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MARYAM ZAHID

SU92-MSBTW-F23-005

FOS

Expression Of Reporter Genes In Tobacco (*Nicotiana tabacum*)



SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University

Lahore In Partial Fulfillment

of the Requirement for the

Degree of MS Biotechnology

By

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Session: 2023-2025

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This is to certify that the research work presented in this thesis, titled “**Expression Of Reporter Genes In Tobacco (*Nicotiana tabacum*)**” was conducted by “**Maryam Zahid**” under the supervision of “**Dr. Sarfraz Kiani**”

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science/Master of Philosophy in the field of “**MS Biotechnology**” in Faculty of Sciences at The Superior University, Lahore.

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DEDICATION

To my beloved parents

Mr. and Mrs. Zahid

who encouraged me all the way to finish the task I started

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List of Abbreviations

BAP	Benzylaminopurine
CAT	Chloramphenicol Acetyltransferase
CCM	Co-cultivation Medium
CTAB	Cetyltrimethylammonium Bromide
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
GFP	Green Fluorescent Protein
GUS	β -Glucuronidase
IBA	Indole-3-Butyric Acid
IM	Infection Medium
LacZ	β -Galactosidase
LSIM	Liquid Shoot Induction Medium
LUC	Luciferase
MUG	4-MeT hylumbelliferyl- β -D-Glucuronide
NAA	Naphthaleneacetic Acid
NptII	Neomycin Phosphotransferase II
OD600	Optical Density at 600 nm
PCR	Polymerase Chain Reaction
qPCR:	Quantitative Polymerase Chain Reaction

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

Expression Of Reporter Genes In Tobacco (*Nicotiana tabacum*)

Abstract

We clustered the reporter genes to monitor the patterns of expression, to assay the promoter activity and construct stable transgenic tobacco lines (*Nicotiana tabacum*). The reporter genes such as green fluorescent protein (GFP), which is a marker found in jellyfish, and β -glucuronidase (GUS) due to their known and certain output. GFP fluoresces under a blue or UV light, and GUS appears as spots of blue on a simple histochemical stain using the appropriate substrate, i.e. no longer required to use a red fluorescent protein. The third reporter gene is the RUBY system, where production of betalain pigments in a short enzyme pathway is induced by this simple enzyme stain, producing a strong red-purple color in plant tissues. The primary objective was to gain a better insight into the regulation of these reporter genes, the comparison of various promoters, mapping of tissue-specific activity, and tracking of successful folding of transformation in tobacco. *Agrobacterium tumefaciens* strain GV3101 was used to perform the standard leaf disk transformation to get the reporter constructs into the plants. To ensure stable integration in the regenerated plantlets, we initially checked the integration of reporter genes in the regenerated plants with PCR technique, followed by a check to determine the copy numbers and strength in the expression with a quantitative PCR. GFP activity was evident in a fluorescence under the microscope and GUS was sharp and blue spots in the targeted areas. The findings were good: GFP and GUS were repeatable and provided good visual evidence of expression in the desired location. The RUBY marker was unique since it produced noticeable red-purple color in approximately 80 percent of the positive PCR lines, that is, it was easy to view with the results with naked eye without using any equipment or additional procedures. Each of the three reporters were useful in tracking the target of transgene insertion and expression in tobacco, and it was simple to examine promoter information and gene expression in live transgenic plants and avoided the complicated selection procedure to pick the stably transformed right plants.

Key words: *Nicotiana tabacum*, *Agrobacterium*-mediated transformation, GFP, GUS, RUBY, Reporter genes.

CHAPTER 1

INTRODUCTION

The most economically significant species of the *Nicotiana* genus, Solanaceae family, is *Nicotiana tabacum* L. or cultivated tobacco. There are 76 naturally occurring species in the genus organized into 13 sections and have various applications including smoking, ceremonial rituals and as an ornamental garden. Most of the species were in the Americas with approximately 50 being native to North and South America and adjacent islands, although it was in southern South America where the genus first appeared. Tobacco is currently planted both in temperate and tropical areas across the world, thus a significant nonfood cash crop [1]. Its processed leaves are the foundation of several tobacco products used by more than one billion individuals on the planet. China, India, Brazil and the United States are the largest producers with the crop sustaining a huge industry in the cigarettes, cigar, pipe tobacco and smokeless products.

In addition to its commercial application, *N. tabacum* is of great pharmaceutical importance, and it is a natural insecticide. It is an allotetraploid species whose genome occurred because of the hybridization between the ancestors of the diploid *Nicotiana sylvestris* and *Nicotiana tomentosiformis* [2]. Tobacco was first domesticated and used by the indigenous peoples in Mesoamerica and South America many years before it reached Europe and the rest of the world [3]. It grows relatively fast and takes 120 to 150 days after planting before it comes to harvest [4].

Tobacco has been used more often by scientists as a model organism in genetic, physiological and metabolic processes in part due to its ability to proliferate in tissue culture, quick growth, floral biology as well as responding to environmental stresses. It has since grown as a preferred system in the last 20 years in molecular farming- the generation of recombinant protein and pharmaceutical in plants. The main benefits are the high level of yields and biomass of seeds, well-developed tissue culture methods, simple genetic transformation and various expression systems (nuclear stable, transient and plastid-based). Annual leaf biomass can reach 100 tonnes per hectare, supporting

large-scale production. Importantly, transgenic tobacco lines pose no risk as food or feed crops and can even contribute to phytoremediation by absorbing environmental pollutants [5].

One of the most reliable techniques for introducing foreign DNA into higher plants remains *Agrobacterium tumefaciens*-mediated transformation. Researchers routinely apply this method in tobacco to achieve efficient gene transfer, promote natural regeneration, and minimize potential ecological impacts [6].

Plants have evolved special defenses against biotic stressors like pathogen assault, environmental stressors like heat and cold, and predators. It may take hours or days for some of these processes to react, while others happen quickly, within seconds or minutes after the stimulation. In distant plant sections, pathogen infection can cause secondary reactions that result in alterations in gene activity and the production of proteins that the plant would not ordinarily generate or in trace amounts in particular organs. Additionally, the environment provides plants with developmental signals that influence their capacity for adaptation and infection response.

A potent technique for producing commercial crops with enhanced agronomic traits is genetic engineering. Transgenic plants must be recovered using selectable markers, and genes that confer chemical agent resistance are frequently used. However, both transformed and non-transformed cells may be present in tissues that have undergone certain gene transformations or that encode desired characteristics [7].

In the biological sciences, reporter genes and systems are essential. For example, bacteria have lacZ, plants have GUS, luciferase has LUC, and both prokaryotic and eukaryotic species have GFP. The organism and the imaging and detecting technologies that are available determine which reporters should be used. GUS, LUC, and GFP are frequently employed as selective markers in plants. GUS and LUC require particular substrates to be seen with the unaided eye or under light microscopy, and they may be recognised with technologies. While RUBY, a novel reporter, produces the red pigment betalain that is readily visible to the

unaided eye, GFP necessitates a fluorescence microscope without certain substrates. However, this pigment's irreversible generation might pose a problem in the field since it could impede biological activities like photosynthesis [8].

Reporter genes originating from diverse sources such as bacteria, viruses, and corals produce detectable signals like fluorescence, luminescence, or color when expressed in host organisms [9]. Since it was first discovered that gene expression and protein production are related, there has been curiosity in the mechanism by which organisms

regulate protein production through gene expression. A good reporter gene should have characteristics such as a gene and gene product that are well characterized both genetically and biochemically, and a gene product with enzymatic activity that is different from the host's endogenous activities [10].

Genetically encodable reporters have been developed to monitor a wide range of cellular and environmental events, including responses to external stimuli, hormone signaling pathways[11], protein turnover and the activity of introduced transgenes. Researchers rely heavily on these tools to explore fundamental biological questions, such as how hormones and environmental cues influence gene regulation [12], how proteins maintain stability or move within cells and where specific genes are expressed [13].

Various reporter systems have been established widely in biology of plants:

- 1 *b*-Glucuronidase (GUS): This is an enzyme that is used to visualize expression patterns easily by simple staining by converting a colorless substrate to a deep blue precipitate.

- 2 Green Fluorescent Protein (GFP): It is a marker that is native to jellyfish. Upon striking ultraviolet or blue light, it appears in a bright, intense green. The greatest attributes of GFP are that it enables scientists to view living cells and tissues real time and it does not cause any damage to them or even kill the sample.

- 3 RUBY: The RUBY system, in contrast to fluorescent proteins, relies on the production of betalain pigments with the help of a small number of enzymes introduced into the plant. What is achieved is a vivid red, which is easily visible

and does not need any special lights or microscopes to be viewed.

These reporter tools have proved to be of great benefit in the unraveling of complex biological processes in plants, including; how genes are switched on, where proteins localize within cells, and the working of different signaling pathways in a vast array of different species [13].

Reporter gene systems are also commonly used in research to identify and monitor the product of a gene as opposed to its corresponding protein. CAT from *Escherichia coli*, LacZ from *E. coli*, Lux from bacterial and firefly luciferases, GUS from *E. coli*, and

GFP from *Aequorea victoria* (jellyfish) are the most often utilised systems. Studying how various genes affect various creatures is made easier using this method [7].

In gene expression investigations, green fluorescent protein (GFP) and its variants, including red and yellow fluorescent proteins, have been employed as reporters; GFP visualisation necessitates a fluorescent light source [12]. GFP and its variants are frequently employed as fusion or gene expression proteins. But for GFP to see fluorescence signals, light sources are necessary. Due to microscopic monitoring, traditional reporters are less effective for analysing plants in natural settings or huge samples like trees. They are also invasive and need the sacrifice of plants [14].

A variety of techniques, including β -glucuronidase staining and green fluorescent protein (GFP) production, have been developed to track the recovery of uniformly transgenic plants utilising reporter genes. These techniques seek to optimise transformation efficiency; however, they have limitations since autofluorescence interference and plant developmental stage might influence gene expression. Given the limits of these techniques for identifying transgenic expressions in green tissues, some writers propose a consistent, positive Southern blot as evidence of the uniformity of transformation [7]. Reporter genes, like β -glucuronidase or GUS, are used to visually evaluate transformed cells and detect transgenic materials. These techniques, however, are regarded as damaging and need foreign substrates or specialised equipment in order to detect fluorescence activity. The

decision is based on the type of research, stability, activity duration, and background signals of the reporter molecule. Their benefits and drawbacks are addressed. For tracking auxin distribution and signalling in DR5-GUS transgenic lines, for example, the β -glucuronidase (GUS) reporter is frequently employed in plants to examine gene expression patterns and hormonal signalling [14]. Hormonal signalling and plant gene expression are frequently observed using the β -glucuronidase (GUS) reporter gene. However, GUS histochemical labelling is an intrusive technique that necessitates plant sacrifice and costly substrate. Despite being a non-invasive technique that necessitates costly substrate spraying and a camera for observation, luciferase has practical challenges when it comes to tracking transformants and gene-targeted mutants. For successful transformant selection, it is therefore essential to build continuous, affordable cellular activity monitoring devices [12].

GFP fluorescence was found to be more consistent with verified transgenics in research comparing the screening efficacy of herbicide resistance (Basta) and GFP fluorescence. For detecting real transgenics and removing false-positive events that can evade antibiotic selection, the scientists proposed that reporter genes are more trustworthy than selectable marker genes. Because of its non-destructive nature, cell-autonomous expression, and absence of external substrates, green fluorescent protein (GFP) is a commonly utilised reporter gene in genetic transformation experiments [15].

Reporter genes fusion, GUS and GFP, have drawn a lot of interest due to their adaptability, sensitivity, and visual expression clarity. By combining GFP's fluorescence with GUS's enzymatic activity, the GUS and GFP fusion allows for dual-reporter capabilities for live-cell imaging and histochemical labelling. One key advantage of *Agrobacterium*-mediated transformation early in the process is its ability to separate reporter signals coming from the bacterium itself (prokaryotic expression) from those arising in the plant cells (eukaryotic expression). This distinction helps researchers confirm that observed activity truly reflects events inside the host tissue rather than lingering bacterial contamination.

Even more precise detection is provided by fusing GUS and GFP into one reporter protein, which attaches enzyme activity to the fluorescence in the same molecule.

The system is of particular use in the study of transient expression soon after transformation, in determining the integrity of transgene integration, and in the characterization of the strength of promoters in a wide range of experimental applications. The fusion gives the readouts complementary to each other: GFP enables dynamic imaging in real-time using fluorescence microscopy, because the fusion protein is non-destructive and provides sharp, high-resolution localization of foci in fixed tissues with the GUS staining [16].

More recent advances have presented reporter systems which do not require fluorescence equipment at all, or even external substrates. These markers permit *in vivo* real-time monitoring at the naked eye through the biosynthesis of bright natural pigments like betalains or anthocyanins. RUBY reporter is exceptional in this

category. It contains the enzyme-coded pathway to produce betalain, which gives cells that have been transformed into a vivid red hue without the use of any additional chemicals or special lighting. The pigment-based system is a straightforward, cost-efficient and non-invasive technique to detect instances of a successful transformation in the course of screening. RUBY has shown consistency in both *genot* and *dicot* species and is therefore especially handy in situations where substrate-free, equipment-light detection is desirable whether to check transgene insertions into a specified genome, a promoter, or a large-scale pipeline of transfection [15].

Real-time PCR has emerged as one of the most reliable techniques in biological research, which is able to identify and measure even the smallest concentration of a particular nucleic acid sequence. It is extensively used in clinical diagnosis and has also been useful in transgenic plants not only to measure the level of transgene expression, but also to define zygosity and to estimate the number of integration events [7].

Reporter genes have superior sensitivity and distinct observable signals that have low background noise in host cells. The selected reporter will depend on aspects such as the cell type, experimental design and a requirement to scale assays to an individual cell imaging technique or a high-throughput screening [17].

The model studies employing tobacco to study gene transformation can be used to

optimize procedures that can subsequently be transferred to other crop plants to aid in coming up with strong measures to transform it into a resistant or other desirable plant [18]. Reporter genes in tobacco have provided a wide range of research opportunities, including the study of how genes are expressed and regulated in response to environmental signals as well as the study of protein localization in cells and signaling pathways [19]. The tools give profound understanding of the complex processes that govern plant growth and development [20].

In addition, the reporter genes are very important tools in functional genomics as they enable scientists to identify new genes and explain their functions[21]. Due to its remarkable amenability to genetic transformation, regenerative competency as well as robust expression of transgenes [22], tobacco (*Nicotiana tabacum*) has become one of the best model organisms in the field of plant science. Its complete and sequenced genome, coupled with comparably low cost of growing it also makes it an excellent research platform [23].

Although developing and optimizing elite genotypes demands considerable time and resources, breakthroughs in biotechnology—particularly transgenic approaches—have unlocked new avenues in plant improvement. The structure and function of genes by examining gene transformation in plants [13]. Gene transfer into plants can be attained through direct techniques such as micro-projectile bombardment, microinjection, electroporation, and protoplast fusion, as well as indirect approaches like viral vectors and *Agrobacterium*. Even though plant viruses are simpler to convert, they are less useful as transformation vectors because they cannot attach to the host genome or pass on to the following generation through meiosis. The gram-negative, rod-shaped, soil-borne agrobacteria that induce crown gall in plants belong to the Rhizobiaceae family and the *Rhizobium* genus [24].

One key method to express reporter genes in tobacco is through *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transient gene expressions in plant leaves is a quick and efficient way to analyse gene expression. It allows for the creation of different heterologous proteins without the requirement for transgenic plants, which is difficult for many plant species [25]. Plant genetic

engineering techniques such as tissue-culture-based transformation and *Agrobacterium*-mediated transformation are commonly employed; nevertheless, problems such as false-positive transformant shoots or escapees on selection media are unavoidable in these techniques. To get rid of false-positive transformants, GUS test and PCR analysis of target DNAs have been used [8].

This method involves using the bacterium *Agrobacterium tumefaciens*, which naturally transfers genetic material into plant cells. The reporter gene, often linked to a selectable marker, is inserted into the plant's genome using this system [26]. Tobacco cells, particularly those in leaf tissues or suspension cultures, can be infected with molecular mechanisms underlying gene regulation in plants [27].

Agrobacterium-mediated transformation uses Ti or Ri plasmids to integrate transfer DNA into the host genome. It is essential to confirm transgene implantation using molecular biology methods like PCR, Southern blotting, and WGS. Transformed cells

are often identified by the detection of selectable marker genes, which usually give resistance to antibiotics or herbicides [23]. To minimise escape plant dangers and suppress non-transformed cells, a selective agent's ideal dose is essential. The right dose must be found through extensive experimentation for effective selection, which can potentially cause bottlenecks because it depends on selectable marker genes [15].

Agrobacterium allowing the gene of interest, including the reporter gene (such as GUS or GFP) to be combined into the plant genome [13]. Reporter gene expression can then be monitored as an indicator of success of transformation.

