Agrobacterium mediated genetic transformation of *Citrus reticulata* cv. Khasi mandarin

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By

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FACULTY OF AGRICULTURE

CERTIFICATE – I

This is to certify that the thesis entitled **"*Agrobacterium* mediated genetic transformation of *Citrus reticulata* cv. Khasi mandarin"** submitted to the Faculty of Agriculture, Assam Agricultural University in partial fulfilment for the degree of **Master of Science (Agriculture)** in **Agricultural Biotechnology** programme is a record of research work carried out by **Sangeeta Bhandari** under my personal supervision and guidance.

All helps received by her have been duly acknowledged.

No part of this thesis has been reproduce elsewhere for any degree.

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Date:…... February, 2020 Major Adviser

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This is to certify that the thesis entitled **"*Agrobacterium* mediated genetic transformation of *Citrus reticulata* cv. Khasi mandarin"** submitted by **Sangeeta Bhandari, Roll No. 2017-AMJ-05** to the Assam Agricultural University, in partial fulfilment of the requirement for the degree of **Master of Science (Agriculture)** in the discipline of **Agricultural Biotechnology** has been examined and approved by the Student's Advisory Committee after viva-voce.

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ABSTRACT

Citrus is number one fruit of the world on accounts of its high nutritional value. India is the fourth largest producer of *Citrus* in the world. The north-eastern region of India is a rich treasure of various *Citrus* species. Khasi mandarin is the most economically important one and plays a vital role in the socio-economic development of the people in this region. Khasi mandarins are declining at a very high rate due to its vulnerability to different pathogen and insect/ pest. Conventional breeding for overcoming these problems are limited in *Citrus* and are directly associated with the reproductive biology of *Citrus*. Recent advances in genetic engineering have made it possible to incorporate desirable genes from elite genotype mainly through *Agrobacterium*-mediated genetic transformation. *Citrus* species showed varied response to *in vitro* regeneration and genetic transformation. Cultivar specific optimization of *in vitro* regeneration and transformation protocol is very important. In the present investigation, *in vitro* regeneration and *Agrobacterium* mediated genetic transformation protocol for Khasi Mandarin was optimized using different explants like epicotyl, hypocotyl, nodal and inter nodal segment obtained from six-week-old *in vitro* grown zygotic seedling. Explants were transformed wih *Agrobacterium* strain LBA4404, harbouring plasmid pBI121-AtSUC-GUS containing *nptII* as a selectable marker and *gus* as a reporter gene. Hypocotyl was found to be the best explants for khasi mandarin transformation and regeneration. MS medium supplemented with BAP (2mg/L), NAA (0.5 mg/L), 2, 4-D (1mg/L), MES (0.5g/L), sucrose (30g/L) and acetosyringone (100μM) was found to be best medium for co-cultivation. Modified MS medium containing BAP (4mg/L), MES (0.5g/L), sucrose (30g/L), phytagel (4g/L), kanamycin (50mg/L) and timentin (150mg/L) showed highest regeneration efficiency (18%). Modified MS medium containing BAP (4mg/L), GA3 (0.5mg/L), MES (0.5g/L), sucrose (30g/L), phytagel (4g/L), kanamycin (50mg/L) and timentin (150mg/L) showed highest multiple shoot induction (6%). *In vitro* regenerated shoots that survived up to 3rd selection cycle were subjected to GUS assay for confirmation of *GUS* expression in the phloem tissues. Present investigation is a preliminary study for optimization of an *in vitro* regeneration and genetic transformation protocol in Khasi Mandarin.

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LIST OF ABBREVIATIONS

%	:	Per cent
/	:	Per
°C	:	Degree centigrade
2,4-D	:	2,4-Dichlorophenoxyacetic acid
BAP	:	6- benzyl aminopurin
bp	:	Base pair
cm	:	Centimeter
cv.	:	Cultivar
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy-ribonucleoside tri-phosphate
e.g.	:	*Exempli gratia* (for example)
EDTA	:	Ethylene diamine tetra acetic acid
et al.	:	*Et alli* (all others)
EtBr	:	Ethidium bromide
g	:	Grams
GA3	:	Gibberellic acid
gus	:	B-Glucouronidase
h	:	Hour(s)
IBA	:	Indole butyric acid
Kb	:	Killo basepair
L	:	Litre
M	:	Molar
mg	:	milligram
min	:	Minute(s)
ml	:	Mililitre(s)
mm	:	Milimeter(s)
mM	:	Milimolar
MES	:	2-(N-morpholino)ethanesulfonic acid
MW	:	Molecular weight
NAA	:	Napthalene acetic acid
ng	:	Nanogram

No.	:	Number
Nos	:	Numbers
OD	:	Optical density
PCR	:	Polymerase chain reaction
pH	:	Hydrogen ion concentration
rpm	:	Revolution per minute
SDS	:	Sodium dodecyl sulphate
Sec.	:	Second(s)
spp.	:	Species
TBE	:	Tris Borate EDTA
U	:	Unit(s)
UV	:	Ultra violet
V	:	Volt
v/v	:	Volume by volume
viz.	:	Videlict (namely)
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar

CHAPTER I

INTRODUCTION

Citrus is the genus of flowering trees and shrubs including more than 162 species. They are grouped under the family Rutaceae and sub family Aurantioideae. *Citrus* (*Citrus* L.) is one of the world's most important fruit crops, with great economic and health values distributed in the tropical and subtropical regions of the world (Randhwa and Srivastava, 1986). It is most widely grown fruit crop throughout the world (Yang *et al.*, 2000). The responsible bioactive components are Vitamin C, beta-carotene, flavonoids, limonoids, folic acid, and dietary fiber. Citrus fruits contain carbohydrates in the form of sugars: sucrose, glucose and fructose. Citrus fruits are originated from southern China and Southeast Asia where they have been cultivated for some 4,000 years.

Brazil is the world leader in citrus production, with approximately 20.2 million tons per year (USDA, 2019). There are about 140 major citrus producing countries according to the UNCTAD. India is the fifth largest producer of citrus after Brazil, the United State, China and Mexico with annual production of 6.2 million tons. India enjoys a remarkable position in the **"Citrus belt of the world"** due to her rich wealth of Citrus genetic resources, both wild and cultivated (Malik *et al*., 2013).

Citrus is the third most important fruit crop of India after mango and banana. Mandarin constitutes about 43.6% of the total citrus produced and occupies nearly 38.2% of the total citrus area in India (National Horticulture Board, 2010-11). The north-eastern region of India is highly diverse in Citrus germplasm, 23 taxa including 68 varieties have been reported by Sharma *et al.* (2004). According to Tanaka (1954 and 1961), Assam was thought to be one of the centers of origin of Citrus. Among the Citrus crops available in northeastern region, Khasi mandarin is the most economically important one and plays a vital role in the socio-economic development of the people in this region. Khasi mandarin is well known for its quality, fruit colour, unique sugar-acid blend and shelf life which make it the most popular citrus cultivar in northeastern region of the country. The significance of this crop is magnified not only because of its nutritional value but also owing to its medicinal use. Assam and Meghalaya have the maximum area for production of Khasi mandarin (Singh *et al*., 2006). Khasi mandarin in north-east India popularly known as Sohniamptra in Khasi, Humoptira or Komola in Assamese, Komla in Bengali and Manipuri (Singh, 1990).

Khasi mandarins are declining at a very high rate due to its vulnerability to many types of pathogen. Insect/ pest, fungal, bacterial and viral diseases are the major constraint in mandarin production. Citrus Tristeza Virus (CTV) is one of the most detrimental pathogens infecting citrus and causes massive crop losses (Ahlawat, 1997; Bar-Joseph *et al.,* 2008; Rocha-Pena *et al.,* 1995). Citrus yellow mosaic disease is a common, widely distributed and severe disease in India and reported to occur especially in sweet orange (*C. sinensis* Osbeck) in southern states and Khasi mandarins (*C. reticulata* Blanco) in northeastern states (Dakshinamurthy and Reddy, 1975; Ahlawat *et al.,* 1985).

The genus *Citrus* presents limitations for improvement *via* conventional breeding, and are directly associated with the reproductive biology of *Citrus*, such as nucellar polyembryony, a high level of heterozygosity and a long juvenile period (Grosser and Gmitter, 1990).

Currently, genetic transformation as a tool for citrus improvement is gaining in popularity. This method of citrus improvement is especially useful in cases where it is not possible to engineer a trait of interest to an otherwise elite cultivar using conventional breeding (Dutt *et al*., 2010). Genetic transformation has become an attractive alternative method for improving *Citrus* and other species because it is possible to maintain cultivar integrity while adding a single trait (Bond and Roose, 1998). Different methods have been used for incorporation of transgenes into citrus. These methods include *Agrobacterium*-mediated transformation using juvenile *in vitro* epicotyl segments (Moore *et al*., 1992; Dutt and Grosser, 2009), mature internode segments obtained from greenhouse-grown plants (Cervera *et al*., 1998a; Almeida *et al*., 2003) or embryogenic callus obtained from unfertilized ovules (Li *et al*., 2002). Direct incorporation of DNA into protoplasts using electroporation (Niedz *et al*., 2003) or PEG mediated (Fleming *et al*., 2000; Omar *et al*., 2007) have also been reported.

