L MS salts and vitamins<br>30 g/L sucrose<br>Set pH to 5.8.<br>8 g/L agar<br>Sterilize by autoclaving and add;<br>0.1 mg/L NAA<br>0.1 mg/L BAP<br>100 μM Acetosynringone |
| Transgenic selection media | LS-PSM | 4.71 g/L MS salts and vitamins<br>30 g/L sucrose<br>Set pH to 5.8.<br>8 g/L agar<br>Sterilize by autoclaving and add;<br>0.1 mg/L NAA<br>0.1 mg/L BAP<br>300 mg/L timentin<br>50 mg/L kanamycin |
| Rooting media | LS-rooting | 4.71 g/L MS salts and vitamins<br>30 g/L sucrose<br>Set pH to 5.8.<br>8 g/L agar<br>Sterilize by autoclaving and add;<br>300 mg/L timentin<br>50 mg/L kanamycin |

## Appendix D

List of major equipment used in this study

| **Machine** | **Purpose** | **Make** |
|---|---|---|
| Nanodrop | Analyzing DNA and RNA Concentration and quality | Thermo-Scientific Nanodrop 2000 Spectrophotometers, USA |
| Gel documentation system | Record and analyze the results of gel electrophoresis. | Bio-Rad, Gel Doc EZ Gel Documentation System, USA |
| PCR machine | Replication of DNA. Genotyping transgenic plant lines | Bio-Rad T100 Thermal Cycler 96 well block PCR, USA |
| Centrifuge | Separate heterogeneous mixtures into their various components | Eppendorf high-speed centrifuge, Germany |
| Vortex | Mixing liquid Samples, Cell disruption, and homogenization | Vortex-Genie 2, Scientific Industries Inc., USA |
| Water bath | Heat shock *E.coli* component cell during transformation | Thermo Scientific, USA |
| Heating block | Incubation of DNA or RNA, enzyme or chain reactions, and denaturation During DNA or RNA extraction. | Thermo Scientific, USA |
| Laminar airflow | provide a controlled and sterile workspace. | Thermo Scientific, USA |
| Microscope | Checking the presence of RFP in transgenic lettuce | Leica M165 FC Fluorescent Stereo Microscope. |
| Incubators | Incubate lettuce explant. during co-cultivation at 22°C | Geneva Scientific. |

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A study aimed to standardize efficient *in vitro* regeneration and Agrobacterium-mediated transformation methods for the *Lactuca sativa* variety 'Paris Island cos.' After analyzing 11 tissue culture media-hormone combinations for shoot regeneration, the study optimized agrobacterium mediate transformation conditions, achieving a 14% transformation efficiency. Transgenic plants expressing the salt and drought tolerance gene SbSI-1 from *Salicornia brachiata* were obtained, demonstrating successful protocol development for genetic improvement in lettuce.

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