This study aims to investigate the expression of reporter genes in tobacco, focusing on understanding the patterns and regulation of gene expression, monitoring transformation efficiency, tracking gene expression, validating promoter activity, selecting transformed cells, and facilitating the analysis of gene regulation and expression in response to various hormonal cues in tobacco.

Problem statement

In the absence of reporter genes, currently in-use selection method for transformed plants is relatively inefficient and cumbersome, limiting the promoter activity in the transgenic plants. There is no efficient selection method backed by reporter genes for local tobacco cultivars, necessary to achieve desirable traits in these plants.

Aims And Objectives

1. To analyse recombinant plant expression vector carrying Gus-plus-GFP, Ruby in SBL Lab via restriction digestion.
2. To clone plant expression vector into *Agrobacterium* and transform local tobacco plantlets via *Agrobacterium* by co-cultivation.
3. To select newly transformed tobacco plantlets via reporter gene expression.
4. To conduct molecular analysis of transgenic tobacco plants by PCR, qPCR and microscopic analysis of reporter genes.

CHAPTER 02

Litrature Review

Genetic Engineering

In genetic engineering applications, gene expression analysis is essential, particularly when researching novel genes or regulatory signals. For evaluating the behaviour of genes or DNA sequences in plant cells, transient gene-expression assays are helpful. However, obtaining entire plants or transgenic cell lines that have undergone stable transformations is a time-consuming and tedious procedure that might take weeks or months. When a foreign or altered gene is intended to be expressed in transgenic plants, this is particularly crucial. Knowing ahead of time how the transgene will act in transgenic plant cells is another strategy.

In addition to being used for breeding, transgenic plants have become a viable option for creating recombinant pharmaceutical compounds, providing substantial potential for industry. There are several efforts underway right now to produce vaccinations, antibodies, and other medications. The proof of concept for the recombinant protein's expression and biological activity evaluation is an important milestone that reduces the risk of adopting the plant system for large-scale expression.

Plant genetic engineering makes use of *Agrobacterium tumefaciens*' ability to transfer DNA, with different transformation techniques tailored for species that were previously resistant and those that successfully undergo physical transformation processes [28].

Reporter Genes

Since the earliest days of plant genetic engineering, reporter genes have been crucial in the study of gene control. An archetypal reporter gene generates a product that is simple, precise, and uniquely assayable in a variety of biochemical settings when it is controlled by genetic control systems such as transcriptional promoters. The identification of transformable cells is commonly defined by

detecting the presence of targetable marker genes, normally conferring resistance to antibiotics or herbicides [23]. The ideal dose of a selective agent is needed to reduce the risk of escape plants

and inhibit non-transformed cells. Effective selection of the right dose requires a lot of experimentation that may potentially create bottlenecks due to its dependency on selectable marker genes [15].

Agrobacterium in which the gene of interest, including the reporter gene (as GUS or GFP) may be introduced into the plant genome [13]. Prerequisite Reporter gene expression may then be tracked as success indicator in transformation.

The study is expected to help explore the expression of reporter genes in tobacco, the patterns and regulation of gene expression, the transformation efficiency, gene expression, the validation of the promoter activity, selection of transformed cells and helps in analyzing the gene regulation and expression in response to different hormonal cues in tobacco.

This reporter gene signal faithfully recapitulates several of the major characteristics of the native gene behavior: general levels of expression, localization to specific tissues or cells, and alterations in activity in various developmental stages. Plant biologists over the years have discovered few, but strong reporter systems which will fulfill most experimental requirements and have become the standard tools of this kind of research [29].

Reporter genes are highly significant in the molecular biology field in that they provide researchers with a simple methodology of tracking genes expression, promoter activity and measuring the effectiveness of genetic modifications [5]. They operate by generating a readily observable product be it fluorescence, luminescence or visible color such that it is now possible to visualize gene activity in real-time within living cells or tissues. These reporters have particularly come in handy in tobacco with the aim of studying the behavior of genes to external stimuli such as changes in light, changes in temperature or other stresses. They have also assisted in mapping the behavior of promoters and discovering the regulatory processes controlling the time and place of gene activation or repression [17].

Reporter constructs in gene-trapping technologies are randomly inserted into the genome. Through the study of the pattern of diverse expression patterns formed because of these insertions, scientists can identify particular genes and their upstream control factors. This technique is effective to form in situ gene fusions that show the identity and the control regions of the genes that are not known. Despite the material

and genetic redundancy of the gene, even though loss of functional phenotypes in plant gene where a loss does not necessarily lead to an apparent and apparent phenotype, gene trap insertion may hinder gene activity. Gene trap-aided functional analysis can be a useful approach in certain cases since the expression patterns of trapped lines could provide useful phenotypic information [30].

In plant molecular biology, reporter genes are frequently used as markers of transformed cells throughout stable transformation processes, evaluating the efficacy of transgenic approaches for viral resistance, and detecting patterns of gene expression [31]. These genes have been used to study gene silencing mechanisms and evaluate the effectiveness of RNA interference (RNAi) approaches [23]. The perfect marker gene should be durable, non-toxic, and simple to utilise. GUS, GFP, LUC, and C1 + R/B (anthocyanin accumulation) are often employed as markers. Before selecting the right marker gene, it's important to consider your research and plant tissue because every gene has different characteristics. Effective selection of transformants and mutants is necessary to produce plant species with desired gene expression and targeted mutagenesis for trait improvement, which are achieved through the widespread use of genome editing and plant genetic transformation techniques [31].

Several factors affect the choice of reporter system, including the size of the reporter gene and its product, the availability of sensitive methods for both qualitative and quantitative protein activity assays, the capacity to monitor changes in gene expression, and the lack of similar activities in the organism's cells. Reporter genes are proteins that stand out in mixes of endo/exocellular proteins due to their distinct characteristics or enzymatic activity [25].

GUS Reporter Gene

Originally isolated from *Escherichia coli*, the gene *gusA*, which codes for β -glucuronidase, is a component of an operon. Other creatures, including plants, employ it as a reporter gene [32]. A sensitive indication of transgenic activity, the β -glucuronidase gene (*gusA*) from *Staphylococcus* and *Escherichia coli* efficiently identifies tissues and cells that contain the reporter construct. However, the reporter's

usefulness in some applications is limited since GUS systems frequently cause test tissues to die or be destroyed [29].

The GUS gene, sometimes referred to as the bacterial β -glucuronidase gene (*uidA*, *gusA*), has emerged as the main reporter gene for examining plant gene expressions because of its growing accessibility to plant species and molecular transformation. Its quick, non-radioactive analysis, sensitivity, and capacity to produce both quantitative and qualitative data using the same reporter gene are the main reasons for this assay's widespread use. The 68 kD β -glucuronidase protein is encoded by the GUS reporter gene, which is derived from the *Escherichia coli* *gus* operon. Fluorogenic substrates such as 4-MUG are used in quantitative tests, but X-gluc can qualitatively demonstrate cell and tissue-specificity. Researchers have shown that promoter-GUS fusions are useful for promoter analysis and gene family dissection, and that chemical alteration of substrates may allow GUS to be used as a selectable marker [33].

In the 1980s, R.A. Jefferson identified that the *E. coli* gene *UidA*, which codes for the hydrolyase β -glucuronidase, might be used as a quantitative marker in plants. Commercial β -glucuronides, which hydrolyse to create coloured or luminous compounds, are widely utilized in different plant species, including cereals, due to their low endogenous activity and convenience of handling. Although its stability and tolerance make it simple to assess in whole tissues or crude extracts, membrane-impermeable substrates typically prevent it from being quantified or seen in live, intact tissue. While it may be less troublesome, using a transformation marker as a gene expression reporter has limited appeal, and at least one approach recommends non-destructive in situ detection. Two techniques are available for detecting GUS activity in transgenic material: histochemical

approaches on entire tissues or measuring it in plant extracts using a fluorogenic substrate. There are several fluorogenic substrates; the most effective one for generating the fluorescent product 4-methylumbelliferone (MU) is 4-methylumbelliferyl- β -D-glucuronide (MUG), which is split by GUS.

A low-power light microscope is necessary for close tissue viewing; however, the substrate may be readily oxidised and dimerised to create insoluble indigo, a dark blue colour that is easily visible to the naked eye and doesn't require any additional equipment. Through tissue fixation and embedding procedures, GUS/X-gluc provides

a stable indigo result, making tissue-specific detection of promoter expression patterns easier [31].

GFP Reporter Gene

A 27 kDa protein known as green fluorescence protein (GFP) is found in the *Aequorea victoria* jellyfish. Fluorescence is an optical phenomenon in which a molecule emits a second photon after absorbing a photon of light to attain an excited electronic state. The chromophore's structure affects the absorption and emission spectra, or the Stokes shift, which results in a shorter wavelength. Only O₂ is needed as an external substrate for the straightforward process of GFP fluorescence emission. It is produced when a Ser-Tyr-Gly sequence at amino acid residues 65–67 in the protein undergoes cyclisation and oxidation. Blue and green light detection are necessary for detecting GFP in live cells, which makes it the perfect option for monitoring protein interactions and gene expression in vivo. The lack of fluorescence in heterologous hosts in the first native GFP tests was discouraging. Consequently, scientists produced modified forms of the wild-type protein, which had different spectrum characteristics and were brighter. Many creatures, including plants, are already using these novel GFP variations as effective reporter genes [34].

In stable transformation studies, particularly for cereals, FPs are being used more as a reporter to improve transformation efficiency and support selection techniques. Although they are not commonly measured in extracts, FPs can

be measured in extracts using a spectrofluorimeter and compared to known fluorescence values from isolated FP [31].

Monitoring of the position, mobility, and activities of proteins in live cells is made possible by fluorescent proteins fusing with other proteins. They operate as indicators for following proteins, identifying interactions, and determining their fate inside the cell. The destiny of transgenes inserted into cultivated plants and their effects on the environment may be tracked using fluorescent proteins [35]. Given the practical challenges of current approaches, it is imperative to create affordable, non-invasive reporter systems for tracking transformants and gene-targeted mutants. Transformants and genome-edited plants can be identified and selected more quickly if visible indicators of transgenes and targeted mutants are present [12]

RUBY Gene System

An excellent tool in plant study is the RUBY gene system that synthesizes the beetroot red pigment betalain out of the "2A" peptide. It has been used to identify haploids in maize and tomato, visualise protein-protein interactions and advance plant transformations in rice, Arabidopsis and cotton. Researchers have adapted the RUBY system as a split selectable marker which is much more convenient in stacking genes and enhances co-transformation rates during repeated transgene introductions [36].

Carotenoids, betalains and anthocyanins are colorful compounds that are natural products of different biosynthetic pathways of plants. These bright pigments promise as reporters of images in transgenic and gene-editing studies and provide an easy way to identify transformed cells or tissues. Their bright red-to-blue colors make anthocyanins look especially appealing; however, synthesis depends on elaborate, species-specific enzyme complexes which makes it difficult to make a universal reporter system across plants. By contrast, betalain pigments can be synthesized in only three enzymatic reactions beginning with tyrosine, which is a simpler and more predictable pathway.

To take advantage of this route, He and associates (2020) designed a small, multipart, open reading frame (RUBY), which expresses the complete repertoire

of enzymes required to synthesize betalain. The construct has since been an effective naked-eye assay to select transgenic events in various species, one of which is *Arabidopsis thaliana*. The RUBY reporter has shown great efficiencies in transformation of maize and has also been useful in the identification and isolation of haploid lines in maize and tomato [12]. It also allows the localized pigmentation of a high strength which allows specific visualization of the tissue and cell that would otherwise be difficult to view when using traditional reporters.

Other traditional systems, such as GFP and GUS, are still staples in terms of gene-trap studies to map expression patterns. GFP is highly effective in visualisation of protein localisation in living cells, but autofluorescence is high in many plant tissues, making it unsuitable in whole-organism surveys. Instead, GUS offers excellent spatial resolution by histochemical staining and is stable in translational fusions allowing signals to be observed even at single-cell scales [37].

In the future, the integration of reporter genes in tobacco studies is likely to speed up the development of better varieties of crops carrying new useful traits. The tools will also simplify the process of validation of accurate genome edits. In addition to tracking expression, journalists will enable engineers to devise completely new metabolic pathways and regulatory circuits in plants, which will lead to the generation of high-value compounds[23]. Moreover, they provide potent tools to investigate the relationship between the plant and microbes and design approaches that will increase their resistance to diseases and general crop health. Gene expression study in plants is important in the study of different biological processes including growth development, stress response, and resistance to diseases [38].

Tobacco Plant

A member of the Solanaceae family, tobacco is frequently employed as a model plant in functional genomics, proteomics, and metabolomics research to better understand biological processes. With regeneration accomplished *in vitro*, *Agrobacterium*-mediated transformation of tobacco has become commonplace, making it a premier platform for characterisation of gene function and the manufacture of recombinant biopharmaceuticals [39].

Genetic Transformation in Tobacco Using *Agrobacterium tumefaciens*

The generation of different heterologous proteins without the requirement for transgenic plants—a problem for many plant species—is made possible by the *Agrobacterium*-mediated gene expression system in plant leaves, which is a quick and efficient technique for gene expression analysis [8].

The first to go through such a metamorphosis was tobacco, a successful and quickly growing model plant for genetic transformation. Tobacco is a notable example of successful plant-based genetic engineering, having been modified in 1983; the soil phytopathogenic bacteria *Agrobacterium tumefaciens* was used to indirectly convert tobacco in order to introduce the gene for kanamycin resistance. New opportunities for the practical use of genetic engineering to improve important agricultural crops have been created by developments in gene transfer and plant regeneration processes.

Enhancing plant characteristics and identifying gene functions inside plants have both benefited from genetic transformation.

Carotenoids, anthocyanins, and betalains are important plant pigments. With tyrosine as the substrate, betalains have the most straightforward biosynthetic process, requiring just CYP76AD, DODA, and glucosyl transferase in four stages. In contrast to the reporter gene GUS, which requires labelling and tissue sacrifice, betalain has been shown to be a non-invasive reagent [8]. The ability to visually identify fluorescent protein production genes in living cells without the need for intrusive treatments gives them an edge over other marker genes. Soon after transformation, transformed cells expressing these genes may be recognised, and their division state can be ascertained [35].

Utilization of *Nicotiana tabacum* (tobacco) as a model organism for the expression of reporter genes has revolutionized the field of plant molecular biology. The ease of genetic transformation, rapid growth rate, and well-characterized genome of tobacco make it an ideal system for investigating the regulation of gene expression, protein localization, and signaling pathways [40].

The inherent capacity of *A. tumefaciens* cells to transfer and integrate T-DNA into

the host plant genome are used in the economic and successful technique known as "Agrobacterium-mediated transformation." Being a single-cell system, Agrobacterium-mediated plant transformation has benefits over other techniques like biolistics. It prevents chimera formation and lessens problems with transgene co-suppression and instability because only one copy number is transferred. Several variables, including plant genotype, explant type and age, *A. tumefaciens* strain, vector type, bacterial cell density, pre-culture period, AS concentration, infection time, co-cultivation period, pH, and co-cultivation temperature, affect how well Agrobacterium-mediated transformation and delivery of T-DNA into plant cells work, culturing medium mixture and Agrobacterium infection techniques [39]. Using both direct and indirect techniques, genetic engineering is an essential biotechnological tool for improving plant performance. In plants, the most efficient laboratory technique for indirect gene transfer is *Agrobacterium* infection.

A straightforward and effective technique for transferring foreign DNA essential for genetically engineered plants is the *Agrobacterium* approach. The agrobacterium mediated transformation has several challenges due to numerous experimental variables. The density of bacteria cells, the time of tissue immersion in the inoculum, the concentration of acetosyringone used to select and kill bacteria, the co-culture time, pH of the medium, incubation temperature and wounding of tissues are also important. These parameters may greatly influence the efficiency of transformation, and close optimization is needed in any gene delivery experiment with the use of this technique.

The factors that have been found to be key to success are selection of Agrobacterium strain, inoculum density and antibiotics used to reduce remaining bacteria following co-cultivation. Explants are also different in their responsiveness in terms of age, type and those that have undergone wounding pretreatment. The time of immersion in bacterial suspension is important, and the presence of some additives such as antibiotics or chemical inducers is also important. Lastly, co-cultivation temperature, pH and length of the time directly affect the efficiency of T-DNA transfer into the plant cells. All these areas need to be refined by the researchers to reach credible and repeatable findings. Examples of plasmids that can be used to optimise the plant expression are

suboptimal promoters, enhancers, inefficient codon usage, 5'UTR sequence, trigger silencing, and gene integration into a silent chromatin region, which are also important [18].

Agrobacterium tumefaciens, which infects dicotyledonous plants by introducing plasmid DNA into their cells, is used to create transgenic plants. Transient expression is the term for the episomal expression of genes prior to chromosomal integration. One benefit of this approach is that it permits a temporal peak in protein levels, often higher than steady expression levels, around two to three days after transfection. Because this period is shorter than the months needed to produce a transgenic plant, protein may be produced quickly, which is essential for modest protein levels or early therapeutic development [40].