Till date there exists no reports on genetic transformation of Khasi mandarin mediated either through *Agrobacterium* or particle bombardment method. Therefore, present investigation is envisaged to carry out with the following objectives.

1. Optimization of different explants for genetic transformation of *Citrus*.
2. Molecular characterization of transgenic *Citrus* for presence and expression of transgene.

CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction

North Eastern (NE) region of India sits in the middle of the Indo- Burma biodiversity hotspot, one of the 25 biodiversity hotspot of the world. NE is also considered as the richest and one of the most endangered places for plant survival in the world. The region is known as one of the centre of origin of various *Citrus* species. *Citrus* species, particularly Khasi mandarin (*Citrus reticulata*), Assam lemon (*Citrus limon*), Rough lemon (*Citrus jambhiri*) and Pummelo (*C. grandis*) are the major commercial horticultural crops grown widely in the North Eastern region. Khasi mandarin is famous for its superior quality in respect of its flavor, juice content, soluble sugar and acidity ratio. Khasi mandarin is the most economically important citrus species and plays vital role in the socio-economic development of the people in this region.

2.2 Problems in Citrus

Citrus fruits are very susceptible to infestation by different types of insects, fungi, bacteria and viruses. In India Citrus cultivation is plagued with various problems including higher incidence of insect and mite pests, which can cause serious quantitative and qualitative losses in Citrus. 250 species of insects and mites have been reported infesting different species of citrus in India (Wadhi *et al*., 1964). About 12 species of major insects and mite pests and many species of nematodes have been recorded in the Khasi mandarin ecosystem of North Eastern India (Hore *et al.*, 2004). Citrus tristeza virus (CTV) is a phloem- limited virus whose natural host range is restricted to citrus and related species."Tristeza" disease is the most devastating disease of the citrus cause by citrus tristeza virus (CTV), an aphid- transmitted, single stranded cloesterovirus that causes phenomenal economic damage to citrus industry. During the last century severe epidemics of CTV caused quick decline that developed in citrus growing regions and destroyed almost 100 million trees worldwide (Ahlawat, 1997; Bar-Joseph *et al.*, 2008; Rocha-Pena *et al.*, 1995). Among bacterial diseases citrus canker (CCK), citrus variegated chlorosis (CVC) and Huanglongbing (HLB) result in significant reductions in citrus production (Bove, 2006).

2.3 Citrus breeding

Despite of substantial genetic diversity and interspecific fertility, the genus *Citrus* includes some of the most difficult species to breed (Grosser *et al.*, 1992). The obstacle for conventional breeding in Citrus is due to various reasons like pollen and/or ovule sterility, long juvenile period and high level of heterozygosity. Most species of Citrus are highly heterozygous and produce progeny that segregate widely for many characters. The presence of adventitious somatic embryos in the nucellus of developing ovule of Citrus also limits hybrid production (Moore *et al.*, 1993). Hence, in traditional breeding, it is difficult to improve the desired traits of Citrus in the short term. Additionally, traditional breeding is mainly restricted to the traits related to fruit quality, such as the fruit ripening time, flesh color, and seed number (Moore *et al.*, 1993).

Recent information regarding various attempts to improve *Citrus* species by using various *in vitro* techniques has been categorically reviewed below:

2.4 Citrus tissue culture and morphogenesis studies

The importance of tissue culture in citrus research was recognized long back, and amply emphasized by (Bittters and Murashige, 1967; Kochba and Spiegel-Roy, 1976). Kochba and Spiegel-Roy (1977) discussed about the significance of tissue culture in citrus breeding for improvement and augmenting production. Conventional methods for Citrus propagation are based on budwood selection and grafting for scion varieties. Citrus propagation through root cuttings, or more frequently nucellar seed propagation and preparation of rootstocks are also reported by Barlass and Skene (1986). Due to increasing demand of Citrus industry and continuous introduction of new improved genotypes, emphasize of the researchers are more towards the use of modern methods to rapidly propagate new and promising plant material.

2.5 Citrus transformation studies

Transformation studies in Citrus have been done for two decades. Somatic hybridization and genetic transformation studies are already integrated in Citrus breeding programs in several countries. Citrus genetic transformation is considered as a promising tool that enables the introduction of desirable traits without altering the genetic background (Marques *et al*., 2011). Genetic transformation of Citrus has been carried out using various methods. The methods are *Agrobacterium* mediated genetic transformation (Miyata *et al*., 2012; Dutt *et al.,* 2009; Pena *et al.,* 2004; Mondal *et al*., 2012), RNA interference (Soler *et al*., 2012) , RNA induced gene silencing (Reyes *et al.,* 2011), Sonication- assisted *A. tumefaciens* (SAAT) (Oliveira *et al*., 2009), PEG (Vardiet *et al.*, 1990), electroporation (Niedz *et al*., 2003) and particle bombardment (Filho *et al*., 2003). *Agrobacterium* mediated transformation using epicotyl explants as target cells for incorporation of the T-DNA has been used more frequently in Citrus genetic transformation (Dutt *et al*., 2009; Pena *et al.,* 2004). *Citrus* species are widely known for their recalcitrance to transformation and subsequent rooting, but constant research has led to the establishment of improved protocols to ensure the production of uniformly transformed plants, albeit with relatively low efficiency, depending upon the genotype (Pena *et al*., 2003). Optimization of *in vitro* regeneration is important in order to carry out successful transformation in Citrus. Many successful transformation events have been achieved and different genes have been introduced into citrus that comprise antibiotic and reporter genes, genes that shorten the juvenile phase, genes that confer stress tolerance and disease resistance, and fruit quality-related genes (Dutt *et al*., 2016; Azevedo *et al*., 2006).

With the aim of accelerating the flowering time of Citrus Pena *et al.* (2004) transformed juvenile citrus seedlings to constitutively express the *Arabidopsi*s *LEAFY* (*LFY*) or *APETALA1* (*AP1*) genes, which promote flower initiation in *Arabidopsis* (Pena *et al*., 2001).

Huanglongbing (HLB) is a serious disease of citrus associated with the phloem limited bacterium *Candidatus* Liberibacter asiaticus (*C*Las). Dutt *et al*. (2012) developed transgenic sweet orange cultivars ‘Hamlin’ and ‘Valencia’ expressing an *Arabidopsis thaliana NPR1* gene under the control of a phloem specific *Arabidopsis* SUC2 (*AtSUC2*) promoter. Over expression of *AtNPR1* resulted in trees with normal phenotypes that exhibited enhanced resistance to HLB.

2.6 *Agrobacterium* mediated genetic transformation of Citrus

Among the several methods available for the genetic transformation of citrus, the most popular method to transform a wide range of citrus cultivars is *Agrobacterium*-mediated transformation using epicotyl explants as target tissue for incorporation of the T-DNA (Dutt *et al*., 2009). *Agrobacterium* has been the most frequently used genetic transformation method in *Citrus* with explants collected from seedlings germinated *in vitro* (Moore *et al*., 1992; Luth and Moore, 1999; Ananthakrishnan *et al*., 2007) or under greenhouse conditions (Cervera *et al*., 1998a; Almeida *et al.*, 2003). In addition to transformation studies via *A. tumefaciens*, recently *A. rhizogenes* has been used (Choi *et al.*, 2000; Nedelkoska *et al*., 2000). The use of *A. rhizogenes* for expression of the *rol* genes and also to deliver foreign genes to susceptible plants has been reported (Choi *et al.*, 2000). *A. rhizogenes*-mediated transformation system was found to be very useful in genetic manipulation of plants for the production of phytochemicals (Shanks *et al*., 1999), large scale secondary metabolite production (Choi *et al.,* 2000), monoclonal antibody production (Wongsamuth *et al*., 1997), and phytoremediation (Nedelkoska *et al*., 2000). GUS and *npt*II have been used as reporter and selection genes, respectively on Citrus genetic transformation.

There are a few reports about the introduction of genes of agronomic importance in *Citrus*, including the gene that encodes for the *Citrus* tristeza virus coat protein (Gutierrez-E. *et al*., 1997; Dominguez *et al*., 2000; Yang *et al*., 2000), the *HAL2* gene that confers tolerance to salinity (Cervera *et al*., 2000), and *CS-ACS1* gene that controls the ethylene biosynthesis in *Citrus* (Wong *et al*., 2001).

Phytophthora species are the most important soil borne problem that affects Citrus trees; they can damage any part of the plant at any age. Azevedo *et al*. (2006) carried out genetic transformation of Rangpur Lime (*Citrus limonia* Osbeck) with the *bO* (*bacterio-opsin*) gene. The authors produced transgenic Rangpur lime plants with the *bO* gene, via *Agrobacterium tumefaciens*-mediated transformation, and evaluated these plants for *Phytophthora nicotianae* resistance. Two transgenic lines were successfully regenerated, and transformation was confirmed by GUS activity assay, PCR analysis, Southern, Northern and Western blot analyses, in addition to detecting the expressed bO protein by an immunological approach (Azevedo *et al*., 2006).

Khawale *et al*. (2006) developed genetic transformation protocol for Nagpur mandarin (*Citrus reticulata* Blanco), a choicest citrus variety grown in India and South East Asia. Dutt *et al.* (2009) optimized an improved protocol for genetic transformation of Carrizo (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.), Ducan

(*Citrus paradisi* Macf.), Hamlin (*Citrus sinensis* (L.) Osbeck) and Mexican Lime (*Citrus aurantifolia* Swingle) cultivars using a vector containing *egfp-nptII* fusion gene.