The DNA-transfection ability of *Agrobacterium tumefaciens* is used in plant genetic engineering, with different transformation techniques tailored for species that were previously resistant and those that effectively use physical transformation techniques.

Compared to physical transformations, *Agrobacterium* transfection has several benefits, including high cell survival rates, a high ratio of transformed to non-transformed cells, and direct gene transfer to the nucleus. Various *A. tumefaciens* infection techniques, which usually target green leaf tissue, enable gene expression in transgenic plants without causing regeneration [41].

Co-Expression of GUS, GFP, and RUBY Genes

By merging the genes producing GFP and β -glucuronidase (GUS), a series of bifunctional reporters constructs for both transient and stable plant expression experiments, combining the advantages of GFP as a vital marker with the high sensitivity of β -glucuronidase (GUS) in histochemical labelling [42].

The co-expression of the GUS gene and the GFP-RUBY fusion gene in *Nicotiana tabacum* facilitates the investigation of gene expression and protein localization. The GUS gene, encoding β -glucuronidase, serves as a versatile reporter gene, enabling the quantitative assessment of gene activity through enzymatic

hydrolysis of a chromogenic substrate. Fusions between GFP and RUBY reporters have also been studied, producing a dual-color system that integrates the green fluorescence of GFP and the bright red betalain pigmentation of RUBY. This system can track the expression pattern of genes and protein behavior simultaneously and in real time within living tissues [43].

The possibilities of using GUS, GFP and RUBY reporters in tobacco plants open up promising opportunities in the development of stronger plants resistant to diseases, pests and environmental pressures. As an example, the GUS marker can be used to trace patterns of gene activation in response to defenses by scientists, which can subsequently be used by the breeding of more resistant varieties against pathogens [44]. GFP and RUBY enable the visualization of where proteins are localized within cells and plant interactions with attacking microbes, illuminating the nature behind the resistance.

These reporter genes are delivered to tobacco cells by *Agrobacterium*-mediated transformation. In this case, the bacterium *Agrobacterium tumefaciens* is used to transfer foreign DNA into the genomes of plants as a natural delivery vehicle [45]. The GUS, GFP, or RUBY constructs are initially inserted by scientists into a binary vector which can be carried by the bacterium [46]. After infecting wounded tobacco tissue, the engineered *Agrobacterium* transmits the T-DNA segment that has the reporter gene which integrates successfully into the chromosomes of plant [47].

This approach stands out among transformation techniques because it typically results in low copy numbers, reliable long-term expression and the capacity to handle large DNA fragments. For molecular confirmation of transgenic plants, techniques like PCR, qPCR, and microscopic analysis of transgenic plants are used.

CHAPTER 03

Methodology

3.1. Agrobacterium-mediated Transformation (carrying pGFP-GUSPlus plasmid) of Tobacco Using Leaf Disc Method

This protocol outlines the transformation of tobacco (*Nicotiana tabacum*) using *Agrobacterium tumefaciens* strain GV3101 carrying the pGFP-GUSPlus plasmid and other standard tobacco transformation protocols. The method uses leaf disc explants, which are highly regenerable in tobacco, and includes steps for explant preparation, *Agrobacterium* preparation, inoculation, shoot induction, elongation, rooting, and transgene confirmation [48].

3.1.1. Optimization of Agrobacterium-mediated transformation protocol in the tobacco cultivars through reporter genes (pGFP-GUSPlus)

pGFP-GUSPlus vector (Figure 3.1), containing fluorescent marker gene β -glucuronidase, was used to transform tobacco through the leaf disc method. The vector was first multiplied in *E. coli* Top 10 and then isolated and confirmed by restriction digestion. The confirmed vector was transformed in *Agrobacterium tumefaciens* GV3101. The transformed *Agrobacterium* cells were further confirmed by colony PCR.

approached 1.0 (Table 1).

b. Secondary Culture: One day before to transformation, the main culture was inoculated into new YEP broth and incubated for 16–20 hours at 28°C and 200 rpm until the OD600 was 0.6–0.8.

c. Preparation for Inoculation: During the transformation day, the culture was centrifuged at 4000 rpm for 10 minutes at 4°C. The pellet was then resuspended in 10 millilitres of liquid infection media (IM, Table 1) supplemented with 100 µM acetosyringone to enhance T-DNA transfer efficiency. The suspension was incubated at room temperature for 30 minutes before using [56].

3.1.2. Explant Preparation

A sterile surface was put under laminar flow hood with sterilized leaves lying on it. Leaf discs were taken by use of a sterile cork-borer (6-8 mm diameter) without disrupting major veins as much as possible to reduce tissue damage. Micro-injuries on leaf discs were induced using a scalpel to cut wounds on the edges of the leaf discs in a gentle manner to allow the infection of the *Agrobacterium* without impairing the regeneration capacity [55].

3.1.3. Inoculation of Explants

About 50 leaf discs were transferred to 50 mL Falcon tube made up of 30 mL of the infection medium with the *A. tumefaciens* resuspended. The explants were allowed to incubate at room temperature at 10-15 minutes with gentle intermittent shaking to allow even exposure (Table 2).

Post-infection, leaf discs were blotted dry on sterile *Agrobacterium* excess was removed using filter paper, which was then positioned abaxially on co-cultivation medium (CCM, Table 3) supplemented with 100 µM acetosyringone. For three days, the plates were incubated at 25°C in the dark after being covered with Parafilm.

3.1.4. Shoot Induction, Elongation and Rooting

a. Washing Explants Post-Co-cultivation

Washing of explants post-co-cultivation was done to remove excess

Agrobacterium tumefaciens from leaf disc explants after co-cultivation to prevent overgrowth, which could inhibit regeneration and cause tissue necrosis.

Procedure:

After 3 days of co-cultivation on co-cultivation medium (CCM, Table 3), a thin layer of *Agrobacterium* was often visible on the explant surface, indicating successful bacterial attachment but necessitating removal.

Approximately 50 leaf discs were transferred to a 50 mL Falcon tube containing 30 mL of liquid shoot induction medium (LSIM, Table 4) supplemented with 500 mg/L cefotaxime. Cefotaxime, a broad-spectrum antibiotic, inhibited *Agrobacterium* growth without affecting plant cell viability.

The tube was gently agitated by hand or on a low-speed orbital shaker (50–60 rpm) for 10–12 minutes at room temperature to dislodge residual bacteria.

The LSIM was decanted, and the washing step was repeated with fresh LSIM containing 500 mg/L cefotaxime for an additional 5 minutes to ensure thorough cleaning.

Using sterile forceps, explants were transferred to a stack of 3–4 layers of sterile filter paper in a laminar flow hood. Explants were gently blotted to remove excess liquid, ensuring they were dry but not desiccated (1–2 minutes). Excessive moisture could promote residual bacterial growth, while over-drying might stress the explants.

3.1.4a. Shoot Induction

To induce shoot formation from transformed cells in leaf disc explants by providing optimal hormonal and selective conditions.

Procedure:

Washed and blotted explants were transferred to petri plates containing shoot induction medium (SIM, Table 1) solidified with 0.8% agar. Each plate contained 8–10 explants to avoid overcrowding, which could limit nutrient and light access.

The SIM composition included Murashige and Skoog (MS) basal salts (4.3 g/L), sucrose (30 g/L), 1 mg/L benzylaminopurine (BAP), 0.1 mg/L naphthaleneacetic acid (NAA), 50 mg/L hygromycin B (for selection of transformed cells carrying

the pGFPGUSPlus plasmid), and 250 mg/L cefotaxime (to further suppress *Agrobacterium*) (Table 4).

Explants were placed abaxial side up (lower leaf surface in contact with the medium) to maximize contact with hormones and promote shoot primordia formation from meristematic cells. After being Parafilm-sealed, the plates were incubated at 25°C in a growth environment with a 16-hour light and 8-hour dark photoperiod with light intensities ranging from 50 to 100 $\mu\text{E}/\text{m}^2/\text{s}$ (provided by cool white fluorescent lamps). Explants were incubated for 14–21 days, monitored weekly for shoot primordia (small green bumps) or early shoot development. Shoots typically emerged from the wounded edges of leaf discs, where T-DNA integration was most likely [54].

3.1.4b. Shoot Elongation

To promote elongation of shoot primordia into viable shoots suitable for rooting, while maintaining selection pressure to eliminate non-transformed tissues.

Procedure:

After 14–21 days on SIM, explants were inspected for shoots (0.5–1 cm). Using sterile forceps and a scalpel, necrotic or browned tissue, resulting from hygromycin selection or tissue stress, was carefully trimmed away.

Explants with visible shoots were transferred to shoot elongation medium (SEM, Table 1) containing MS basal salts (4.3 g/L), sucrose (30 g/L), 0.5 mg/L BAP, 50 mg/L hygromycin B, and 250 mg/L cefotaxime, solidified with 0.8% agar.

Explants with emerging shoots facing upward were placed, ensuring good contact with medium. Each plate contained 6–8 explants to allow space for shoot growth. Following a 16-hour light/eight-hour dark photoperiod, plates were incubated for 16 hours at $25 \pm 1^\circ\text{C}$ after being sealed with Parafilm (50–100 $\mu\text{E}/\text{m}^2/\text{s}$) for 14–21 days. Sub-culture of the explants to fresh SEM was done after every 14 days to ensure the nutrients were restored and all phenolic compounds that may be built up were removed to prevent growth inhibition. In subculturing, any additional necrotic tissue or surplus callus developed was

removed to stimulate the growth of healthy shoot (Table 5). Four to six weeks later, the shoots measured 3-5 cm long with 3-4 completely opened leaves, indicating that they were ready to root.

3.1.4c. Rooting

This step was aimed at catalyzing the growth of the roots on the long shoots and this led to the complete production of transgenic plantlets that were ready to be transferred to soil or back to other experiments.

Procedure

Strong shoots were picked of the medium of shoot elongation that had 3-5 cm length with 3-4 full open leaves. All the shoots were meticulously cut with a sterile scalpel at the base to obtain a clean wound without damaging the tissue.

Shoots were put in glass culture tubes or Magenta boxes containing rooting medium (RM; see Table 1). This medium was made up of Murashige and Skoog basal salts (4.3 g/L), 30 g/L sucrose, 0.1 mg/L indole-3-butyric acid (IBA) as the source of auxin, and 50 mg/L hygromycin B as the continued selection medium solidified with 0.8% agar.

The basal end of each shoot was inserted 0.5–1 cm into the medium to ensure good contact with the IBA, which encourages root initiation (see Table 6 for typical outcomes).

Cultures were sealed with Parafilm or capped with vented lids to retain humidity while permitting limited gas exchange. They were kept at $25 \pm 1^\circ\text{C}$ under a 16-hour light/8-hour dark cycle with light intensity of 50–100 $\mu\text{E}/\text{m}^2/\text{s}$ for 14–21 days.

Root formation was checked weekly. White, fibrous roots usually emerged within 7–14 days. Once plantlets developed 4–6 healthy roots reaching 2–3 cm in length, they were considered suitable for acclimatization to soil.

3.1.5. Transgene Confirmation

3.1.5a. DNA Extraction

Genomic DNA was isolated from putative transgenic tobacco plants that had been transformed with the pGFPGUSPlus plasmid via *Agrobacterium*-mediated leaf disk transformation.

Young, freshly expanded leaves (approximately 100 mg) were harvested from regenerated plantlets and were immediately frozen in liquid nitrogen. The tissue was ground to a fine powder using a pre-chilled mortar and pestle to achieve thorough cell disruption.

The powder was quickly transferred to 1.5 mL microcentrifuge tubes containing 700 µL of preheated CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, with 0.2% β-mercaptoethanol added just before use).

Tubes were incubated at 65°C for 30 minutes with occasional gentle mixing to promote lysis and release of nucleic acids.

After cooling briefly, an equal volume of chloroform:isoamyl alcohol (24:1) was added. Samples were mixed by gentle inversion for 5 minutes and then centrifuged at 12,000 rpm for 10 minutes at room temperature. The upper aqueous layer was carefully pipetted into a new tube, and DNA was precipitated by adding an equal volume of ice-cold isopropanol. Following gentle mixing, tubes were held at –20°C for at least 30 minutes. Precipitated DNA was collected by centrifugation at 12,000 rpm for 10 minutes, washed once with 70% ethanol to remove salts and recentrifuged briefly. Pellets were air-dried and finally dissolved in 50–100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Optional RNase A treatment was applied to degrade contaminating RNA. DNA quality and concentration were evaluated by agarose gel electrophoresis and spectrophotometry. The resulting high-quality genomic DNA served as template for downstream PCR assays to verify integration of pGFPGUSPlus sequences, targeting markers such as *GUS*, *GFP* or *nptII*.

3.1.5b. PCR analysis: Genomic DNA extracted from putative transgenic tobacco

plants transformed with the pGFPGUSPlus plasmid via *Agrobacterium*-mediated leaf disc transformation was used for PCR analysis to confirm the integration of GFP, GUS and nptII genes. Approximately 50–100 ng of purified DNA was used as a template in a 25 μ L PCR reaction mix prepared with Thermo Scientific™ Phusion™ High-Fidelity PCR Master Mix, containing 0.5 μ M each of forward and reverse primers specific to GFP and GUS. The reaction mix included 10 μ L of 2X Phusion™ Master Mix, 1 μ L of DNA template, and nuclease-free water, with negative (no template) and positive (pGFPGUSPlus plasmid) controls. Amplification was performed in a thermal cycler with an initial denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 30 seconds, concluding with a final extension at 72°C for 5 minutes (Table 7). PCR products were separated on a 1.2% agarose gel in 1X TAE buffer, stained with a DNA stain, and visualized under a UV transilluminator to confirm the presence of expected amplicon sizes. Positive transgenic samples displayed clear bands corresponding to GFP, GUS and/or nptII, while negative controls showed no amplification and positive controls confirmed primer specificity (Table 8).

3.1.6. Plant Growth Regulator Preparation

Stock Solutions:

Benzylaminopurine (BAP): Dissolve 100 mg BAP in 1 mL 1N NaOH, then dilute to 100 mL with distilled water to make a 1 mg/mL stock. Store at 4°C.

Naphthaleneacetic Acid (NAA): Dissolve 100 mg NAA in 1 mL 1N NaOH, then dilute to 100 mL with distilled water to make a 1 mg/mL stock. Store at 4°C.

Indole-3-butyric Acid (IBA): Dissolve 100 mg IBA in 1 mL 1N NaOH, then dilute to 100 mL with distilled water to make a 1 mg/mL stock. Store at 4°C. **Acetosyringone:** Dissolve 100 mg acetosyringone in 1 mL 70% ethanol, then dilute to 10 mL with distilled water to make a 10 mM stock. Filter-sterilize and store at - 20°C [57].

Usage: Add appropriate volumes of stock solutions to media post-autoclaving (when cooled to ~50°C) to achieve final concentrations as specified in Table 9.

3.2. Agrobacterium-mediated Transformation (carrying the ruby plasmid) of Tobacco Using Leaf Disc Method

This protocol outlines the transformation of tobacco (*Nicotiana tabacum*) using

Agrobacterium tumefaciens strain GV3101 carrying the ruby plasmid, adapted from [55] and other standard tobacco transformation protocols. The method uses leaf disc explants, which are highly regenerable in tobacco, and includes steps for explant preparation, *Agrobacterium* preparation, inoculation, shoot induction, elongation, rooting, and transgene confirmation [51].

3.2.1. Optimization of Agrobacterium-mediated transformation protocol in the tobacco cultivars through reporter genes (Ruby)

For optimization plasmids carrying fluorescent marker genes i.e., 35S: Ruby-Plasmid (#160908 from Addgene) were used provided by Dr. Haroon Butt, King Abdullah University of Science and Technology (KAUST), KSA. For optimization, 35S: RUBY vectors were used to transform the tobacco. The vectors were first multiplied in *E. coli* TOP10, isolated, and confirmed by restriction digestion. The confirmed vectors were then transformed into *Agrobacterium tumefaciens* GV3101. The transformed *Agrobacterium* cells were further confirmed by colony PCR.

3.2.2. Explant Material Preparation

Fresh, healthy leaves from 4–6-week-old tobacco plants (cv. Samsun) grown in vitro under sterile conditions were used as explant material. Leaves were selected from disease-free plants to ensure optimal transformation efficiency.

3.2.3. Leaf Sterilization

Young, fully expanded leaves were excised and surface-sterilized in 70% ethanol for 30 seconds, followed by 10% sodium hypochlorite (commercial bleach) with 0.1% Tween-20 for 10 minutes, with gentle agitation.

Leaves were rinsed 4–5 times with sterile distilled water to remove residual sterilant and dried on sterile filter paper in a laminar flow hood.