Bunnag *et al.* (2012) carried out genetic transformation of *Citrus sinensis* L by *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA 1305.1) with antisense *ACC oxidase* gene. Transgenic Citrus with ethylene resistance trait developed by introducing an antisense *ACC oxidase* gene has been widely used in order to overcome the stress caused by ethylene (Hamilton *et al*., 1990; Oeller *et al*., 1991).

2.7 Phloem specific promoter

In plant transformation studies, constitutive promoters like CaMV35S promoter (Odell *et al.,* 1985), FMV (Figwort mosaic virus) promoter (Maiti *et al.,* 1997), *Agrobacterium tumefaciens* Ti plasmid mannopine synthetase promoter (DiRita and Gelvin, 1987) are commonly used to target gene expression throughout the plant. In certain cases constitutive expression of a transgene may not be necessary, especially in cases where gene expression in a particular tissue and/or organ is necessary (Guo *et al*., 2004). Targeting transgenes in vascular organ is sufficient to express defense related protein which could particularly confer resistance to pathogens that attacks the vascular tissues. AtSUC (*Arabidopsis thaliana* sucrose H+ symporter) has been described to be a phloem loading transporter (Sauer and Stolz, 1994) and has been observed to target phloem specific gene (Truernit and Sauer, 1995; Zhao *et al*., 2004). Interest in phloem specific promoter for the engineering of transgenic plants has been increasing in recent years (Graham *et al*., 1997; Nagadhara *et al.,* 2003; Saha *et al.,* 2006; Sadeghi *et al*., 2007; Liu and Liu, 2008; Benyon *et al*., 2013; Krapp *et al*., 2014; Miyata *et al*., 2017).

Dutt *et al*. (2012) transformed *Citrus aurantifolia* with constructs that contained phloem specific promoter to drive the expression of *GUS* gene. There are several promoters which drives phloem-specific gene expression. *Agrobacterium rhizogenes rolC* promoter (Schmulling *et al*., 1989) is a strong bacterium-derived phloem promoter. Singer *et al.* (2011) isolated two similar, but distinct, alleles of the *Citrus sinensis sucrose synthase-1 promoter (CsSUS1p)* and inserted them upstream of the β- glucuronidase (GUS) gene to test their ability to drive expression in the phloem of transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. Both promoter variants were capable of conferring localized GUS expression in the phloem. Therefore the use of promoters preferentially expressed in specific plant tissues is a desirable strategy to search for resistance for pathogens that colonize these tissues.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial transformation of pBI121-AtSUC-GUS in *Agrobacterium*

In the present investigation, *Agrobacterium* strain LBA4404 was used. The already cloned pBI121-AtSUC-GUS containing *gus* as a reporter gene driven by phloem specific promoter and *nptII* as a selectable marker gene was used (Fig. 1).

3.1.1 Preparation of LBA4404 electro-competent cell

For the preparation of the electro-competent cells of LBA4404 strain, a single colony of LBA4404 strain was inoculated into 10ml of LB (Appendix I) medium containing kanamycin (50mg/L) and rifampicin (10mg/L) as a starter culture. The culture was incubated at 28°C overnight. Next day 3ml of starter culture was inoculated to 10ml of liquid LB (Appendix I) containing kanamycin (50mg/L) and rifampicin (10mg/L) and allowed to grow at 28°C until the OD600 0.3-0.4 was attained. The cells were harvested by centrifugation at 4500 rpm at 4°C. The pellet was resuspended in 4ml of ice cold 10% glycerol. The cells were harvested by centrifugation at 4500 rpm at 4°C. The pellet was finally resuspended in 10% glycerol by gentling swirling. 200µl of cells were aliquoted and stored at -80°C.

3.1.2 Bacterial transformation of pBI121-AtSUC-GUS into electro-competent LBA4404 strain

In 200µl of electro-competent LBA4404 cells 1µl (100ng/µL) plasmid DNA was added and transferred into an electrocuvette. Electroporation was performed in MicroPulse^{TM} (BioRad, USA) as per the manufacturer's instruction. In the electrocuvette 1ml of Mgl medium (Appendix II) was added, mixed properly and transferred to an eppendorf tube and cultured at 28°C for 2h. 100µl of the culture was spread on Mgl plate containing kanamycin (50mg/L) and rifampicin (10mg/L) and incubated at 28°C for overnight.

Fig. 1

3.1.3 Isolation of plasmid pBI121-AtSUC-GUS from the transformed colonies of LBA4404

Single transformed colony of LBA4404 was picked up and grown in 3ml of Mgl (Appendix II) broth containing kanamycin (50mg/L) and rifampicin (10mg/L). Plasmid DNA was isolated from transformed colonies of LBA4404 using alkaline lysis method as described by Sambrook *et al.* (1989). The cells were harvested by centrifugation at 10,000 rpm for 2 min at 4°C. The supernatant was discarded and the cells were resuspended in 200µl of alkaline lysis solution I and kept on ice for 5min. 400µl of alkaline solution II was added, mixed properly and kept on ice for 10 min. 300µl of alkaline lysis III was added and mixed well by gently inversion and kept on ice for 5min. The composition of solution I, II and III are present in Appendix III. The mixture was centrifuged at 12,000 rpm for 10 min and supernatant was collected. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently. The mixture was then centrifuged at 12,000 rpm for 10min and aqueous phase was collected. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the aqueous phase and centrifuged at 12,000 rpm for 10 min at 4°C. The aqueous phase was collected and isopropanol was added in equal amount and kept at 20°C for 1h. The plasmid DNA was pelleted by centrifugation at 15,000 rpm for 15min at 4°C. The pellet was washed with 70% ethanol and air dried. The pellet was dissolved in 20µl of TE buffer (100mM Tris-HCl, 1mM EDTA, pH 7.4) containing 1µl RNAse (10mg/ml). It was incubated at 37°C for 20min. The plasmid DNA was stored at -20°C.

3.1.4 Polymerase chain reaction of pBI121-AtSUC-GUS using gene specific primer

The isolated plasmid DNA was quantified and the purity was estimated with the help of Nanodrop spectrophotometer. To confirm the presence of the isolated plasmid pBI121-AtSUS-GUS PCR was carried out using AtSUS promoter specific primer (Table 1). PCR amplification was performed with (100ng/µl) of plasmid DNA as template in a 20µl action using Eppendorf thermocycler. The reaction mixture consisted of 2µl 10XTaq buffer, 0.5µl of dNTPs (10mM), 0.5µl (10µM) forward primer, 0.5µl (10µM) reverse primer, 0.2µl *Taq* DNA polymerase (5U), 2µl plasmid DNA (100ng/µl) and molecular biology grade water was used to make up the volume of master mix to 20µl. The PCR assay is presented in Table 1.

Table 1

3.2 *Agrobacterium* mediated genetic transformation of Khasi mandarin

Agrobacterium strain LBA4404 harboring pBI121-AtSUC-GUS was used for genetic transformation of Khasi mandarin.

3.2.1 Material

Citrus reticulata cv. Khasi Mandarin was used as a source of plant material. Fruits of Khasi Mandarin were obtained from Citrus Research station, AAU, Tinsukia (Plate 1). Seeds were extracted and used for *in vitro* generation of explants (Plate 2).

3.2.2 Establishment of explants under *in vitro* conditions

The seeds of *Citrus reticulata* cv. Khasi mandarin were extracted from fresh fruit, soaked in distilled water for 3 hours and then dried in room temperature. The seed coat was removed. The seeds were surface sterilized with 70% ethanol for 2min followed washing three times with sterilized distilled H_2O followed by 0.1% Hg_2Cl for 3min and washing with sterilized distilled H_2O for three times. The seed were blot dried and inoculated on basal MS medium (Murashige and Skoog medium, 1962) (Appendix IV) (Plate 3). The plates were cultured at 28°C for 4 weeks in dark (Plate 4) followed by transferred to jar bottles and maintained at 16h light/ 8h dark for another two weeks (Plate 5). Six weeks old *in vitro* regenerated zygotic seedlings were used as explants (Plate 6).

3.2.3 Preparation of *Agrobacterium tumefaciens* culture for co-cultivation

The *Agrobacterium* strain LBA4404 harbouring plasmid pBI121-AtSUC-GUS was grown in broth Mgl (Appendix II) medium containing kanamycin (50mg/L) and rifampicin (10mg/L) for 2 days at 28°C. After two days the 2ml of primary culture was sub cultured in 48 ml Mgl containing kanamycin (50mg/L), rifampicin (10mg/L) and acetosyringone (100µM) and grown at 28°C until the optical density OD600 reached 0.3. The cells were harvested by centrifugation at 5000g for 6 min at 25°C. The pellet was resuspended in CM medium (Table 2) containing 100µM acetosyringone. The *Agrobacterium* suspension was then used for co-cultivation.

3.2.4 Explants used for genetic transformation

Six weeks old zygotic seedling was used to prepare the explants. Under microscope the explants like epicotyl, hypocotyl, node and inter-node segments were

Plate 1&2

Plate 3&4

Plate 5&6

Table 2

Plate 7&8

excised carefully (Plate 7). The excised explants were incubated in broth CM medium (Table 2) for 3h before infection with *Agrobacterium*.