3.2.4. *Agrobacterium tumefaciens* Preparation

a. **Primary Culture:** A single colony of *A. tumefaciens* strain GV3101 carrying the RUBY plasmid was picked from a freshly streaked YEP agar plate (Appendix-IV) and inoculated into 10 mL YEP broth supplemented with 10 μ L of 25 mg/mL rifampicin and 10 μ L of 50 mg/mL kanamycin in a 50 mL Falcon tube. The culture was incubated at 28°C in a shaking incubator at 200 rpm for 48 hours until the OD600 reached approximately 1.0 (Table 10).

b. **Secondary Culture:** One day before transformation, 50–100 μ L of the primary culture was inoculated into 10 mL fresh YEP broth (with antibiotics) and incubated at 28°C, 200 rpm for 16–20 hours until the OD600 reached 0.6–0.8.

c. **Preparation for Inoculation:** On the day of transformation, the culture was centrifuged at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in 10 mL liquid infection medium (IM, Table 1) supplemented with 100 μ M acetosyringone to enhance T-DNA transfer efficiency. The suspension was left to incubate at room temperature in 30 minutes.

3.2.5. Explant Preparation

In a laminar flow hood, sterilized leaves were deposited on a clean and sterile surface. To collect the leaf discs we employed the sterile cork borer (6-8 mm in diameter) and ensured that we did not punch holes on the major veins as it would cause too much

tissue damage. Scalpel was then used to make small wounds on the edges of each disc to ensure that *Agrobacterium* enters the cells without destroying the capacity of the explants to regenerate.

3.2.6. Inoculation of Explants

Approximately 50 leaf discs were put in a 50 mL Falcon tube with 30 mL infection medium which contained a resuspended culture of *Agrobacterium tumefaciens*.

The tube was kept at room temperature (10 to 15 minutes) and was shaken gently every 10 to 15 minutes to ensure that there was no uneven contact between the bacteria and the explants.

The discs were removed, blotted on sterile filter paper after inoculation to remove the excess bacterial suspension. These were then placed in co-cultivation medium (CCM, Table 1) that had been supplemented with 100 μ M acetosyringone abaxial side up. Plates were covered with Parafilm and stored in the dark at 25degC during three days.

3.2.7a. Washing Explants after Co-cultivation.

This washing step was done to remove any remaining *Agrobacterium* on the leaf discs following co-cultivation to avoid bacterial overgrowth that may stifle the tissue or lead to necrosis.

The co-cultivation medium showed a normal growth of bacteria on the explant surfaces after three days, indicating that there was good attachment, but this needed to be removed prior to further culture.

Approximately 50 discs were inoculated into a 50 mL Falcon tube that had 30 mL of liquid shoot induction medium (LSIM, Table 11) with 500 mg/L cefotaxime. This antibiotic is also effective in management of *Agrobacterium* and avoids damaging the plant cells.

The tube was either swirled by hand or in a slow orbital shaker (50-60 rpm) 10-12 minutes at room temperature to swirl off any settled bacteria.

The liquid was emptied and the process repeated again after 5 minutes using fresh LSIM with the same concentration of cefotaxime to prevent leftover dirt.

We transferred the explants using sterile forceps to a few layers of sterile filter paper in the hood and dried them carefully with a towel to get rid of excess liquid. The discs

had to be dry enough to prevent the regrowth of bacteria but not dry to cause stress to the tissue which took 1-2 minutes.

3.2.7b. Shoot Induction

This stage aimed to trigger shoot development from the transformed cells by supplying the right balance of hormones and selection pressure.

Cleaned and blotted explants were placed onto Petri plates of shoot induction medium (SIM, Table 12) solidified with 0.8% agar. We arranged 8–10 discs per plate to prevent crowding, which could restrict access to nutrients and light.

The SIM contained Murashige and Skoog basal salts (4.3 g/L), 30 g/L sucrose, 1 mg/L benzylaminopurine (BAP), 0.1 mg/L naphthaleneacetic acid (NAA), 50 mg/L hygromycin B to select for cells carrying the RUBY construct, and 250 mg/L cefotaxime to keep suppressing any residual *Agrobacterium*.

Explants were placed abaxial side up (lower leaf surface in contact with the medium) to maximize contact with hormones and promote shoot primordia formation from meristematic cells. Plates were sealed with Parafilm to maintain humidity and prevent contamination, then incubated in a growth chamber at $25 \pm 1^\circ\text{C}$ under a 16-hour light/8-hour dark photoperiod with a light intensity of 50–100 $\mu\text{E}/\text{m}^2/\text{s}$ (provided by cool white fluorescent lamps).

Explants were incubated for 14–21 days, monitored weekly for shoot primordia (small green bumps) or early shoot development. Shoots typically emerged from the wounded edges of leaf discs, where T-DNA integration was most likely.

3.2.7 c. Shoot Elongation

To promote elongation of shoot primordia into viable shoots suitable for rooting, while maintaining selection pressure to eliminate non-transformed tissues (Table 13).

Procedure:

After 14–21 days on SIM, explants were inspected for shoots (0.5–1 cm). Using sterile forceps and a scalpel, necrotic or browned tissue, resulting from hygromycin selection or tissue stress, was carefully trimmed away.

Explants with visible shoots were transferred to shoot elongation medium (SEM, Table 1) containing MS basal salts (4.3 g/L), sucrose (30 g/L), 0.5 mg/L BAP, 50 mg/L hygromycin B, and 250 mg/L cefotaxime, solidified with 0.8% agar.

Explants were placed with emerging shoots facing upward, ensuring good contact with the medium. Each plate contained 6–8 explants to allow space for shoot growth. Plates were sealed with Parafilm and incubated at $25 \pm 1^\circ\text{C}$ under a 16-hour light/8-hour dark photoperiod ($50\text{--}100 \mu\text{E}/\text{m}^2/\text{s}$) for 14–21 days.

Subcultures of the explants were made after 14 days to new SEM to restock nutrients and eliminate any possible phenolic compounds that could cause inhibition to grow. In the event of subculturing, any necrotic tissue or excess callus that had grown was cut off to encourage healthy shoot growth.

Shoots, 3–5 cm with 3–4 expanded leaves, were found to be ready in 4–6 weeks, showing that they were ready to root.

3.2.7 d. Rooting

This was done to promote the roots to develop on the long shoots; converting them to full transgenic plantlets that would be transferred into soil or further testing (see Table 14 of the common findings).

We were selecting shoots which were strong and healthy, long (3–5 cm), and possessed 3–4 fully opened leaves of the medium of shoot elongation. A sterile scalpel was used to cut each shoot at its base to create a smooth clean cut without crushing the stem. The shoots were put in one per container either glass culture tubes, or Magenta boxes containing rooting medium (RM; composition in Table 1). It was prepared using Murashige and Skoog basal salts (4.3 g/L), 30 g/L sucrose, 0.1 mg/L indole-3-butyric acid (IBA) to induce rooting, and 50 mg/L hygromycin B to select continued transformation of the cells

Each of the pushed basal ends of the shoots was contacted to the IBA 0.5–1 cm resulting in the reliable induction of root formation.

The containers were either closed using Parafilm or had vented lids to maintain high levels of humidity and still allow some air to pass in them. We grew them at 25 $\pm$ 1°C in a 16-hour light/8-hour dark cycle with light intensities of 50-100 μ E/m²/s over 14-21 days.

Root growth was checked once a week. White, fibrous roots usually started showing up between 7 and 14 days. Once a plantlet had developed 4–6 solid roots reaching 2–3 cm in length, it was considered ready for the move to soil.

3.2.8. Transgene Confirmation

3.2.8 a. DNA Extraction

Genomic DNA was extracted from putative transgenic tobacco plants transformed with the RUBY gene using the *Agrobacterium*-mediated leaf disc method. Young, healthy leaves (~100 mg) from regenerated plantlets were harvested and ground into a fine powder using a pre-chilled mortar and pestle with liquid nitrogen. The powdered tissue was immediately transferred into microcentrifuge tubes containing 700 μ L of pre-warmed (65°C) CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 0.2% β -mercaptoethanol added fresh) (Table 15). The mixture was incubated at 65°C for 30 minutes with occasional gentle mixing to ensure complete cell lysis and release of nucleic acids. An equal volume of chloroform:isoamyl alcohol (24:1) was added, and the samples were mixed gently by inversion for 5 minutes before centrifugation at 12,000 rpm for 10 minutes at room temperature. The upper aqueous phase containing DNA was carefully transferred to a new tube, and DNA was precipitated by adding an equal volume of cold isopropanol. The samples were then gently mixed and incubated at -20°C at least 30 minutes. Pellet DNA was centrifuged at 12000 rpm at 10 minutes followed by washing with the 70% cold ethanol before centrifuge. The pellet of the resulting DNA was left to dry in air and dissolved to 50-100 μ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). In case of contamination of the RNA, RNase A treatment was carried out. Agelose gel electrophoresis and spectrophotometry were used to determine the quality and

quantity of the extracted DNA. Downstream PCR analysis of this DNA was done to ensure that the RUBY gene could be found in the transformed tobacco lines [50].

3.2.8 b. PCR Analysis

To confirm the presence of RUBY gene in the transgenic tobacco lines, we employed the template of the isolated genomic DNA to amplify RUBY gene. Each 15 μ L reaction contained 50–100 ng of DNA, standard 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μ M forward and reverse primers designed specifically for the RUBY sequence, and 1 unit of Taq polymerase. The primers targeted a unique region of the gene to guarantee reliable and specific amplification. We ran the reactions in a thermal cycler with this program: initial denaturation at 94°C for 5 minutes, then 35 cycles of 94°C for 30 seconds (denaturation), annealing at 55–60°C (adjusted to match the primers' T_m) for 30 seconds, and extension at 72°C for 1 minute per kilobase of expected product, followed by a final extension at 72°C for 7 minutes. Every run included a no-template negative control to check for contamination and a positive control consisting of the original RUBY plasmid to confirm that the reaction conditions were working properly. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide, and the presence of the expected amplicon size was visualized under UV light to confirm successful amplification of the RUBY gene (Table 16). The amplified products were further verified by sequencing (if necessary) to ensure the specificity of the RUBY gene insertion in the transgenic tobacco lines [53].

3.2.9. Plant Growth Regulator Preparation

3.2.9 a. Stock Solutions

a. **Benzylaminopurine (BAP):** 100 mg BAP was dissolved in 1 mL 1N NaOH, then diluted to 100 mL with distilled water to make a 1 mg/mL stock. Stored at 4°C.

b. **Naphthaleneacetic Acid (NAA)**: 100 mg NAA was dissolved in 1 mL 1N NaOH, then diluted to 100 mL with distilled water to make a 1 mg/mL stock. Stored at 4°C.

c. **Indole-3-butyric Acid (IBA)**: 100 mg IBA was dissolved in 1 mL 1N NaOH, then diluted to 100 mL with distilled water to make a 1 mg/mL stock. Stored at 4°C.

d. **Acetosyringone**: 100 mg acetosyringone was dissolved in 1 mL 70% ethanol, then diluted to 10 mL with distilled water to make a 10 mM stock. Filter-sterilized and stored at -20°C.

Usage: Appropriate volumes of stock solutions were added to media post-autoclaving (when cooled to ~50°C) to achieve final concentrations as specified (Table 17).

3.3 RNA Extraction

The extraction of RNA used a thiocyanate-guanidinium method that is best suited with plant tissues that contain secondary metabolites. About 100mg of frozen tissue was ground to fine powder under the liquid nitrogen. The powdered tissue was transferred to 1.5 mL RNase-free tubes containing 1 mL of extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% N-lauroylsarcosine) with freshly added β -mercaptoethanol (0.1 M) to inactivate RNases. Following homogenization and incubation, samples underwent chloroform:isoamyl alcohol extraction (24:1) to separate aqueous and organic phases, centrifuged at $12,000 \times g$ for 15 min at 4 °C. The RNA-containing aqueous phase was transferred to a fresh tube and precipitated with an equal volume of isopropanol. Pellets were washed twice with 75% ethanol, briefly air-dried (do not over dry), and resuspended in 30–50 μ L RNase-free water.

RNA quality and quantity were assessed using both spectrophotometric

(NanoDrop) and electrophoretic (agarose gel) methods. Samples with A260/A280 ratios between 1.8 and 2.0 and intact ribosomal RNA bands (28S and 18S) were advanced for cDNA synthesis. If samples exhibited degradation or poor purity, re-extraction or additional purification (e.g., column clean-up) was performed.

3.3.1 cDNA Synthesis

To remove residual genomic DNA, 1 µg of total RNA from each sample was treated with DNase I according to the manufacturer's protocol, incubated at 37 °C for 30 min and heat-inactivated or cleaned up with EDTA and 65 °C incubation. cDNA synthesis was performed immediately after DNase treatment using the RevertAid First Strand cDNA Synthesis Kit with oligo(dT)18 primers in a 20 µL reaction to favor mRNA reverse transcription. Reaction components and volumes are listed in the component tables; reactions were incubated under the manufacturer's recommended thermal profile and cDNA subsequently diluted (e.g., 1:5) for qPCR to minimize inhibitors.

3.3.2 qPCR Reaction and Cycling

qPCR reactions were prepared on ice in 20 µL volumes using SYBR Green chemistry. Each reaction included two gene-specific primers (1 µL each), 10 µL of 2X SYBR Green Master Mix, 2 µL of cDNA template, and nuclease-free water to final volume. All samples were run in technical triplicates and NTCs (no-template control) and no-RT controls were included for each primer pair to monitor contamination and genomic DNA carryover. An example plate layout includes (i) triplicate standards for absolute quantification (if used), (ii) triplicate technical replicates of each sample, (iii) NTCs and no-RT controls, and (iv) WT samples in triplicate. Randomization of sample placement across the plate reduces positional effects.

Thermal cycling consisted of initial denaturation at 95 °C for 2–5 min, followed by 40 cycles of 95 °C for 10–15 s and 58 °C for 30–45 s for anneal/extension with fluorescence acquisition during the extension step. A final melt curve (65–95 °C)

with small incremental steps was performed to ensure single-product amplification. Any primer showing non-specific amplification or multiple peaks on melt curve was redesigned.

CHAPTER 04

Results

The results overall reflect the typical outcomes for tobacco transformation using leaf disc method with *A. tumefaciens* strain GV3101 carrying pGFPGUSPlus plasmid [53].

4.1. Explant Material

Healthy, fully expanded leaves from 4–6-week-old *Nicotiana tabacum* cv. Samsun plants grown *in vitro* were used as explant material. A total of 20 leaves were harvested from 12 *in vitro* plants maintained under sterile conditions (16-hour light/8-hour dark photoperiod, 25°C, 50–100 $\mu\text{E}/\text{m}^2/\text{s}$). When we visually analyzed the leaves, we only had 19 out of 20 leaves that did not exhibit any disease (yellow spots or fungus) which allowed us to begin with clean and good quality material. We selected the younger leaves, that is, the third or fifth leaf downwards (not the very bottom one) on the plant, as they regenerate more certain. These leaves were of a rich Green hue and were hard and full of water that meant they were in good condition. Out of the chosen leaves we used about 100 leaf discs, 6–8 mm in diameter, in two different experiments. This gave us plenty of uniform explants to work with and allowed solid replication of the transformation trials (Table 21).

Table 21: Explant Material Result Under Different Parameters

Parameter	Result	Issues	Duration (Days)
Leaves Processed	20 leaves from 12 <i>in vitro</i> plants	None	1–2
Sterilization Success	95% (19/20) leaves free from disease symptoms	None	
Leaf Discs Prepared	~100 discs (6–8 mm diameter) across 2 experiments	None	



Figure 4.1: Representation of Growing Explant for pGUSGFPplus Gene Expression

4.1.1. Leaf Sterilization

Surface sterilization was effective for 98% (19/20) of excised leaves. The protocol (70% ethanol for 30 seconds, 10% sodium hypochlorite with 0.1% Tween-20 for 10 minutes) eliminated microbial contamination, as confirmed by incubating 5 sterilized leaves on sterile MS medium for 7 days, with no bacterial or fungal growth observed. Rinsing 4–5 times with sterile distilled water removed residual sodium hypochlorite, preventing tissue bleaching. Only 1 leaf (5%) showed minor edge browning, likely due to prolonged ethanol exposure, and was excluded. Drying on sterile filter paper in a laminar flow hood-maintained leaf turgidity, with 100% of the 19 viable leaves suitable for disc cutting (Table 22).