3.2.5 Co-cultivation of explants with *Agrobacterium*

Excised explants were infected with *Agrobacteium* suspension for 5 min. Blot dried explants on sterile filter paper and cultured on solid CM medium for 3 days in dark at 25°C (Plate 8). After 3 days of co-cultivation explants were washed with sterilized dH_2O for 3 times at 1 min interval and final wash with timentin (200mg/L) for 1 min. Explants were blot dried on sterile filter paper.

3.2.6 Regeneration and selection of explants after co-cultivation

The explants were then transferred to regeneration (selection medium) RM II (Table 3) containing kanamycin (50mg/L) and timentin (150mg/L). Three different media were tried in the present investigation. The culture plates were maintained at 28°C in dark for 15 days. After 15 days the explants that survived in the first selection were transferred to fresh RM II medium containing kanamycin (50mg/L) and timentin (150mg/L) and transferred to light. After 3 round of selection, the survived explants were transferred to fresh RMII medium containing appropriate antibiotics as described above.

3.2.7 Multiple shoot induction from explants

After 3 round of selection the regenerated explants were transferred to shooting medium RMG (Table 4). The regenerated shoots were excised carefully along with some portion of original explant and inoculated on RMG (Table 4) shooting medium containing kanamycin (50mg/L) and timentin (150mg/L) and maintained at 28°C under continuous light for 5 weeks.

3.3 GUS histochemical assay

The putative transformants were subjected to GUS assay as described by Jefferson *et al.* (1989). The X-gluc staining solution used for the assay was prepared as mentioned in Appendix V. Transformed shoots and leaves along with non-transformed shoots and leaves were immersed in 0.1M X-Gluc solution (Appendix V). The solution was then incubated for 24 hours at 37°C in water bath. After incubation shoots and leaves were washed with 70% ethanol for several times to de-chlorophylle the tissues. The presence of blue colour staining in the phloem region was observed under microscope and documented.

Table 3&4

CHAPTER IV

EXPERIMENTAL FINDINGS

4.1 Bacterial transformation of pBI121-AtSUC-GUS in *Agrobacterium*

4.1.1 Transformation of plasmid pBI121-AtSUC-GUS into *Agrobacterium* strain LBA4404

The binary plasmid pBI121-AtSUC-GUS was mobilized into *Agrobacterium tumefaciens* strain LBA4404 through electroporation and transformed colonies were observed in the Mgl plate containing kanamycin (50mg/L) and rifampicin (10mg/L) antibiotics whereas in control plates no colonies were observed (Plate 9).

4.1.2 Isolation of plasmid pBI121-AtSUC-GUS from the transformed colonies of LBA4404

Plasmid pBI121-AtSUC-GUS was isolated from transformed colonies of LBA4404 using alkaline lysis method as described by Sambrook *et al.* (1969). The plasmid DNA was resolved in 1% agarose gel (Plate 10).

4.1.3 Polymerase chain reaction of pBI121-AtSUC-GUS using gene specific primer

The construct (pBI121-AtSUS-GUS) used in the current study was subjected to PCR analysis for confirming the presence of the AtSUC promoter using AtSUC specific primers. The PCR amplified product using gene specific primers were resolved in 1% agarose gel and expected size of fragment (954 bp) indicated the presence of the AtSUS promoter in the construct employed in the study (Plate 11).

4.2 *Agrobacterium* mediated genetic transformation of Khasi mandarin

4.2.1 Explants used for genetic transformation

In the present investigation, *in vitro* grown 6 week old zygotic seedlings were used to excise the explants like epicotyl, hypocotyl, node and internode segment for genetic transformation study.

A total of 100 epicotyl segments, 150 hypocotyl segments, 100 nodal and 150 inter-nodal segments were inoculated on regeneration medium supplemented with BAP (4mg/L), MES (0.5g/L) and sucrose (30g/L) (RMII). The regeneration percentage

Plate 9&10

Plate 11

Table 5

found for all the four explants were 53% for epicotyl, 58% for hypocotyl, 45% for nodal and 26% for inter-nodal segment, respectively (Table 5). Hypocotyl explants showed highest percentage of regeneration (58%) and was selected for genetic transformation study.

4.2.2 Preparation of *Agrobacterium tumefaciens* culture for co-cultivation

The *Agrobacterium* strain LBA4404 harbouring plasmid pBI121-AtSUC-GUS was grown in broth Mgl (Appendix III) medium containing kanamycin (50mg/L) and rifampicin (10mg/L) for 2 days at 28°C. In the present investigation, it was observed that sub culturing of *Agrobacterium* for 5 hours before co-cultivation (OD600 0.3) containing acetosyringone (100µM) showed better transformation efficiency in Khasi mandarin.

4.2.3 Effect of Co-cultivation duration and medium on genetic transformation of Khasi mandarin

In the present investigation, co-cultivation (CM) medium containing BAP (2mg/L), NAA (0.5mg/L), 2, 4-D (1mg/L), MES (0.5g/L) and sucrose (30g/L) having 100µM acetosyringone was found to be best medium for co-cultivation. Excised explants were infected with *Agrobacteium* for 5 min on CM medium. In order to determine the optimum duration of co-cultivation, 50 explants were subjected to two different durations (3 days and 5 days) (Table 6). Out of 50 explants subjected to 3 days of co-cultivation duration, 35 explants were transferred to regeneration medium (RMII) and 15 explants survived after 3 weeks on RMII medium containing kanamycin (50mg/L) and timentin (150mg/L). Whereas in 5 days of co-cultivation duration out of 50 explants, 10 explants were transferred to regeneration medium (RMII) and 3 explants survived after 3 weeks on RMII medium containing appropriate antibiotics. Co-cultivation duration of 3 days at 27°C was found to be optimum for *Agrobacterium* mediated genetic transformation of khasi mandarin (Table 6).

4.2.4 Optimization of kanamycin concentration for transformation

Optimization of kanamycin concentration is essential for *Agrobacterium* mediated genetic transformation of khasi mandarin using plasmid pBI121-AtSUC-GUS having *nptII* as a selectable marker gene. In the present investigation, three different concentrations of kanamycin (50mg/L, 70mg/L and 100mg/L) were used. RMII medium with no kanamycin was used as control. After 4 weeks of culture on regeneration medium it was observed that in control, there was no inhibition of shoot growth.

Table 6

Table 7

However, RMII medium containing kanamycin (100mg/L) showed 100% mortality. RMII medium containing kanamycin (70mg/L) showed 93.3% mortality and in kanamycin (50mg/L) showed 70% mortality rate (Table 7). RMII medium containing kanamycin (50mg/L) was further used as optimum concentration of kanamycin for selection of putative transformed shoots during *Agrobacterium* mediated genetic transformation of Khasi mandarin.

4.3 *In vitro* regeneration study

4.3.1 Optimization of Regeneration medium

In the present investigation, three different regeneration medium were evaluated. The basis of selection of the regeneration medium was based on Dutt *et al.* (2009) work with slight modification (Table 3).

In order to determine the best medium for regeneration, 100 explants were inoculated on each regeneration medium (Table 8). Regeneration efficiency was found to be 10% for RMI, 45% for RMII and 15% for RMIII (Table 8). Based on the observation RMII was selected as regeneration medium in the current study.

4.3.2 Multiple shoots induction from explants

The frequency of multiple shoot induction was found to be highest (6%) in RMG medium supplemented with BAP (4mg/L), GA3 (0.5mg/L), MES (0.5g/L) and Sucrose (30g/L) containing kanamycin (50mg/L) and timentin (150mg/L). The basis of the selection of this medium was based on Dutt *et al.* (2009) work with slight modifications. RMG medium showed highest shoot proliferation (3 shoots/explants) within 5 weeks of culture (Table 9).

4.4 Regeneration and selection of explants after co-cultivation

The RMII which was found best for shoot induction was used as regeneration (selection) medium for culturing explants after 3 days of co-cultivation with *Agrobacterium* on CM medium supplemented with acetosyringone (100µM). Regeneration (selection) medium RMII was supplemented with kanamycin (50mg/l) and timentin (150mg/l). Out of 400 explants 280 explants that were survived in 1st selection (Plate 12) cycle of 15 days in dark were transferred to fresh RMII medium containing appropriate antibiotics and transferred to light for 2nd selection (Plate 13). In 2nd selection 150 explants survived, the survived green shoots were transferred to fresh RMII medium containing kanamycin (50mg/l) and timentin (150mg/l) for 3rd round of

Table 8

Table 9

Plate 12

Plate 13

Plate 14

Plate 15

Plate 16

selection (Plate 14). In 3rd selection 90 explants survived. The regeneration efficiency was calculated after 3 round of selection and was found to be 18% (Table 10). After three rounds of selection the regenerated shoots were excised along with some portion of the original explants and cultured on RMG medium supplemented with BAP (4mg/L), GA3 (0.5mg/L), MES (0.5g/L), sucrose (30g/L), kanamycin (50mg/L) and timentin (150mg/L) for multiple shoot induction. Out of 30 regenerated explants survived in RMG medium 19 explants induced multiple shoot proliferation. Highest shoot proliferation of 3 shoots/explants was found within 5 weeks of culture (Plate 15 and Plate 16).

4.5.1 Transgenic detection through GUS histochemical assay

Putative transformed shoots and leaves along with control shoots and leaves were subjected to GUS assay. The hypocotyl shoots and leaves showed GUS expression in the phloem region (Plate 17). In the present investigation, transformation efficiency of 0.6% was observed for *Citrus reticulata* cv khasi mandarin mediated through *Agrobacterium* (Table 10).

Plate 17

Table 10

CHAPTER V

DISCUSSION

Genetic transformation allows the release of improved cultivars with desirable characteristics in a shorter period of time and therefore may be useful in citrus breeding programs. Genetic transformation of Citrus has been carried out using various methods. *Agrobacterium*-mediated transformation using epicotyl explants as target tissue for incorporation of the T-DNA (Dutt *et al*., 2009) is a most popular method of genetic transformation in citrus. There is high variation in the transformation efficiency among different citrus species, the best results being achieved in sweet oranges (*Citrus sinensis* L. Osbeck), especially the Hamlin cultivar (Mendes *et al*., 2002). Mandarins (*Citrus reticulata*) are relatively difficult citrus cultivar to transform.

Citrus genetic transformation has been preferentially obtained from explants collected from juvenile tissue primarily because of their high morphogenetic potential and low contamination rates (Munoz *et al.*, 1999). Some authors used epicotyl segments excised from seedlings germinated in the dark for 3-6 weeks and transferred to 16h light condition (Kaneyoshi *et al*., 1994; Bonde and Roose, 1998; Luth and Moore, 1999; Yang *et al*., 2000, Azevedo *et al.,* 2006). Some authors used internodal segments excised from seedlings cultivated in the greenhouse for 6-12 weeks (Pena *et al*., 1995; Dominguez *et al*., 2000) for transformation. In the present investigation four different explants were used. The explants were obtained from 6 weeks old *in vitro* grown seedling of *Citrus reticulata* cv. Khasi mandarin. The seed of Khasi mandarin were used for establishment of explants. Hypocotyl was found to be the best explant which showed higher percentage of regeneration followed by epicotyl, nodal and inter nodal segments. Dutt *et al*. (2009) found epicotyl segments to be the most effective explant for *Agrobacterium* mediated genetic transformation of Carrizo (*Citrus sinensis* Osb. x *Poncirus trifoliata* L.Raf.), Duncan (*Citrus paradisi* Macf.), Hamlin (*Citus sinensis* (L.) Osbeck) and Mexican Lime (*Citus aurantifolia* Swingle) cultivars. Cervera *et al*. (1998) reported that mature internode segments obtained from greenhouse-grown plants were found to be the best explants for genetic transformation of Citrange (*Citrus sinensis* x *Poncirus trifoliata*). Dutt and Grosser (2011) used embryogenic cell suspension culture for *Agrobacterium* mediated genetic transformation of *Citrus sinensis* 'Hamlin', 'Valencia' and 'OLL8' and *Citrus reticulata* 'Ponkan' and 'W. Murcott' (tangor).

In the current study, the *Agrobacterium* strain LBA4404 harbouring plasmid pBI121-AtSUC-GUS was grown in broth Mgl medium containing kanamycin (50mg/L) and rifampicin (10mg/L) for 2 days at 28°C. In the present study, OD600 0.3 was found to be optimum for genetic transformation. Similarly Dutt *et al*. (2009) also reported OD600 0.3 to be optimum for genetic transformation of Carrizo (*Citrus sinensis* Osb. x *Poncirus trifoliata* L.Raf.), Duncan (*Citrus paradisi* Macf.), Hamlin (*Citus sinensis* (L.) Osbeck) and Mexican Lime (*Citus aurantifolia*). The correlation between OD600 and transformation efficiency has also been reported in almond (Archilletti *et al*., 2001), tea (Mondal *et al*., 2001) and chestnut (Corredoira *et al*., 2005). In the present investigation, sub culturing of *Agrobacterium* for 5 hours until the optical density reaches to OD600 0.3 with the addition of acetosyringone (100μM) lead to improved transformation efficiency of Khasi mandarin. Addition of acetosyringone, a potent inducer molecule results in the activation of *vir* gen (Stachel *et al*., 1985). Similar effect of sub culturing was also reported by Dutt *et al*. (2009).

The other important factors in the genetic transformation of citrus are the period of inoculation of explants with *Agrobacterium*, the co-cultivation medium and duration of co-cultivation. In the present investigation, submergence of explants in the *Agrobacterium* cell suspension for 5 min followed by co-cultivation for 3 days was found to be optimum for genetic transformation of Khasi mandarin. An overgrowth of the *Agrobacterium* after three days of co-cultivation was observed similar to that reported by Cervera *et al.* (1998b). Pena *et al*. (2004) found co-cultivation for 3 days optimum for genetic transformation of Carrizo citrange. Dutt *et al.* (2011) used cell suspension culture for *Agrobacterium* mediated genetic transformation of *Citrus sinensis, Citrus reticulata* cv Ponkan and found co-cultivation for 5 days optimum for efficient transformation. In the current study, MS medium supplemented with BAP (2mg/L), NAA (0.5mg/L), 2, 4-D (1mg/L) was found to be best co-cultivation medium for Khasi mandarin. Almeida *et al*. (2003) reported that for *Citrus* genetic transformation, temperatures between 26-28°C is optimum temperature for co-cultivation. In our study, co-cultivation of explants at 27°C was found to be best for Khasi mandarin. However, few studies showed that best temperature for co-cultivation was 22°C for *Phaseolus acutifolius* and *Nicotiana tabacum* (Dillen *et al*., 1997) and 22-24°C for *Lycopersicum esculentum* (Costa *et al*., 2000).

In the present investigation, modified MS medium supplemented with BAP (4mg/l), MES (0.5mg/l), showed highest percentage of regeneration (18%). Similar result was reported by Dutt *et al.* (2009) where stable transgenic lines were produced when explants were cultured on modified MS medium supplemented with BAP (4.4μM) and MES (0.5mg/l). Mendez *et al.* (2002) reported similar hormone level to be optimum for Hamlin transformation. Azevedo *et al.* (2006) used epicotyl segments of Rangpur lime (*Citrus limonia* Osbeck) for genetic transformation and found explants cultured on regeneration EME medium supplemented with sucrose (25 g/L) and BAP (2.0 mg/L) showed higher frequency of regeneration.

MS medium supplemented with BAP (4mg/L), GA3 (0.5mg/L), MES (0.5g/L) and sucrose (30g/L) containing kanamycin (50mg/L) and timentin (150mg/L) showed higher percentage of shoot proliferation and highest number of shoots development (3 shoots/explants) in the current study. Kitto and Young, (1981) found BAP (5mg/L) optimum for multiple shoot inductions in Carrizo citrange (Washington navel x *Poncirus trifoliata* L. Raf). Moore, (1986) reported highest multiple shoot induction with 22μM BAP in Carrizo citrange (*C. sinensis* L. Osbeck X *Poncirus trifoliata* L. Raf.) and Cleopatra Mandarin (*C. reshni* Hort. cv. Tanaka). MS medium supplemented with 1μM BAP and 2.89 μM GA3 was reported to be best for multiple shoot induction by Dutt *et al*. (2009). Pena *et al.* (2004) found BAP (3mg/L) to be most effective, for multiple shoot induction in Carrizo citrange (*Citrus sinensis* (L.) Osbeck X *Poncirus trifoliata* (L.) Raf). Almeida *et al.* (2003) carried out genetic transformation of Valencia, Natal sweet oranges (*Citrus sinensis* L. Osbeck) and Rangpur lime (*Citrus limonia* L. Osbeck) using epicotyls segments as explants and found EME medium supplemented with GA3 (1mg/L) showed highest number of multiple shoot induction.

In the present study, the effect of various concentration of kanamycin (50, 70, 100 mg/L) were tried to determine the appropriate dose of antibiotic for regeneration of putative transformed shoots during three round of selection. Kanamycin concentration (70 mg/L) and (100mg/L) resulted in 93.3% and 100% mortality rate, respectively. Kanamycin (50mg/L) was found to be optimum for selection of putative transformed shoots of Khasi mandarin. Dutt *et al*. (2009) reported that kanamycin (70mg/L) was found optimum for selection of transformed shoots in Carrizo (*Citrus* *sinensis* Osb. x *Poncirus trifoliata* L.Raf.), Duncan (*Citrus paradisi* Macf.), Hamlin (*Citus sinensis* (L.) Osbeck) and Mexican Lime (*Citus aurantifolia* Swingle) using epicotyl as explant. Kanamycin (100mg/L) found to be best for selection of transformed shoots of Rangpur Lime (*Citrus limonia* Osbeck) (Azevedo *et al.*, 2006). Thus the concentration of kanamycin for selection of transformed shoots might be varied from species to species and type of explants used for genetic transformation.

Strong transgene expression without tissue discrimination can result in unnecessary energy expenditure by the plant and possible side effects due to putatively harmful transgene products (Altipeter *et al*., 2005; Wang *et al*., 2005; Zhao *et al*., 2004). One method to reduce such energy expenditure is to use tissue-specific promoters (Barbosa-Mendes *et al*., 2009; Dutt *et al.*, 2012; Miyata *et al*., 2012; Benyon *et al*., 2013; Attilio *et al*., 2013; Zou *et al*., 2014). Currently, one of the most commonly used types of phloem-specific promoters in plant biotechnology are those derived from homologs of genes encoding sucrose synthase (SUS), such as AtSUS1 from *Arabidopsis* (Martin *et al*., 1993). In the present investigation, phloem specific promoter AtSUC was used to drive the expression of GUS gene. “Tristeza’’ disease is the most devastating disease of the citrus cause by citrus tristeza virus (CTV). The virus is readily transmitted with infected buds and spread locally by several aphid species in a semi-persistent mode (Bar-Joseph *et al*., 1989). Citrus tristeza virus (CTV) is phloem restricted in natural citrus hosts. The 23-kDa protein (p23) encoded by the virus is an RNA silencing suppressor and a pathogenicity determinant.