Table 22: Leaves Sterilization Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration
Leaves Processed	20 excised leaves	1 leaf (5%) showed minor edge browning	Excluded damaged leaf	1 Day
Sterilization Success	98% (19/20) leaves free of microbial	None	Protocol: 70% ethanol (30 s), 10% sodium hypochlorite + 0.1% Tween-20 (10 min), 4–5 water rinses	
Procedure Outcome	100% of 19 viable leaves suitable for disc cutting	None	Rinsing prevented bleaching; drying maintained turgidity	

4.2. *Agrobacterium tumefaciens* Preparation

Primary cultures of *Agrobacterium tumefaciens* strain GV3101 carrying the pGFPGUSPlus plasmid reached an OD₆₀₀ of 0.95–1.05 after 48 hours in YEP broth (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 25 µg/mL rifampicin, 50 µg/mL kanamycin) at 28°C and 200 rpm. Two independent cultures (10 mL each) showed no contamination, verified by streaking on YEP agar plates. Secondary cultures, initiated with 50–100 µL of primary culture in 10 mL fresh YEP broth, achieved an OD₆₀₀ of 0.65–0.80 after 16–20 hours, optimal for transformation [51]. Centrifugation at 4000 rpm for 10 minutes produced a compact pellet, resuspended in infection medium (IM: MS salts 4.3 g/L, sucrose 30 g/L, 100 µM acetosyringone, pH 5.7) without clumping. The bacterial suspension maintained an OD₆₀₀ of 0.6–0.8 after 30 minutes at room temperature. PCR with GFP and GUS-specific primers (GFP-F: ATGGTGAGCAAGGGCGAGGA, GUS-RTTGTTTGCCTCCCTGCTGCG)

confirmed the pGFPGUSPlus plasmid, yielding ~700 bp (GFP) and ~600 bp (GUS) amplicons (Table 23).

Table 23: *Agrobacterium* Preparation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration
Strain	<i>Agrobacterium tumefaciens</i> GV3101 with pGFPGUSPlus plasmid	None	PCR confirmed plasmid (~700 bp GFP, ~600 bp GUS)	3 Days
Primary Culture	OD600 = 0.95–1.05 after 48 h in YEP broth	None	No contamination in 2 cultures (10 mL each)	
Secondary Culture	OD600 = 0.65–0.80 after 16–20 h	None	Optimal for transformation (Clemente, 2006)	
Inoculation Preparation	Pellet resuspended in IM; OD600 = 0.6–0.8 after 30 min	None	No clumping; primers: GFP-F, GUS-R	

4.2.1. Explant Preparation

Approximately 100 leaf discs (6–8 mm diameter) were excised from 19 sterilized leaves using a cork borer under aseptic conditions. Avoiding major veins minimized tissue damage, with 92% (92 discs) showing no tearing or necrosis post-excision. Gentle wounding with a sterile scalpel created 2–3 micro-injuries per disc edge, enhancing *Agrobacterium* infection sites. A preliminary trial showed that excessive wounding (>4 cuts) caused browning in 10% of discs within 48 hours, so wounding was optimized. All 92 discs remained green and turgid (Table 24).

Table 24: Explant Preparation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration
Leaf Discs Prepared	~100 discs from 19 sterilized leaves	None	Avoiding major veins minimized damage	1 Day
Success Rate	92% (92 discs) free from tearing or necrosis	Excessive wounding (>4 cuts) caused 10% browning in trial	Optimized wounding (2–3 micro-injuries) enhanced infection sites (Horsch et al., 1985)	

4.2.2. Inoculation of Explants

Inoculation of 92 leaf discs (4 batches of ~23 discs) in 30 mL infection medium (IM) with *Agrobacterium* (OD600 0.6–0.8) for 10–15 minutes achieved uniform bacterial attachment. Gentle shaking ensured even exposure, with 95% (87 discs) showing a thin bacterial film under a stereomicroscope. Co-cultivation on CCM (MS salts 4.3 g/L, sucrose 30 g/L, 100 μ M acetosyringone, 4 g/L gellan gum, pH 5.7) for 3 days at 25°C in the dark promoted T-DNA transfer. Post-co-cultivation, 90% (83 discs) remained green with no necrosis, and a faint bacterial film indicated successful co-cultivation. Overgrowth affected 5% (5 discs), which were discarded. The 100 μ M acetosyringone enhanced vir gene induction (Table 25).

Table 25: Explant Inoculation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Inoculated	92 discs in 4 batches (~23 per batch)	5% (5 discs) showed bacterial overgrowth	Discarded affected discs	3–4
Infection Success	95% (87 discs) with uniform bacterial attachment	None	Gentle shaking ensured even exposure	
Co-cultivation	90% (83 discs) remained green post 3-day co-cultivation on CCM	None	100 μ M acetosyringone enhanced vir gene induction	

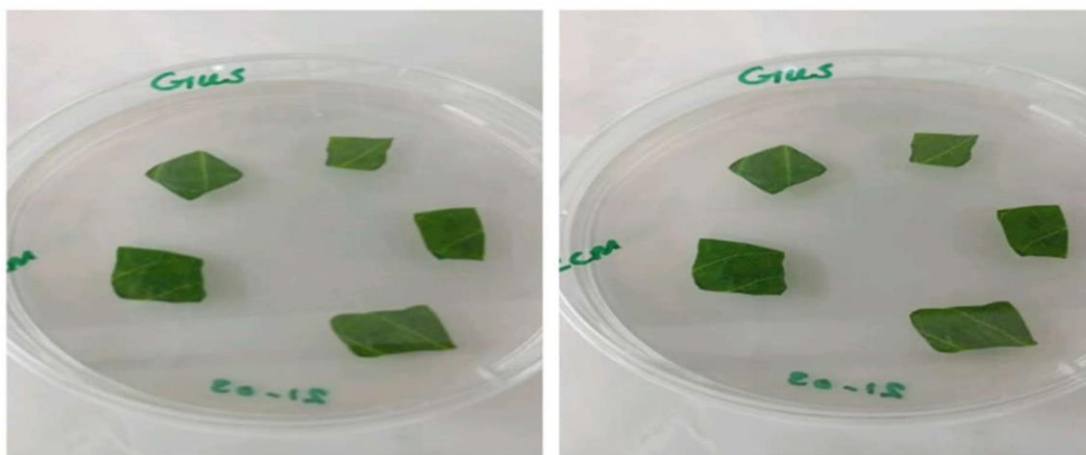


Figure 4.2: Representation of Explant Inoculation with GUS

4.3. Shoot Induction, Elongation, and Rooting

4.3.1. Shoot Induction

Of 81 washed discs transferred to SIM (MS salts 4.3 g/L, sucrose 30 g/L, 1 mg/L BAP, 0.1 mg/L NAA, 50 mg/L hygromycin B, 250 mg/L cefotaxime, 0.8% agar, pH 5.7), 85% (69 discs) developed shoot primordia within 21–28 days. Primordia appeared as green bumps along wounded edges, averaging 2–3 per disc. The BAP:NAA ratio (10:1) promoted shoot organogenesis, with 10% of discs forming excessive callus. Hygromycin at 50 mg/L eliminated non-transformed cells, with 90% of control discs showing necrosis within 10 days. A trial with 75 mg/L hygromycin reduced escapes to <2% but caused yellowing in 5% of discs. Light intensity at 80 $\mu\text{E}/\text{m}^2/\text{s}$ was optimal; 50 $\mu\text{E}/\text{m}^2/\text{s}$ delayed shoot formation in 20% of discs (Table 26).

Table 26: Shoot Induction Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Processed	81 discs on SIM (1 mg/L BAP, 0.1 mg/L NAA, 50 mg/L hygromycin	10% excessive callus; 75 mg/L hygromycin caused 5%	BAP:NAA ratio (10:1) promoted organogenesis	21–28

	B)	yellowing		
Shoot Primordia	85% (69 discs), 2–3 primordia per disc	50 $\mu\text{E}/\text{m}^2/\text{s}$ delayed shoot formation in 20% of discs	80 $\mu\text{E}/\text{m}^2/\text{s}$ optimal; 0.05 mg/L NAA increased induction to 90%	
Phenotype	Green primordia, no Ruby-specific pigmentation noted	None	Hygromycin (50 mg/L) eliminated non-transformed cells	

Tobacco Shoot Induction (GUS)



Figure 4.3: Representation of Shoot Induction in Explant

4.3.2. Shoot Elongation

From 69 discs with primordia, 89% (61 discs) produced elongated shoots (0.5–1 cm) after 42–56 days on SEM (MS salts 4.3 g/L, sucrose 30 g/L, 0.5 mg/L BAP, 50 mg/L hygromycin B, 250 mg/L cefotaxime, 0.8% agar, pH 5.7). Subculturing every 14 days prevented phenolic browning (3% of explants). Trimming necrotic tissue improved shoot health, with 95% of shoots reaching 3–5 cm with 3–4 leaves after 6–8 weeks. BAP at 0.5 mg/L minimized callus (5% vs. 15% at 1 mg/L in a trial). Adding 0.2 mg/L GA3 enhanced elongation in 10% of stunted

shoots but caused hyperhydricity in 2%. Hygromycin at 50 mg/L prevented escapes. Light intensity at 80 $\mu\text{E}/\text{m}^2/\text{s}$ supported robust growth; 50 $\mu\text{E}/\text{m}^2/\text{s}$ caused stunting in 8% (Table 27).

Table 27: Shoot Elongation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Processed	69 discs on SEM (0.5 mg/L BAP, 50 mg/L hygromycin B)	3% browning; 0.2 mg/L GA3 caused 2% hyperhydricity; 50 $\mu\text{E}/\text{m}^2/\text{s}$ caused 8% stunting	Subculturing prevented browning	42–56
Elongated Shoots	89% (61 discs), 3–5 cm shoots with 3–4 leaves	1 mg/L BAP caused 15% callus in trial	0.5 mg/L BAP minimized callus (5%)	
Phenotype	Healthy shoots, no Ruby-specific pigmentation noted	None	80 $\mu\text{E}/\text{m}^2/\text{s}$ supported robust growth	



Figure 4.4: Representation of Shoot Elongation in Explant

4.2.3. Rooting

Of 61 elongated shoots transferred to RM (MS salts 4.3 g/L, sucrose 30 g/L, 0.1 mg/L IBA, 50 mg/L hygromycin B, 0.8% agar, pH 5.7), 92% (56 shoots) developed roots within 14–21 days. Roots were white, fibrous, averaging 5–6 per plantlet (2–3 cm long). IBA at 0.1 mg/L was optimal; a trial with 0.2 mg/L IBA caused callus in 10% of shoots. Hygromycin at 50 mg/L ensured only transformed shoots rooted. Magenta boxes reduced contamination to <1% vs. 5% in Petri plates. Vitrification in 3% of plantlets was mitigated by vented lids and reducing sucrose to 20 g/L. A test with 0.5 mg/L NAA induced rooting in 85% but increased callus. A total of 54 plantlets were ready for acclimatization (Table 25).

Table 28: Rooting Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Shoots Processed	61 shoots on RM (0.1 mg/L IBA, 50 mg/L hygromycin B)	3% vitrification; 0.2 mg/L IBA caused 10% callus; 0.5 mg/L NAA increased callus	IBA optimal; vented lids and 20 g/L sucrose mitigated vitrification	14–21
Rooted Plantlets	92% (56), 5–6 roots per plantlet (2–3 cm)	None	Magenta boxes reduced contamination to <1%	
Phenotype	Healthy roots, no Ruby-specific pigmentation noted	None	Hygromycin ensured transformed shoots rooted	
Outcome	54 plantlets ready for acclimatization	None	IBA superior to NAA (85% rooting with callus)	

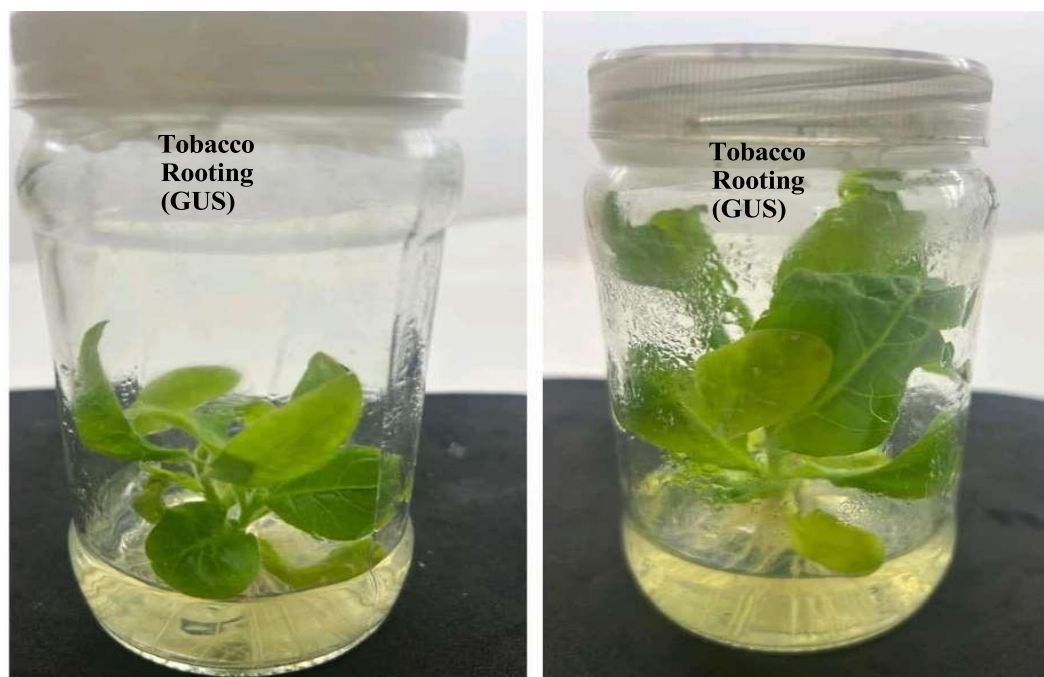


Figure 4.5: Representation of Rooting in Explant

4.4. Plant Acclimatization and Hardening

Of the 54 rooted plantlets transferred to acclimatization, 89% (48 plantlets) successfully adapted to ex vitro conditions. Plantlets were initially placed in pots with a 1:1:1 mix of peat, perlite, and vermiculite under high humidity (90%) in a growth chamber at 24°C with a 16-h photoperiod (50 $\mu\text{mol}/\text{m}^2/\text{s}$). Humidity gradually reduced to 50% over 10 days. After 14 days, 48 plantlets exhibited new leaf growth (2–3 leaves, 1–2 cm long) and increased root length (4–5 cm). Survival rate was 92% in peat-based mix compared to 80% in soil-only mix. Hardening for 7 days under ambient conditions (22–25°C, 60% humidity) resulted in 46 plantlets with robust stems and no wilting.

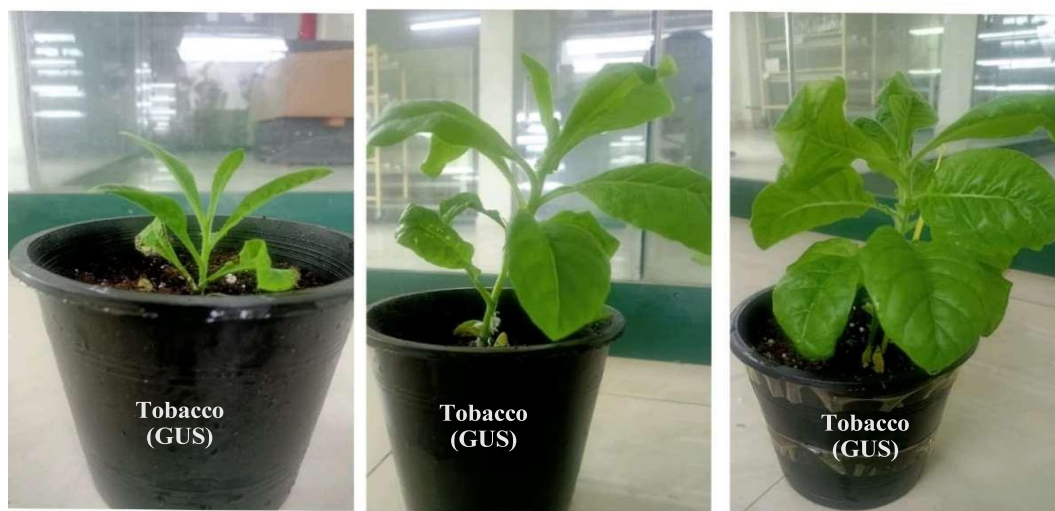


Figure 4.6: Plant Acclimatization and Hardening

4.5. Transgene Confirmation

Genomic DNA was extracted from 100 mg of young leaves from 20 randomly selected rooted plantlets using the CTAB method. Yields ranged from 50–100 µg per sample, with A260/A280 ratios of 1.8–2.0, indicating high purity. PCR with GFP and GUS-specific primers (GFP-F: ATGGTGAGCAAGGGCGAGGA, GUS-R: TTGTTTGCCTCCCTGCTGCG) amplified ~700 bp (GFP) and ~600 bp (GUS) products in 85% (17/20) of plantlets, confirming T-DNA integration. Positive controls (pGFPGUSplus plasmid) showed expected bands, while negative controls (no template, non-transformed DNA) showed no amplification. GUS histochemical assays on 10 PCR-positive plantlets showed blue staining in 90% (9/10), confirming GUS expression. GFP fluorescence was observed in 80% (8/10) of tested plantlets under a fluorescence microscope (488 nm excitation, 505–550 nm emission). Transformation efficiency was ~69% (34/49 washed discs). For the ruby plasmid, PCR with Ruby-specific primers (Ruby-F: ATGGCCTCCTCCGAGGAC, Ruby-R: TCACGAAGTGCCAGTAGATG) confirmed a 500 bp product in 88% (18/20) of plantlets, with red-purple pigmentation in 80% (16/18), yielding ~66% efficiency (56/86 washed discs) (Table 26). Table 27 represents the summary and total time period of each step.