Huanglongbing (HLB) disease commonly known as citrus greening is also highly destructive disease of Citrus (Bove, 2006). Huanglongbing affects all cultivated citrus varieties and cannot be currently controlled due to a lack of resistant cultivars. Three species of Gram negative phloem-limited bacteria *Candidatus* Liberibacter are associated with this disease (Bove, 2006). Because *Candidatus* Liberibacter, the Gram-negative bacterium associated with HLB, infects only the phloem tissue, it may be advantageous to limit transgene expression to the vascular tissue and reduce expression in the fruit. Transgenic solutions are being widely explored to develop huanglongbing (HLB) resistance in citrus. Liberibacter-free transgenic plants were developed using *Agrobacterium tumefaciens* with three binary vectors containing the *uid*A gene under control of three different phloem-specific promoters: AtPP2 (*Arabidopsis thaliana* phloem protein 2), AtSUC2 (*A. thaliana* sucrose transporter) and CsPP2 (*Citrus* phloem protein 2) (Miyata, 2009). In the present investigation, *GUS* gene expression driven by phloem specific promoter (AtSUC) was observed in transgenic hypocotyl shoots and transgenic leaves. Similarly Dutt *et al.* (2012) reported GUS expression driven by AtSUC2 promoter in phloem tissues of 6-week-old transgenic 'Mexican' lime stem cross-section.

Several reports have demonstrated a strong influence of the genotype in Citrus organogenesis (Gutierrez *et al.,* 1997, Vila *et al*., 1989 and Ghorbel *et al.,* 1998) and genetic transformation (Pena *et al*., 1995 and Moore *et al*., 1992) suggesting the need for optimizing species specific *in vitro* regeneration as well as genetic transformation protocols. This work was an attempt to optimize an *in vitro* regeneration and transformation protocol for *Citrus reticulata* cv. Khasi mandarin.

CHAPTER VI

SUMMARY AND CONCLUSION

Citrus reticulata cv. Khasi mandarin was utilized for the present investigation with following objectives:

1. Optimization of different explants for genetic transformation of *Citus.*
2. Molecular characterization of transgenic *Citrus* for presence and expression of transgene.

In *Agrobacterium* mediated genetic transformation, *Agrobacterium* strain LBA4404 harbouring the plasmid pBI121-AtSUC-GUS containing *nptII* as a selectable marker gene and GUS as a reporter gene driven by phloem specific promoter (AtSUC) was used.

The salient findings of the present study are:

- The protocol for *Agrobacterium tumefaciens* mediated genetic transformation of *Citrus reticulata* cv. khasi mandarin was optimized.
- Hypocotyl was found to be the best explants for Khasi mandarin transformation and regeneration.
- Modified MS medium supplemented with BAP (2mg/L), NAA (0.5mg/L), 2, 4-D (1mg/L), MES (0.5g/L), sucrose (30mg/L) and acetosyringone (100µM) was found to be the best medium for co-cultivation.
- Optical density OD600 0.3 of *Agrobacterium* cell suspension was found best for co-cultivation of explants for genetic transformation of Khasi mandarin.
- Co-cultivation duration of 3 days was found optimum for Khasi mandarin transformation.
- Highest regeneration efficiency of 18% was observed in RMII medium {modified MS medium supplemented with BAP (4mg/L), MES (0.5g/L), sucrose (30g/L), kanamycin (50mg/L) and timentin (150mg/L)}.

- Highest multiple shoot induction (6%) was observed in RMG medium {modified MS medium supplemented with BAP (4mg/L), GA3 (0.5mg/L), MES (0.5g/L), sucrose (30g/L), kanamycin (50mg/L) and timentin (150mg/L)}.

- Regeneration and selection medium containing kanamycin concentration (50mg/L) showed best result for selecting putative transformed shoot during *Agrobacterium* mediated genetic transformation of *Citrus reticulata* cv Khasi mandarin.

- GUS expression in the phloem tissues was observed in the transgenic shoots and leaves with transformation efficiency of 0.6%.

CONCLUSION

In the present investigation an *in vitro* regeneration and transformation protocol for *Citrus reticulata* cv. Khasi mandarin have been optimized. Phloem-specific promoters are becoming essential for use in a range of applications, such as conferring resistance to phloem-limited pathogens and phloem-feeding insects. Citrus tristeza virus (CTV) is a phloem- limited virus whose natural host range is restricted to citrus and related species."Tristeza" disease is the most devastating disease of the citrus cause by citrus tristeza virus (CTV), causes phenomenal economic damage to citrus industry. The use of phloem specific promoter in citrus will be very beneficial for introduction of resistance genes in the Citrus genome.

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APPENDIX I

Composition of LB medium

Ingredients	Per litre
Tryptone	10
Yeast extract	5
NaCl	10
pH	7

For solidification: Agar (15g/L)

APPENDIX II

Composition of Mgl Medium

Chemicals	**For 1L**
Mannitol	5g
L- Glutamic acid	1g
KH_2PO_4	0.25g
NaCl	0.1g
$MgSO_4.7H_2O$	0.1g
Tryptone	5g
Yeast extract	25g
Biotin	10µl (Stock: 0.1 mg/ml)
pH	7.0 adjusted using 1N KOH and 1N HCl

For solidification: Agar (15g/L)

APPENDIX III

A. Preparation of Alkaline lysis solutions

1. Alkaline lysis solution I

    50 mM glucose

    25 mM Tris-Cl

    10mM EDTA

2. Alkaline lysis solution II

    0.2N NaOH

    1% SDS

3. Alkaline lysis solution III

    5mM potassium acetate

    Glacial acetic acid

B. RNase A preparation

10 mg of pancreatic Ribonuclease A (Sigma, USA) was dissolved in 1ml of 10 mM Tris-HCl, 15 mM NaCl and pH 7.5. The mixture was incubated in a water bath (100°C) for 15 min. After 15 min incubation, cooled at room temperature and stored at -20°C.

APPENDIX IV

Composition of MS medium (Murashige and Skoog, 1962)

Chemical	Final conc. per L of MS medium	Stock/250ml	Quantity to be dispensed for 1 L of MS medium
NH_4NO_3	1.65 g	16.5 g	25 ml
KNO_3	1.90 g	19 g	
$MgSO_4.7H_2O$	0.37 g	3.70 g	
KH_2PO_4	0.170 g	1.70 g	
$CaCl_2$	0.440 g	4.4 g	25 ml
$MnSO_4$	22.3 mg	223 mg	25 ml
$ZnSO_4$	8.60 mg	86 mg	
H_3BO_3	6.2 mg	62 mg	
KI	0.83 mg	8.3 mg	
Na_2MoO_4	0.25 mg	2.5 mg	
$CuSO_4$	0.025 mg	0.25 mg	
$CoCl_2.6H_2O$	0.025 mg	0.25 mg	
FeEDTA	40 mg	1 g	10 ml
Nicotinic acid	0.5 mg	12.5 mg	10 ml
Pyridoxine	0.5 mg	12.5 mg	
Thiamine	0.1 mg	2.5 mg	
Glycine	2.0 mg	50 mg	
Myo-inositol	100 mg Added as solid		
Sucrose	30.0 g Added as solid		
pH	5.8 Adjust with 1N KOH and 1N HCl		

For solidification: Phytagel (4g/L)

APPENDIX V

Composition of 0.1 M X-gluc staining solution

Stock solution	Volume per 1 ml of staining buffer	Final concentration
1 M sodium phosphate (pH 7.0)	100µl	0.1 M
0.5 M EDTA (pH 8.0)	20µl	10 mM
50 mM K3Fe (CN)6	20µl	1 mM
10% triton X-100	10µl	0.1% (v/v)
0.1 M X-gluc (50mg/ml) in dimethylformamide	20µl	2 mM
Sterile water, nuclease- free	830µl	-

Table 1. Nucleotide sequence of primers and PCR assay profile used in the current study

Sl No.	Promoter used	Forward primer and Reverse primer (5′ – 3′)	Size of the amplicon (bp)	PCR Profile
1.	AtSUC2	AT-F AAGCTTGCAAAATAGCACACCATTTATG AT-R AGGATCCTTTGACAAACCAAGAAAGTAAG	954	94°C for 1 min: 1 cycle 94°C for 30 sec: 64°C for 30sec: } 30 cycle 72°C for 1.30 min: 72°C for 5 min:

Table 2. Composition of co-cultivation (CM) medium used in the present study

MS	6-Benzylaminopurine (mg/L)	Napthalene acetic acid (mg/L)	2, 4-Dichlorophenoxyacetic acid (mg/L)	Sucrose (g/L)	MES (g/L)	Acetosyringone (µM)
MS Basal	2	0.5	1	30	0.5	100

pH=5.8, Phytagel 4gm/L

Table 3. Composition of modified MS medium used for regeneration of shoots from different explants

Medium	BAP (mg/L)	NAA (mg/L)	Kinetin (mg/L)	MES (g/L)	Sucrose (g/L)
RMI	1	0.5	Nil	0.5	30
RMII	4	Nil	Nil	0.5	30
RMIII	1	0.5	2	0.5	30

pH= 5.8, Phytagel (4g/L)

Table 4. Composition of modified MS medium used for shoot multiplication

Medium	BAP (mg/L)	GA3 (mg/L)	MES (g/L)	Sucrose (g/L)
RMG	4	0.5	0.5	30

pH= 5.8, Phytagel (4g/L)

Table 8. Regeneration efficiency of different media used in the current study

Media	No. of explants inoculated	No. of explants regenerated	Regeneration efficiency (%)
RMI	100	10	10
RMII	100	45	45
RMIII	100	15	15

Table 5. ***In vitro*** **regeneration efficiency of different explants**

Explant	**Total no. of explants inoculated on RMII medium**	**Explants regenerated**	**Regeneration efficiency (%)**
Epicotyl	100	53	53
Hypocotyl	150	87	58
Node	100	45	45
Internode	150	40	26

Table 7. Optimization of kanamycin concentration for selection of putative transformed shoots

Sl No.	Kanamycin concentration (mg/L)	Total no. of explants used	Total no. of explants died after 4 weeks	Mortality rate (%)
1	Nil (Control)	150	30	20
2	50	150	105	70
3	70	150	140	93.3
4	100	150	150	100

Table 6. Effect of co-cultivation duration

Co-cultivation duration (days)	No. of explants co-cultivated	Explants transferred to regeneration medium	No. of explants survived after 3 weeks
3	50	35	15
5	50	10	3

Table 9. Induction of multiple shoots from different explants on RMG medium

Media	No. of explants on RMG medium	No. of explants inducing shoots after 5 weeks of culture	Average no. of shoots developed per shoot after 5 weeks of culture
RMG	30	19	3

Table 10. ***Agrobacterium*** **mediated genetic transformation of** ***Citrus reticulata*** **cv khasi mandain**

Citrus cv.	No. of explants taken for co-cultivation	No. of explants survived during co-cultivation	No. of explants survived after 1st regeneration and selection	No. of explants survived after 2nd regeneration and selection	No. of explants survived after 3rd regeneration and selection	Regeneration efficiency (%)	No. of explants survived on shooting medium	No. of multiple shoots developed after 5 weeks	No. of GUS positive transformed shoots	Transformation efficiency (%)
Khasi mandarin	500	400	280	150	90	18	30	3	3	0.6

Transformation efficiency = (No. of GUS positive transformed shoots/ Total no. of explants taken) X 100%

= 3/500 X 100 %

= 0.6 %

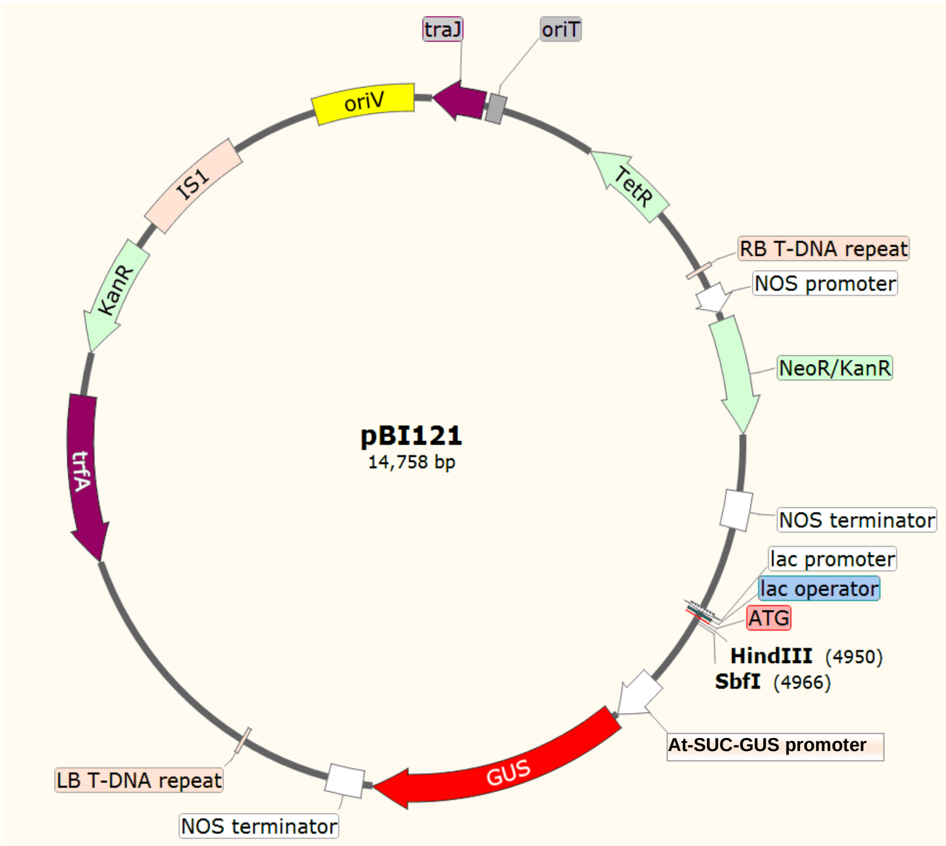

Fig. 1. Physical map of pBI121-AtSUC-GUS

Plate 1: Fresh Khasi mandarin fruits collected from Citrus research station, AAU, Tinsukia for present investigation

Plate 2: Seeds of *Citrus reticulata* cv. khasi mandarin

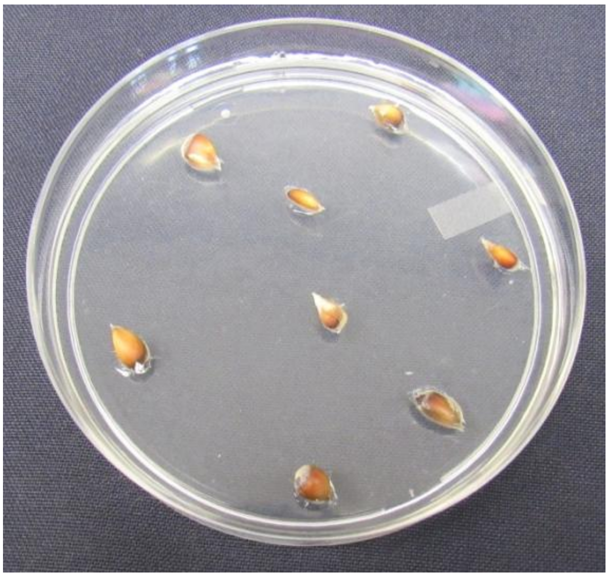

Plate 3: Seeds inoculated on MS basal medium

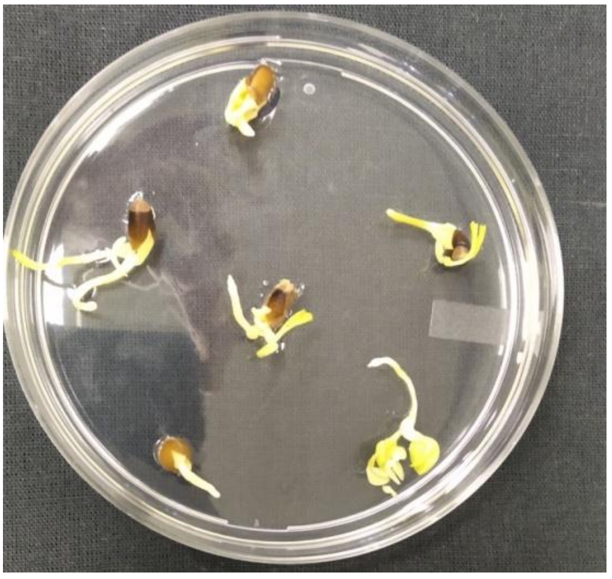

Plate 4: Germinated Khasi mandarin seeds on MS medium cultured at 28°C in dark for 4 weeks

Plate 5: Germinated seedling maintained at 16h light/8h dark for 2 weeks

Plate 6: Six weeks old Khasi mandarin seedling used for preparation of different explants for genetic transformation study

Plate 7: Different explants like epicotyl, hypocotyl, node and inter-node segments used for genetic transformation

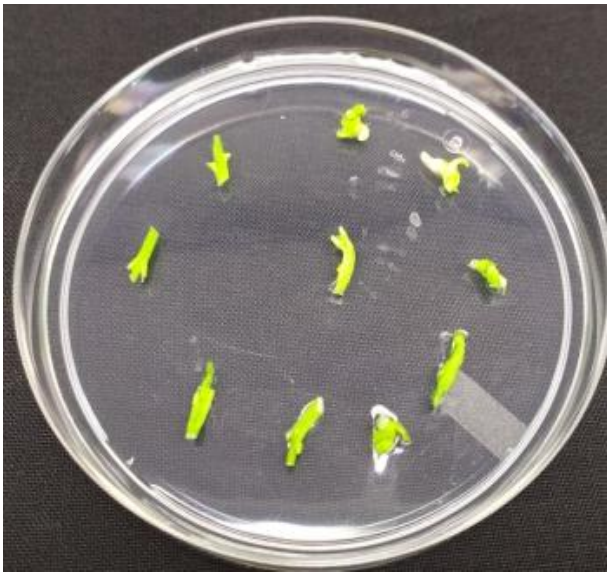

Plate 8: Co-cultivation of explants with *Agrobacterium* on CM medium (100µM acetosyringone) for 3 days at 27°C in dark