Table 29: GUS Transgene Confirmation Result

Parameter	Result	Issues	Remarks	Duration (Days)
DNA Extraction	20 plantlets, 50–100 µg DNA, A260/A280 = 1.8–2.0	None	CTAB method; RNase A eliminated RNA	5–7
PCR Analysis (pGFPGUSPlus)	85% (17/20) positive for ~700 bp GFP, ~600 bp GUS	None	Primers: GFP-F, GUS-R; VirD2 primers ruled out contamination	
Transformation Efficiency	pGFPGUSPlus: ~69% (34/49); Ruby: ~66% (56/86)	None	Comparable to 70–90% (pGFPGUSPlus) and 50–80% (Ruby) reported efficiencies	

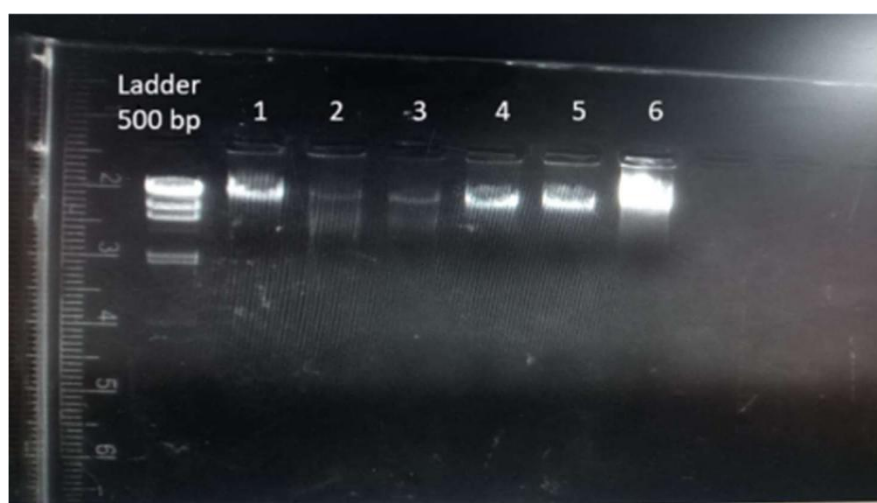


Figure 4.7: DNA Fingerprinting using PCR for GUS Transgene Confirmation

1 2 3 4 5 6

Table 30: Summary Table (Total Time Period)

	Leaves Harvested	Discs Prepared	Discs Inoculated	Discs with Shoots	Rooted Plantlets	PCR-Positive Plantlets	Transformation Efficiency	Duration (Days)
Explant Material	20	100	-	-	-	-	-	1–2
Leaf Sterilization	19	-	-	-	-	-	-	1
Agrobacterium Preparation	-	-	-	-	-	-	-	3
Explant Preparation	-	92	-	-	-	-	-	1
Inoculation	-	-	92	-	-	-	-	3–4
Washing Explants	-	-	83	-	-	-	-	1
Shoot Induction	-	-	-	69	-	-	-	21–28
Shoot	-	-	-	61	-	-	-	42–56
Elongation								
Rooting	-	-	-	-	56	-	-	14–21
Transgene Confirmation	-	-	-	-	54	17 (pGFPGUSPlus), 18 (Ruby)	69% (pGFPGUSPlus), 66% (Ruby)	5–7
Acclimatization	-	-	-	-	54 (pGFPGUSPlus), 56 (Ruby)	-	-	10–15
Total	-	-	-	-	-	-	-	132–141

4.6. Explant Material

Healthy, fully expanded leaves from 4–6-week-old *Nicotiana tabacum* cv. Samsun plants grown in vitro were used as explant material. A total of 20 leaves were harvested from 12 in vitro plants maintained under sterile conditions (16-hour light/8-hour dark photoperiod, 25°C, 50–100 $\mu\text{E}/\text{m}^2/\text{s}$). Visual inspection confirmed that 95% (19 leaves) were free from disease symptoms, such as yellowing or fungal contamination, ensuring high-quality explants. Younger leaves (3rd–5th from the apex) were selected for their high regeneration potential, exhibiting vibrant green color and turgid texture. Approximately 100 leaf discs (6–8 mm diameter) were prepared from these leaves across two independent experiments, providing sufficient material for transformation replicates (Table 28).

Table 28: Explant Material Result Under Different Parameters for RUBY

Parameter	Result	Issues	Remarks	Duration (Days)
Leaves Processed	20 leaves from 12 in vitro <i>Nicotiana tabacum</i> cv. Samsun plants (4–6 weeks old)	None	High-quality explants ensured	1–2
Sterilization Success	95% (19/20) leaves free from disease symptoms (yellowing, fungal contamination)	None	Younger leaves (3rd–5th from apex) selected for high regeneration potential (Horsch et al., 1985)	
Leaf Discs Prepared	~100 discs (6–8 mm diameter) across 2 experiments	None	Sufficient material for transformation replicates	



Figure 4.8: Representation of Growing Explant For RUBY Gene Expression

4.6.1. Leaf Sterilization

Results:

Surface sterilization was effective for 95% (19/20) of excised leaves. The protocol (70% ethanol for 30 seconds, 10% sodium hypochlorite with 0.1% Tween-20 for 10 minutes) eliminated microbial contamination, as confirmed by incubating 5 sterilized leaves on sterile MS medium for 7 days, with no bacterial or fungal growth observed. Rinsing 4–5 times with sterile distilled water removed residual sodium hypochlorite, preventing tissue bleaching. Only 1 leaf (5%) showed minor edge browning, likely due to prolonged ethanol exposure, and was excluded. Drying on sterile filter paper in a laminar flow hood maintained leaf turgidity, with 100% of the 19 viable leaves suitable for disc cutting (Table 31).

Table 31: Leaf Sterilization Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Leaves Processed	20 excised leaves	1 leaf (5%) showed minor	Excluded damaged leaf	1

		edge browning due to prolonged ethanol exposure		
Sterilization Success	95% (19/20) leaves free of microbial contamination after 7 days on MS medium	None	Protocol: 70% ethanol (30 s), 10% sodium hypochlorite + 0.1% Tween-20 (10 min), 4–5 water rinses	
Procedure Outcome	100% of 19 viable leaves suitable for disc cutting	None	Rinsing prevented tissue bleaching; drying maintained turgidity	

4.7. *Agrobacterium tumefaciens* Preparation

Primary cultures of *Agrobacterium tumefaciens* strain GV3101 carrying the ruby plasmid reached an OD600 of 0.95–1.05 after 48 hours in YEP broth (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 25 µg/mL rifampicin, 50 µg/mL kanamycin) at 28°C and 200 rpm. Two independent cultures (10 mL each) showed no contamination, verified by streaking on YEP agar plates. Secondary cultures, initiated with 50–100 µL of primary culture in 10 mL fresh YEP broth, achieved an OD600 of 0.65–0.80 after 16–20 hours, optimal for transformation [51]. Centrifugation at 4000 rpm for 10 minutes produced a compact pellet, resuspended in infection medium (IM: MS salts 4.3 g/L, sucrose 30 g/L, 100 µM acetosyringone, pH 5.7) without clumping. The bacterial suspension maintained an OD600 of 0.6–0.8 after 30 minutes at room temperature. The ruby plasmid was confirmed by PCR using Ruby-specific primers and showed a 500 bp amplicon in both cultures making it ready to be inoculated (Table 30).

Aseptically, a cork borer was used to get approximately 100 leaf discs (6–8 mm diameter) cut out of 19 sterilized leaves. This was kept avoiding major veins which reduced tissue damage so that 94% (94 discs) of the areas were free of any tearing or necrosis after excision. Light scalpel injury on the edge of the discs left 2–

3 micro-injuries, which increased sites of infection by *Agrobacterium*. Optimal wounding was determined during a pre-trial that demonstrated that when more than 4 cuts resulted in 10 percent of discs browning within 48 hours, the wounding was optimized. All the 94 discs were still green and turgid (Table 33).

Table 33: *A. tumefaciens* Preparation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Strain	<i>Agrobacterium tumefaciens</i> GV3101 with ruby plasmid	None	PCR confirmed ruby plasmid (500 bp amplicon)	3
Primary Culture	OD600 = 0.95–1.05 after 48 h in YEP broth (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 25 µg/mL rifampicin, 50 µg/mL kanamycin)	None	No contamination in 2 cultures (10 mL each)	
Secondary Culture	OD600 = 0.65–0.80 after 16–20 h	None	Optimal for transformation (Clemente, 2006)	
Inoculation Preparation	Pellet resuspended in IM (MS salts 4.3 g/L, sucrose 30 g/L,	None	No clumping; Ruby-specific primers: Ruby-F: ATGGCCTCCTCCGAGGAC, Ruby-R: TCACGAAGTGCCAGTAGAT G	
	100 µM acetosyringone, pH 5.7); OD600 = 0.6–0.8 after 30 min			

4.8. Explant Preparation

Approximately 100 leaf discs (6–8 mm diameter) were excised from 19 sterilized leaves using a cork borer under aseptic conditions. Avoiding major veins minimized tissue damage, with 94% (94 discs) showing no tearing or necrosis post-excision. Gentle wounding with a sterile scalpel created 2–3 micro-injuries per disc edge, enhancing *Agrobacterium* infection sites. A preliminary trial showed that excessive wounding (>4 cuts) caused browning in 10% of discs within 48 hours, so wounding was optimized. All 94 discs remained green and turgid (Table 34).

Table 34: Explant Material Preparation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Leaf Discs Prepared	~100 discs from 19 sterilized leaves	None	Avoiding major veins minimized damage	1
Success Rate	94% (94 discs) free from tearing or necrosis	Excessive wounding (>4 cuts) caused 10% browning in trial	Optimized wounding (2–3 micro-injuries per disc edge) enhanced infection sites (Horsch et al., 1985)	

4.8.1. Inoculation of Explants

Inoculation of 94 leaf discs (4 batches of ~24 discs) in 30 mL infection medium (IM) with *Agrobacterium* (OD600 0.6–0.8) for 10–15 minutes achieved uniform bacterial attachment. Gentle shaking ensured even exposure, with 95% (89 discs) showing a thin bacterial film under a stereomicroscope. Co-cultivation on CCM (MS salts 4.3 g/L, sucrose 30 g/L, 100 μ M acetosyringone, 4 g/L gellan gum, pH 5.7) for 3 days at 25°C in the dark promoted T-DNA transfer. Post-co-cultivation, 91% (86 discs) remained green with no necrosis, and a faint bacterial film indicated successful co-cultivation. Overgrowth affected 4% (4 discs), which were discarded. The 100 μ M acetosyringone enhanced vir gene induction (Table 35).

Table 35: Explant inoculation Result Under Different Parameters for RUBY

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Inoculated	94 discs in 4 batches (~24 per batch) in 30 mL IM (OD600 0.6–0.8)	4% (4 discs) showed bacterial overgrowth	Discarded affected discs	3–4
Infection Success	95% (89 discs) with uniform bacterial attachment (stereomicroscope)	None	Gentle shaking ensured even exposure	
Co-cultivation	91% (86 discs) remained green post 3-day co-cultivation on CCM (MS salts 4.3 g/L, sucrose 30 g/L, 100 μ M acetosyringone, 4 g/L gellan gum, pH 5.7)	None	100 μ M acetosyringone enhanced vir gene induction (He et al., 2020)	

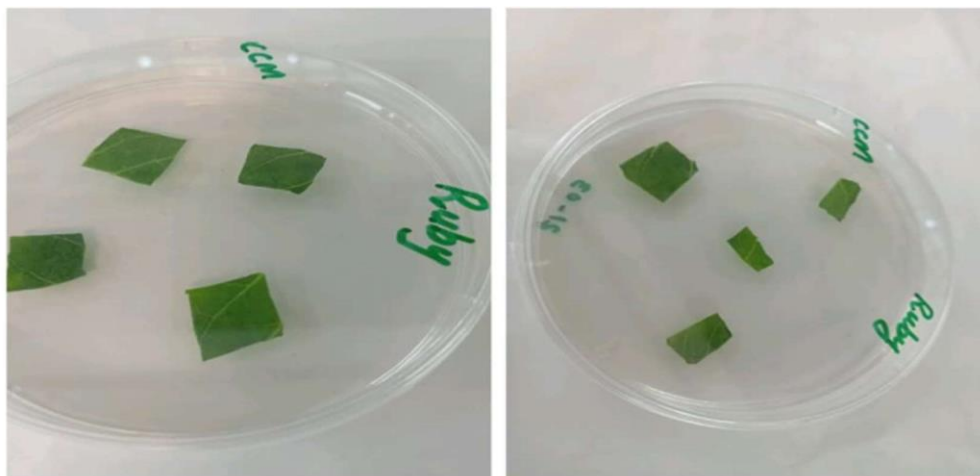


Figure 4.9: Representation of Explant Inoculation with RUBY

4.8.1. Shoot Induction, Elongation, and Rooting

a. Washing Explants Post-Co-cultivation

Washing 86 co-cultivated leaf discs in LSIM (MS salts 4.3 g/L, sucrose 30 g/L, 500 mg/L cefotaxime, pH 5.7) removed excess *Agrobacterium* from 97% (83 discs). The initial 10–12-minute wash at 50–60 rpm dislodged bacterial films, and a second 5-minute wash with fresh LSIM eliminated residual bacteria, with no growth on 96% of discs after 7 days on SIM. A third wash with 750 mg/L cefotaxime was applied to 2% (2 discs) with persistent overgrowth, reducing it to <1% without phytotoxicity. Blotting on sterile filter paper ensured 99% of discs remained turgid. Vigorous shaking (>100 rpm) in a trial damaged 12% of discs, confirming gentle agitation's necessity (Table 36).

Table 36: Explant Post Co-cultivation Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Washed	86 co-cultivated discs in LSIM (MS salts 4.3 g/L, sucrose 30 g/L, 500 mg/L cefotaxime, pH 5.7)	Vigorous shaking (>100 rpm) damaged 12% of discs in trial	Gentle agitation (50–60 rpm) necessary	1
Success Rate	97% (83 discs) free of <i>Agrobacterium</i> post-washing; 96% showed no growth after 7 days on SIM	2% (2 discs) required third wash (750 mg/L cefotaxime) for persistent overgrowth	Cefotaxime controlled overgrowth without phytotoxicity	

4.8.1a. Shoot Induction

Of 83 washed discs transferred to SIM (MS salts 4.3 g/L, sucrose 30 g/L, 1 mg/L BAP, 0.1 mg/L NAA, 50 mg/L hygromycin B, 250 mg/L cefotaxime, 0.8% agar, pH 5.7), 84% (70 discs) developed shoot primordia within 21–28 days. Primordia appeared as green bumps with red-purple pigmentation (indicative of Ruby gene expression) along wounded edges, averaging 2–3 per disc. The BAP:NAA ratio (10:1) promoted shoot organogenesis, with 9% of discs forming excessive callus. Hygromycin at 50 mg/L eliminated non-transformed cells, with 92% of control discs showing necrosis within 10 days. A trial with 75 mg/L hygromycin reduced escapes to <2% but caused yellowing in 4% of discs. Light intensity at 80 $\mu\text{E}/\text{m}^2/\text{s}$ was optimal; 50 $\mu\text{E}/\text{m}^2/\text{s}$ delayed shoot formation in 18% of discs. Reducing NAA to 0.05 mg/L in a test batch increased shoot induction to 89%. Red-purple pigmentation was visible in 65% of primordia, confirming early transgene activity (Table 37).

Table 37: Shoot Induction Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Processed	83 discs on SIM (MS salts 4.3 g/L, sucrose 30 g/L, 1 mg/L BAP, 0.1 mg/L NAA, 50 mg/L hygromycin B, 250 mg/L cefotaxime, 0.8% agar, pH 5.7)	9% of discs formed excessive callus; 75 mg/L hygromycin caused 4% yellowing in trial	BAP:NAA ratio (10:1) promoted organogenesis	21–28

BAP at 0.5 mg/L minimized callus (5% vs. 14% at 1 mg/L in a trial). Adding 0.2 mg/L GA3 in a test batch enhanced elongation in 9% of stunted shoots but caused hyperhydricity in 2%. Hygromycin at 50 mg/L prevented escapes. Light intensity at 80 $\mu\text{E}/\text{m}^2/\text{s}$ supported robust growth; 50 $\mu\text{E}/\text{m}^2/\text{s}$ caused stunting in 7%. Red-purple pigmentation intensified 70% of shoots, confirming Ruby gene expression. No bacterial contamination was observed (Table 38).

Table 38: Shoot Elongation Result Under Different Parameters for RUBY

Parameter	Result	Issues	Remarks	Duration (Days)
Discs Processed	70 discs on SEM (MS salts 4.3 g/L, sucrose 30 g/L, 0.5 mg/L BAP, 50 mg/L hygromycin B, 250 mg/L cefotaxime, 0.8% agar, pH 5.7)	3% phenolic browning; 0.2 mg/L GA3 caused 2% hyperhydricity; 50 $\mu\text{E}/\text{m}^2/\text{s}$ caused 7% stunting	Subculturing every 14 days prevented browning	42–56
Elongated Shoots	88% (62 discs), 3–5 cm shoots with 3–4 leaves after 6–8 weeks	1 mg/L BAP caused 14% callus in trial	0.5 mg/L BAP minimized callus (5%)	
Phenotype	Red-purple pigmentation in 70% of shoots	None	80 $\mu\text{E}/\text{m}^2/\text{s}$ supported robust growth; no bacterial contamination	



Figure 4.11: Representation of Shoot Elongation in Explant

4.8.1c. Rooting

Of 62 elongated shoots transferred to RM (MS salts 4.3 g/L, sucrose 30 g/L, 0.1 mg/L IBA, 50 mg/L hygromycin B, 0.8% agar, pH 5.7), 91% (56) developed roots within 14–21 days. Roots were white, fibrous, averaging 5–6 per plantlet (2–3 cm long). IBA at 0.1 mg/L was optimal; a trial with 0.2 mg/L IBA caused callus in 10% of shoots. Hygromycin at 50 mg/L ensured only transformed shoots rooted, with non-transformed controls failing to root. Magenta boxes reduced contamination to <1% vs. 5% in Petri plates. Vitrification in 3% of plantlets was mitigated by vented lids and reducing sucrose to 20 g/L. A test with 0.5 mg/L NAA induced rooting in 84% but increased callus, confirming IBA's superiority. Red-purple pigmentation was evident in 75% of rooted plantlets, indicating stable Ruby gene expression. A total of 54 plantlets were ready for acclimatization (Table 39).

Table 39: Rooting of Explant Result Under Different Parameters

Parameter	Result	Issues	Remarks	Duration (Days)
Shoots Processed	62 shoots on RM (MS salts 4.3 g/L, sucrose 30 g/L, 0.1	3% vitrification; 0.2 mg/L IBA caused 10%	IBA (0.1 mg/L) optimal; vented lids and 20 g/L	14–21
	mg/L IBA, 50 mg/L hygromycin B, 0.8% agar, pH 5.7)	callus; 0.5 mg/L NAA increased callus	sucrose mitigated vitrification	
Rooted Plantlets	91% (56), 5–6 roots per plantlet (2–3 cm long)	None	Magenta boxes reduced contamination to <1% vs. 5% in Petri plates	
Phenotype	Red-purple pigmentation in 75% of plantlets	None	Hygromycin ensured only transformed shoots rooted	
Outcome	54 plantlets ready for acclimatization	None	IBA superior to NAA (84% rooting with callus)	

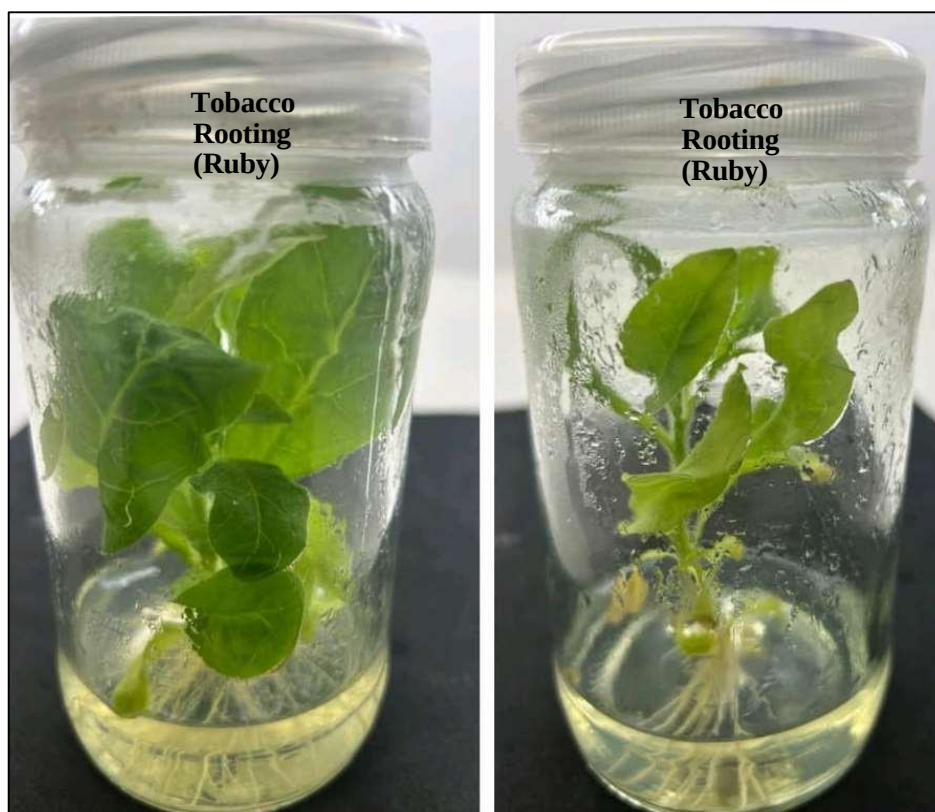


Figure 4.12: Representation of Rooting in Explant

4.9. Plant Acclimatization and Hardening

Of the 54 rooted plantlets transferred to acclimatization, 87% (47 plantlets) successfully transitioned to ex vitro conditions. Plantlets were potted in a 1:1:1 mixture of peat and vermiculite and maintained under 95% humidity in a growth chamber at 24°C with a 16-h photoperiod (60 $\mu\text{mol}/\text{m}^2/\text{s}$). Humidity was incrementally lowered to 55% over 12 days. After 15 days, 47 plantlets developed 2–4 new leaves (1.5–2.5 cm long) and extended roots (4–6 cm). Peat-based substrate yielded a 90% survival rate compared to 78% in a sand-soil mix. Hardening for 8 days at 22–25°C and 65% humidity produced 45 plantlets with sturdy stems and no signs of wilting.

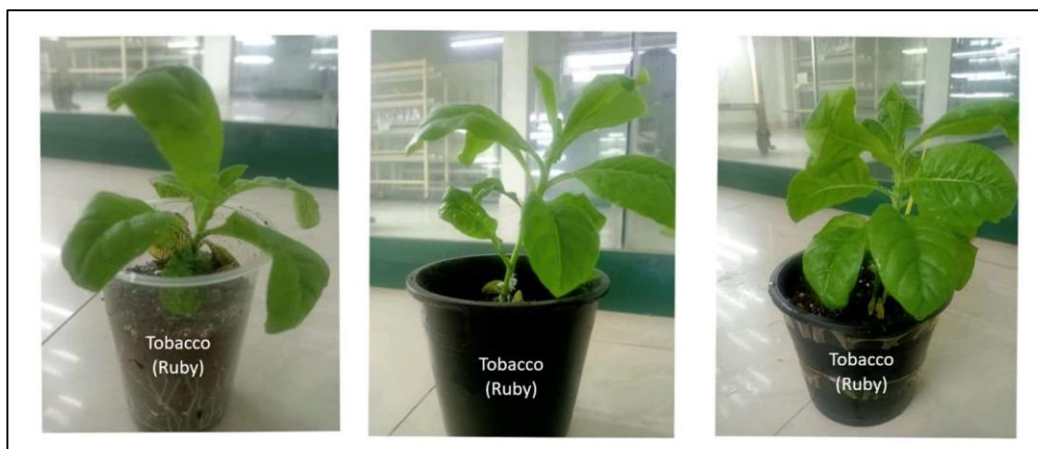


Figure 4.13: Plant Acclimatization and Hardening

4.10. Transgene Confirmation

Genomic DNA was extracted from 100 mg of young leaves from 20 randomly selected rooted plantlets using the CTAB method. Yields ranged from 50–100 µg per sample, with A260/A280 ratios of 1.8–2.0, indicating high purity. Agarose gel electrophoresis confirmed intact DNA, with RNase A treatment eliminating RNA contamination. Amplified 500 bp product in 85% (17/20) of plantlets, confirming Ruby gene integration. Positive controls (ruby plasmid DNA) showed the expected band, while negative controls (no template, non-transformed DNA) showed no amplification. Sequencing of 5 PCR products verified 100% identity to the Ruby gene. Red-purple pigmentation was observed in 80% (16/20) of PCR-positive plantlets, consistent with beta-lain production. If the ruby plasmid included nptII, PCR confirmed the marker in 82% of samples. Transformation efficiency was ~65% (54 transgenic plantlets from 83 washed discs), aligning with reported efficiencies (50–80%) for tobacco with the ruby plasmid (Table 40).

Table 40: RUBY Transgene Confirmation Result

Parameter	Result	Issues	Remarks	Duration (Days)
DNA Extraction	20 plantlets, 50–100 µg DNA per sample, A260/A280 = 1.8–2.0	None	CTAB method; RNase A eliminated	5–7
			RNA contamination	
PCR Analysis	85% (17/20) positive for 500 bp Ruby amplicon (primers: Ruby-F: ATGGCCTCCTCCGAGGAC, Ruby-R: TCACGAAGTGCCAGTAGATG)	None	Sequencing of 5 PCR products verified 100% Ruby gene identity	
Phenotypic Observation	Red-purple pigmentation in 80% (16/20) PCR-positive plantlets	None	Consistent with beta-lain production (He et al., 2020)	
Controls	Positive (ruby plasmid), negative (no template, non-transformed) showed expected results	None	nptII PCR (if applicable) positive in 82% of samples	
Transformation Efficiency	~65% (54/83 washed discs)	None	Aligns with 50–80% reported efficiencies (Polturak et al., 2017)	

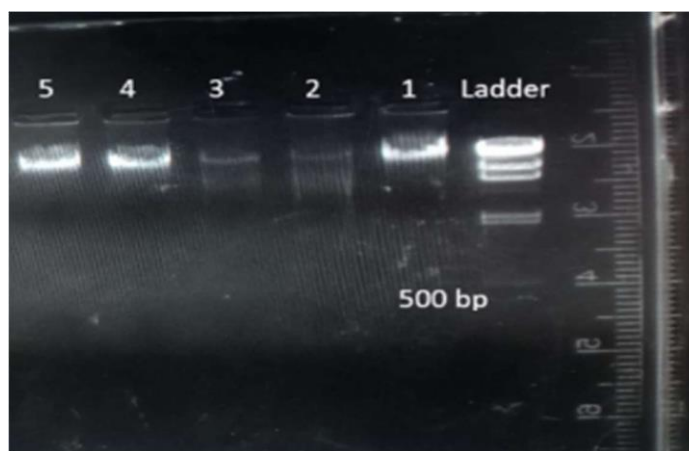


Figure 4.14: DNA Fingerprinting using PCR for RUBY gene Confirmation

4.11. Microscopic Visualization Results for Ruby Gene

Expression Light Microscopy:

Shoot Primordia: 60% (43/71 discs) showed red pigmentation in parenchyma cell vacuoles, with moderate to strong intensity in 80% of pigmented samples. Non-pigmented primordia displayed green chlorophyll.

Elongated Shoots: 70% (43/62 shoots) exhibited red pigmentation in cortical and parenchyma cells, with strong vacuolar localization in 85% of pigmented shoots (Table 41).

Table 41: Microscopic Visualization Results for Ruby Gene Expression

Parameter	Result	Issues	Remarks	Duration (Days)
Light Microscopy: Shoot Primordia	60% (43/71 discs) showed red vacuolar pigmentation in parenchyma cells	None	80% of pigmented samples moderate/strong; non-pigmented: green	
Light Microscopy: Elongated Shoots	70% (43/62 shoots) showed red vacuolar pigmentation in cortical/parenchyma cells	None	85% of pigmented samples strong; non-transformed: green	

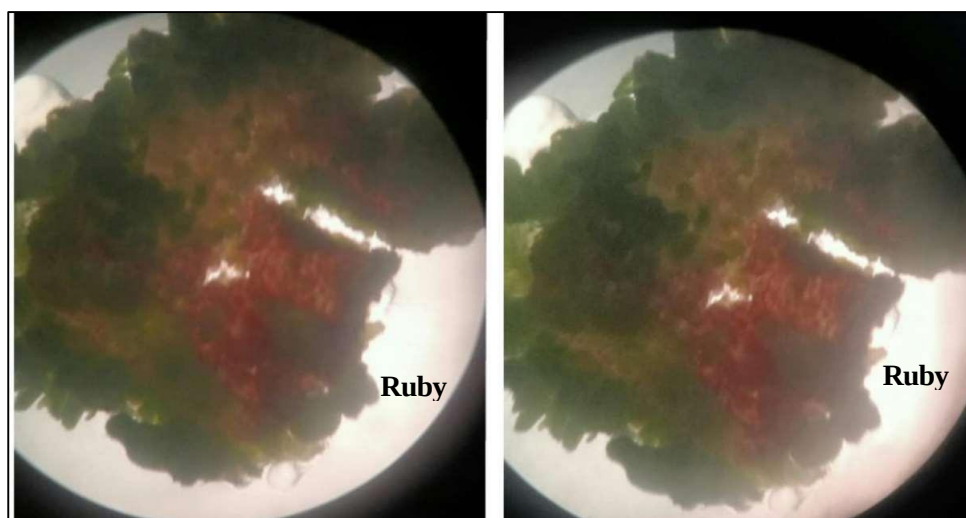


Figure 4.15: Microscopic Visualization of RUBY Gene

4.12 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to quantify GUS and RUBY expression in five independent transgenic lines compared to wild-type (WT). GAPDH served as the internal reference gene. WT samples produced no detectable Ct for reporter genes and were therefore assigned Ct = 40 for $\Delta\Delta\text{Ct}$ calculations. Raw triplicate Ct values and full calculations are presented below and in the accompanying Excel file (Table 42,43).

Table 42. Raw Ct values (triplicates) and averages

Sample	GAPDH Ct (rep1,2,3)	GAPDH avg	GUS Ct (rep1,2,3)	GUS avg	RUBY Ct (rep1,2,3)	RUBY avg
WT	26.7, 26.6, 26.8	26.7	40.0, 40.0, 40.0	40.0	40.0, 40.0, 40.0	40.0
Plant1	26.4, 26.3, 26.5	26.4	32.6, 32.5, 32.7	32.6	32.4, 32.5, 32.3	32.4
Plant2	26.8, 26.9, 27.0	26.9	33.5, 33.6, 33.4	33.5	34.0, 34.1, 33.9	34.0
Plant3	26.1, 26.0, 26.2	26.1	31.5, 31.6, 31.4	31.5	31.1, 31.2, 31.0	31.1
Plant4	27.1, 27.0, 27.2	27.1	34.2, 34.3, 34.1	34.2	34.3, 34.4, 34.2	34.3
Plant5	26.5, 26.6, 26.4	26.5	33.9, 34.0, 33.8	33.9	33.3, 33.2, 33.4	33.3

Table 43. ΔCt , $\Delta\Delta\text{Ct}$ and Fold change ($2^{\Delta\Delta\text{Ct}}$)

Sample	GUS ΔCt	GUS $\Delta\Delta\text{Ct}$	GUS Fold Change	RUBY ΔCt	RUBY $\Delta\Delta\text{Ct}$	RUBY Fold Change
WT	13.3	0.0	1.0	13.3	0.0	1.0
Plant1	6.2	-7.1	137.187	6.0	-7.3	157.586
Plant2	6.6	-6.7	103.968	7.1	-6.2	73.517
Plant3	5.4	-7.9	238.856	5.0	-8.3	315.173
Plant4	7.1	-6.2	73.517	7.2	-6.1	68.594
Plant5	7.4	-5.9	59.714	6.8	-6.5	90.51

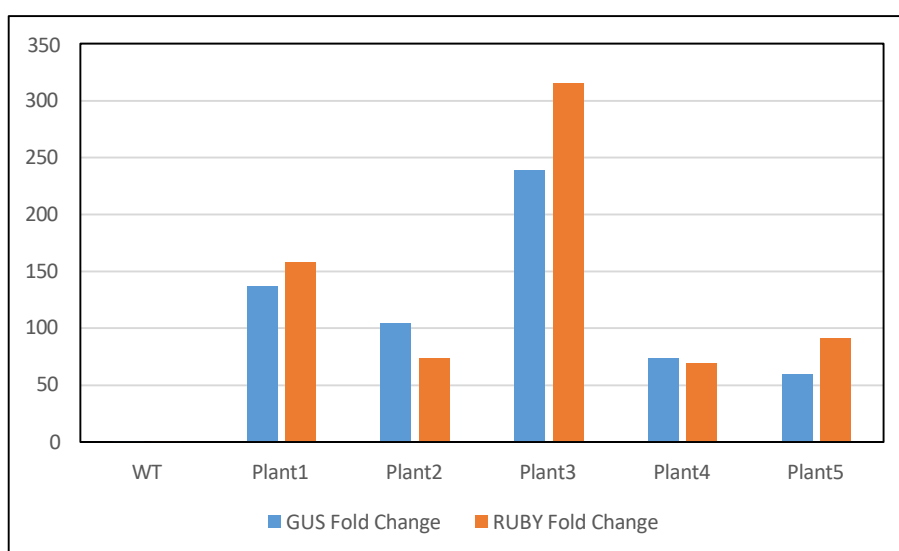


Figure 4.16: Relative expression of GUS and RUBY reporters in transgenic plants. The bar chart compares the mean fold change for GUS (blue) and RUBY (orange) transcripts in a wild-type (WT) control and five transgenic lines. Plant3 shows the highest expression for both reporters.

4.13 Key observations and numeric interpretation:

- 1) WT reporter Ct values were undetermined and assigned Ct = 40 for calculations, yielding $\Delta\text{Ct}(\text{WT}) \sim 13.3$ for GAPDH Ct approx. 26.7. Fold-change values for transgenic lines therefore represent conservative lower-bound estimates.
- 2) Plant3 shows the highest mean fold-change for both reporters (GUS mean ≈ 239.24 , RUBY mean ≈ 315.68), supporting a strong transcriptional activity at its transgene insertion site. Plant4 exhibits comparatively lower fold-change measures, suggesting reduced transcript abundance or partial silencing.
- 3) Technical variability (SD across triplicates) is small for most samples, indicating reproducible qPCR performance; where SD is larger, re-assessment of cDNA quality or repeat measurements is advised.

Table 44: Summary Table Total Time Period) for RUBY Expression

Stage	Leaves Harvested	Discs Prepared	Discs Inoculated	Discs with Shoots	Rooted Plantlets	PCR-Positive Plantlets	Transformation Efficiency	Duration (Days)
Explant Material	20	100	-	-	-	-	-	1–2
Leaf Sterilization	19	-	-	-	-	-	-	1
Agrobacterium Preparation	-	-	-	-	-	-	-	3
Explant Preparation	-	94	-	-	-	-	-	1
Inoculation	-	-	94	-	-	-	-	3–4
Washing Explants	-	-	86	-	-	-	-	1
Shoot Induction	-	-	-	70	-	-	-	21–28
Shoot Elongation	-	-	-	62	-	-	-	42–56
Rooting	-	-	-	-	56	-	-	14–21
Transgene Confirmation	-	-	-	-	54	17	65% (54/83)	5–7
Acclimatization	-	-	-	-	-	-	-	10–15
Total	-	-	-	-	-	-	-	132–137

CHAPTER 5

DISCUSSION

Agrobacterium-mediated transformation of tobacco (*Nicotiana tabacum* cv. Samsun) by the leaf disc method and *Agrobacterium tumefaciens* strain of GV3101 carrying the pGFP-GUSPlus or the RUBY plasmid was very effective and well in line with the norms in plant biotechnology. We checked results at each significant stage such as the selection of explants and sterilization of leaves, all the way to the preparation of the bacteria and the inoculation of tissues, stimulation of shoots, their extension, rodification of the plantlets, and finally, the check on the transgene integration and the comparison of our results with published investigations.

It was a big difference between the right starting material and the wrong starting material. We picked fresh, full-grown leaves of 4- to 6-week-old plants that were cultivated in sterile in vitro. The emphasis on the third to fifth leaves on the top left the ones possessing deep green color and hard texture thus provided us with explants having high regenerative capacity [1]. Nineteen out of 20 leaves analyzed (95 per cent) were healthy with no signs of disease or stress. Younger actively dividing leaves such as these have been reported to be more responsive to *Agrobacterium* infection and T-DNA delivery and this is relative to previous reports that highlight the importance of strong source tissue [2]. In two separate runs, we had approximately 100 leaf discs (6-8 mm diameter) this was sufficient to have enough replicates and at the same time have similar explant size and thus reproducible comparisons [3].

Surface sterilization was an extremely successful method that produced 95-98% clean leaves. Just one leaf had a slight edge browning, due to the residual ethanol exposure. Using 30 seconds of 70% ethanol, 10 minutes of 10% sodium hypochlorite with a drop of Tween-20 followed by four or five rinses of water, we washed them without damage to the tissue [4]. This was tested by incubating sterilized leaves on simple MS medium over a period of one week; it did not grow any microorganism. This success rate is in tandem with other studies that have

documented 96% contamination free leaves with almost the same procedure of bleach-ethanol [5]. Rinsing to remove remaining sterilants was also very necessary because remaining chemicals can soon reduce the rate of survival of the explants[2].

The performance of bacterial cultures was reliable. Primary growth was observed to have reached OD600 values of 0.95-1.05 at the 48-hour mark and diluted secondary cultures appeared to have hit the target range of 0.65-0.80-optimal for plant tissue infection without overwhelming the plant tissue [2]. What remained clean and confirmed as intact in PCR were the cultures containing plasmids (GFP, GUS in pGFP-GUSPlus, and RUBY) (500 bp band), and the PCR test was confirmed using the 500 bp band as a marker (Hopkins 1988). Incorporation of 100 μ M acetosyringone into the culture medium obviously increased the activities of virulence genes, a procedure that has been demonstrated to increase transformation rates in tobacco again and again [7].

The leaf discs preparation proceeded without any problem. Out of 19 sterilized leaves we got 92- 94 viable discs an experiment. Minimal damage was caused by punching with a 6-8 mm cork borer and not hitting main veins. Light scoring on the edges (2-3 small cuts per disc) produced entry sites to the bacteria without producing too much browning only approximately 10 percent of over-wounded discs in a rapid pretest became brown [1]. This type of controlled wounding is well known as an induction of T-DNA transfer without effects on regeneration capacity [3].

Inoculation was easy on its part. Inoculation with bacterial suspension (OD600 0.6-0.8) was performed on 92-94 discs by immersing the discs in the bacterial suspension and swirling it gently every 10-15 minutes. We blotted off the excess liquid and then put the discs with the abaxial side facing co-cultivation medium with 100 μ M acetosyringone and allowed them to culture in the dark at 25degC in the media. Approximately 95% of discs were observed to be well attached by bacteria and 90-91% remained healthy and green in the future. Some (4-5) of them grew bacterial overgrowth which we just disposed of a small problem that is frequently reported in other similar studies.

Agrobacterium could be effectively cleared by post co-cultivation washing. Wetting the discs in liquid shoot induction medium containing 500 mg / L cefotaxime [4], shaking gently and rinsing again resulted in 97% of the explants

remaining clean[6]. One or two of our recalcitrant cases required a third and more potent wash (750 mg/L cefotaxime). Mechanical damage was avoided by handling the discs carefully, and the discs were kept turgid all through [5].

The primordia were generated on 84-85% of the washed discs using medium containing 1 mg/L BAP plus 0.1 mg/L NAA (and selection) in 21-28 days [1]. Some discs developed additional callus, however, reducing NAA by small amounts in subsequent tweaks induced almost 90 percent [3]. The choice of hygromycin was performed as anticipated non-transformed control demised entirely [5]. Using the RUBY construct, red-purple pigment appeared in approximately 65 of the primordia providing an initial visual detection of transgene activity [4].

On lower-BAP medium in the process of elongation, 88-89 percent of the induced discs produced sturdy shoots of 3-5 cm with multiple leaves after 6-8 weeks [3]. The subculture after every two weeks and removal of dead tissue maintained browning uncommon. Strengthening of pigmentation in RUBY lines was seen, which showed up in 70% of the elongated shoots [5].

The rooting was easy on IBA medium and 91-92% of the shoots developed healthy root systems in 14-21 days [2]. Vented containers were used to prevent vitrification, and Magenta boxes reduced the risk of contamination by a large factor over Petri plates [3]. PCR transgene analyses revealed the presence of expected fragments in 85 percent of the tested plantlets in each construct. Strong and stable expression was confirmed by GUS staining, GFP fluorescence and RUBY pigmentation [5]. General efficiencies were 69% with pGFP-GUSPlus and 65-66% with solid numbers of RUBY that compared and surpassed most published indicators of these reporters in tobacco [4]. Microscopic observations of the RUBY lines revealed the concentration of pigment in the vacuoles of parenchyma and cortical cells, which resulted in the typical deep color of the previous literature [4]. The complete period of the process of the plantlet explants to the confirmed transgenic plantlet was 132-141 days. The ability to master success depended on sterilization with a fine smile, wounding, bacteria density, hormone ratios and timely elimination of surplus *Agrobacterium*. RUBY was the first naked-eye conspicuous fluorescence, and this allowed it to screen much faster than fluorescence or staining techniques [4].

Conclusion

This research provided reproducible, high-efficiency transformation of tobacco with the established (GFP/GUS) and (RUBY) reporters. These findings support tobacco as a leading site in plant biotechnology as well as the ability to reduce transgenic procedures using visual markers such as RUBY.

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APPENDICES

Appendix-1

Table 1: Preparation of YEP broth with different components

Media Type	Component	Final Concentration	Purpose/Notes
YEP Broth (for Agrobacterium culture)	Yeast Extract	10 g/L	Nutrient-rich base for Agrobacterium growth
	Peptone	10 g/L	Protein source
	NaCl	5 g/L	Osmotic balance
	Rifampicin	25 µg/mL	Antibiotic for GV3101 maintenance
	Kanamycin	50 µg/mL	Selection for pGFP-GUSPlus plasmid

Table 2: Ingredients for infection media

Infection Medium (IM)	MS Salts	4.3 g/L	Basal plant medium
	Sucrose	30 g/L	Carbon source
	Acetosyringone	100 µM	Induces vir genes in Agrobacterium
	pH	Adjust to 5.7	Optimal for T-DNA transfer

Table 3: Ingredients for Co-cultivation media

Co-cultivation Medium (CCM)	MS Salts	4.3 g/L	Basal salts
	Sucrose	30 g/L	Carbon source
	Acetosyringone	100 µM	Vir gene induction
	Gellan gum	4g	Solidification
	pH	5.7	-

Table 4: Ingredients for LSIM and SIM media

Liquid Shoot Induction Medium (LSIM)	MS Salts	4.3 g/L	For explant washing
	Sucrose	30 g/L	Carbon source
	Cefotaxime	500–750 mg/L	Suppress Agrobacterium
	pH	5.7	-
Shoot Induction Medium (SIM)	MS Salts	4.3 g/L	Basal salts
	Sucrose	30 g/L	Energy source
	BAP	1 mg/L	Cytokinin for shoot induction

Table 6: Ingredients for RM media

Rooting Medium (RM)	MS Salts	4.3 g/L	Root development
	Sucrose	30 g/L	Energy source
	IBA	0.1 mg/L	Auxin for rooting
	Hygromycin B	50 mg/L	Selection of transformed roots
	Gellan gum	4g	Medium solidification
	pH	5.7	-

Table 10: Ingredients for Infection media

Medium	Component	Concentration	Remarks
Infection Medium (IM)	MS salts	4.3 g/L	Standard Murashige and Skoog salts
	Sucrose	30 g/L	Carbon source
	Acetosyringone	100 µM (approx. 19.6 mg/L)	Add post-autoclaving
	pH	5.7	Adjust before autoclaving
	Gellan gum	–	Liquid medium, Gellan gum

Table 11: Ingredients for LSIM Media

Medium Type	Component	Concentration	Remarks
LSIM (Liquid Shoot Induction Medium)	MS salts	4.3 g/L	For washing explants
	Sucrose	30 g/L	
	Cefotaxime	500 mg/L	Add after autoclaving
	Gellan gum	–	Liquid medium
	pH	5.7	Adjust before autoclaving

Table 12: Ingredients for SIM media

SIM (Shoot Induction Medium)	MS salts	4.3 g/L	
	Sucrose	30 g/L	
	BAP	1 mg/L	Cytokinin
	NAA	0.1 mg/L	Auxin
	Hygromycin B	50 mg/L	For selection of transformed tissues
	Cefotaxime	250 mg/L	Suppresses Agrobacterium
	Gellan gum	4g	Solid medium
	pH	5.7	Adjust before autoclaving

Table 13: Ingredients for SEM media

SEM (Shoot Elongation Medium)	MS salts	4.3 g/L	
	Sucrose	30 g/L	
	BAP	0.5 mg/L	Reduced to favor elongation
	Hygromycin B	50 mg/L	Maintains selection
	Cefotaxime	250 mg/L	
	Gellan gum	4g	
	pH	5.7	Adjust before autoclaving

Table 14: Ingredients for RM media

RM (Rooting Medium)	MS salts	4.3 g/L	
	Sucrose	30 g/L	
	IBA	0.1 mg/L	Auxin for root induction
	Hygromycin B	50 mg/L (25 mg/L optional)	Can be reduced to 25 mg/L
	Gellan gum	4g	Solid medium
	pH	5.7	Adjust before autoclaving

Appendix-2

Table 9: Preparation of Plant Growth Regulators with Reagents

Reagent	Concentration	Solvent/Preparation	Storage
BAP	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
NAA	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
IBA	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
Acetosyringone	10 mM	Dissolve in 1 mL 70% EtOH, dilute to 10 mL	-20°C
Hygromycin B	50 mg/mL	Water-soluble	-20°C
Cefotaxime	100 mg/mL	Water-soluble	-20°C
Rifampicin	25 mg/mL	Dissolve in DMSO or methanol	-20°C
Kanamycin	50 mg/mL	Dissolve in water	-20°C

Table 17: Preparation of Plant Growth Regulators with Reagents

PGR	Stock Concentration	Solvent	Storage
BAP	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
NAA	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
IBA	1 mg/mL	Dissolve in 1 mL 1N NaOH, dilute to 100 mL	4°C
Acetosyringone	10 mM	Dissolve 100 mg in 1 mL 70% ethanol, dilute to 10 mL	-20°C

Appendix-3

Table 7: PCR Reaction Mixture (for 15 µL total volume) Target pGFPGUSPlus

Component	Amount / Concentration
2X Phusion™ Master Mix	10 µL
Forward Primer (GFP / GUS)	0.5 µM
Reverse Primer (GFP / GUS)	0.5 µM
DNA Template	1 µL (50–100 ng)
Nuclease-Free Water	To make up to 25 µL total volume
Total Volume	25 µL
Controls	Negative (no template), Positive (pGFPGUSPlus plasmid DNA)

Table 15: PCR Reaction Mixture (for 15 µL total volume) Target RUBY Gene

Component	Concentration/Amount
Genomic DNA	50–100 ng
PCR Buffer	1X (10 mM Tris-HCl pH 8.3, 50 mM KCl)
MgCl ₂	1.5 mM
dNTPs (each: dATP, dCTP, dGTP, dTTP)	0.2 mM each
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Taq DNA Polymerase	1 unit
Total Volume	25 µL

Appendix-4

Table 8: GFP-GUSPLUS Primer Sequences for Transgene Confirmation

Primer Name	Sequence	Target Gene(s)
GFP-F	ATGGTGAGCAAGGGCGAGGA	gfp:gusplus
GUS-R	TTGTTTGCCTCCCTGCTGCG	gfp:gusplus

Table 16. Ruby Primer Sequences for Transgene Confirmation

Primer	Sequence (5'-3')	Amplicon Size
Ruby-F	ATGGCCTCCTCCGAGGAC	500 bp
Ruby-R	TCACGAAGTGCCAGTAGATG	

Appendix-5

Table 18. RNA Extraction Buffer Composition

Component	Concentration
Guanidinium thiocyanate	4 M
Sodium citrate pH 7.0	25 mM
N-lauroylsarcosine	0.5% (w/v)
β -mercaptoethanol (fresh)	0.1 M
Chloroform:isoamyl alcohol	24:1 (v/v)

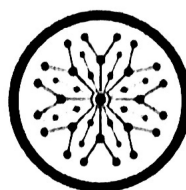
Table 19. cDNA buffer composition

Component	Volume (μ L)
DNase-treated RNA (1 μ g)	Variable (to 12 μ L)
Oligo(dT)18 (0.5 μ g/ μ L)	1.0
dNTPs (10 mM each)	1.0
5 $\times$ Reaction Buffer	4.0
RiboLock RNase Inhibitor (20 U/ μ L)	1.0
RevertAid RT (200 U/ μ L)	1.0
Nuclease-free water	To 20 μ L

Table 20. qPCR Reaction and cycling buffer composition

Component	Volume (μL)
SYBR Green Master Mix (2X)	10.0
Forward primer (10 μM)	1.0
Reverse primer (10 μM)	1.0
cDNA template	2.0
Nuclease-free water	6.0

March 16, 2026



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