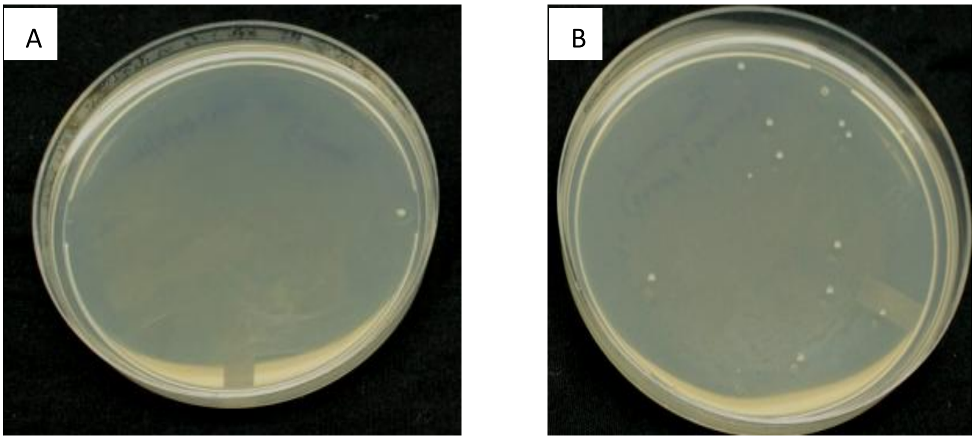

Plate 9: Bacterial transformation of plasmid pBI121-AtSUC-GUS A. Control; B. Transformed colonies of LBA4404 strain

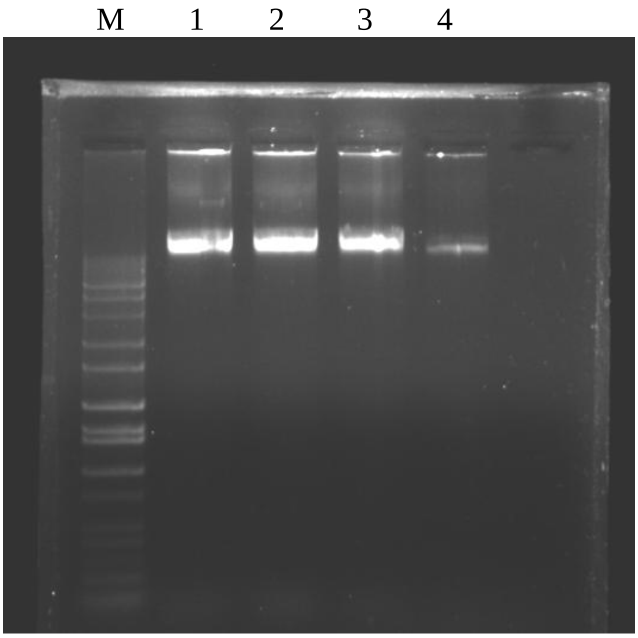

Plate 10: pBI121-AtSUC-GUS isolated from transformed LBA4404 and resolved on 0.8% agarose gel. Lane M: 1Kb ladder, Lane 1-4: Isolated plasmid DNA

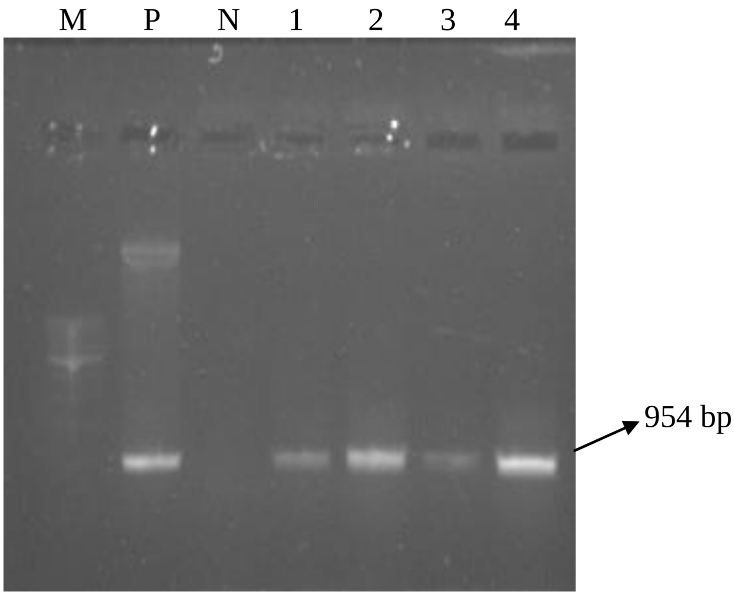

Plate 11: PCR amplification using At-SUC promoter specific primer. The amplified fragment of 954 bp resolved on 1% agarose gel. Lane M- 500bp step up ladder, Lane P: Positive control, Lane N: Negative control, Lane 1-4: Plasmid

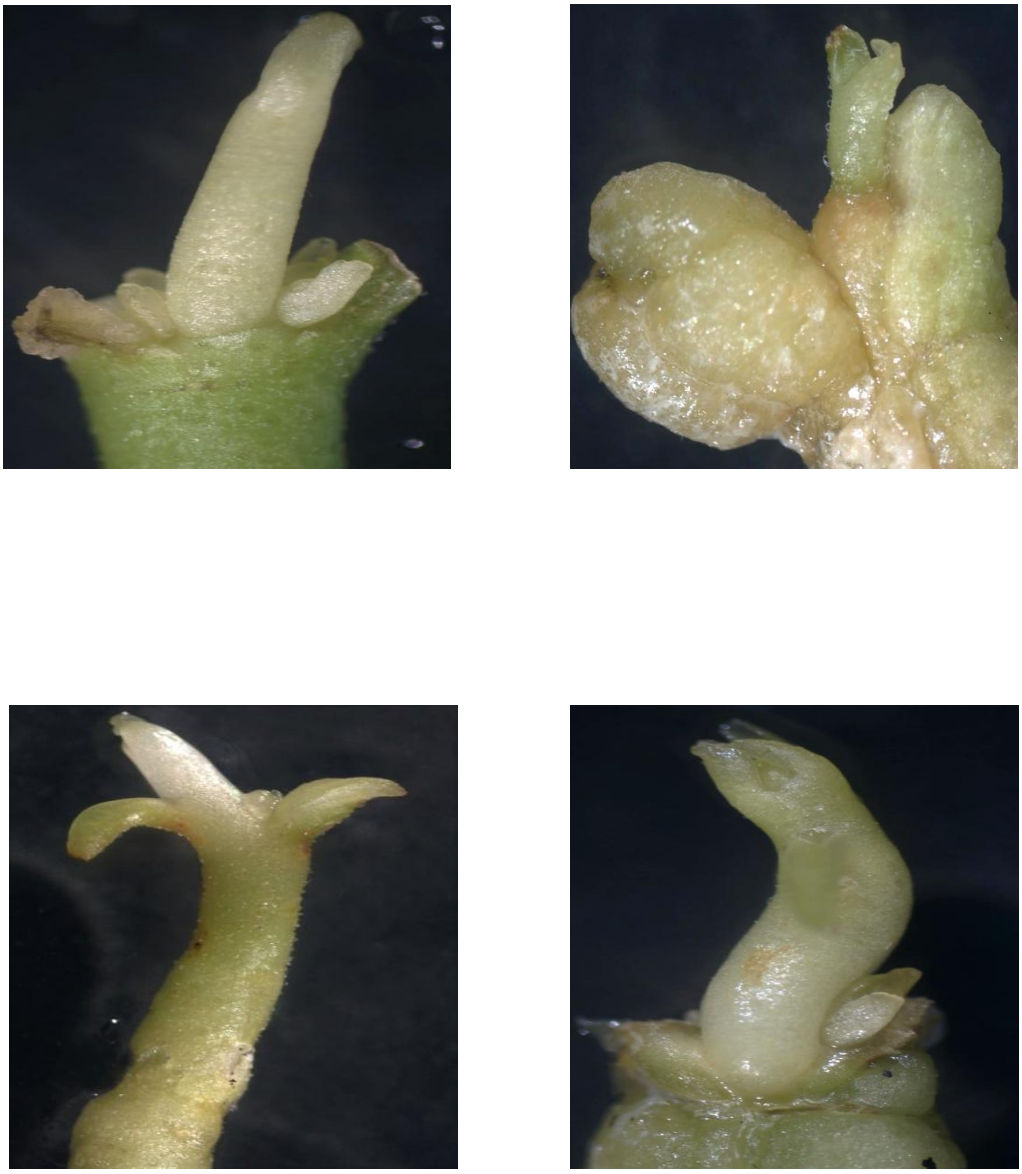

Plate 12: Regenerated shoots (50X magnification) in 1st selection after 15 days of culture on RMII medium containing kanamycin (50mg/L) and timentin (150mg/L)

Plate 13: Regenerated shoots (50X magnification) in 2nd selection after 3 weeks of culture on RMII medium containing kanamycin (50mg/L) and timentin (150mg/L)

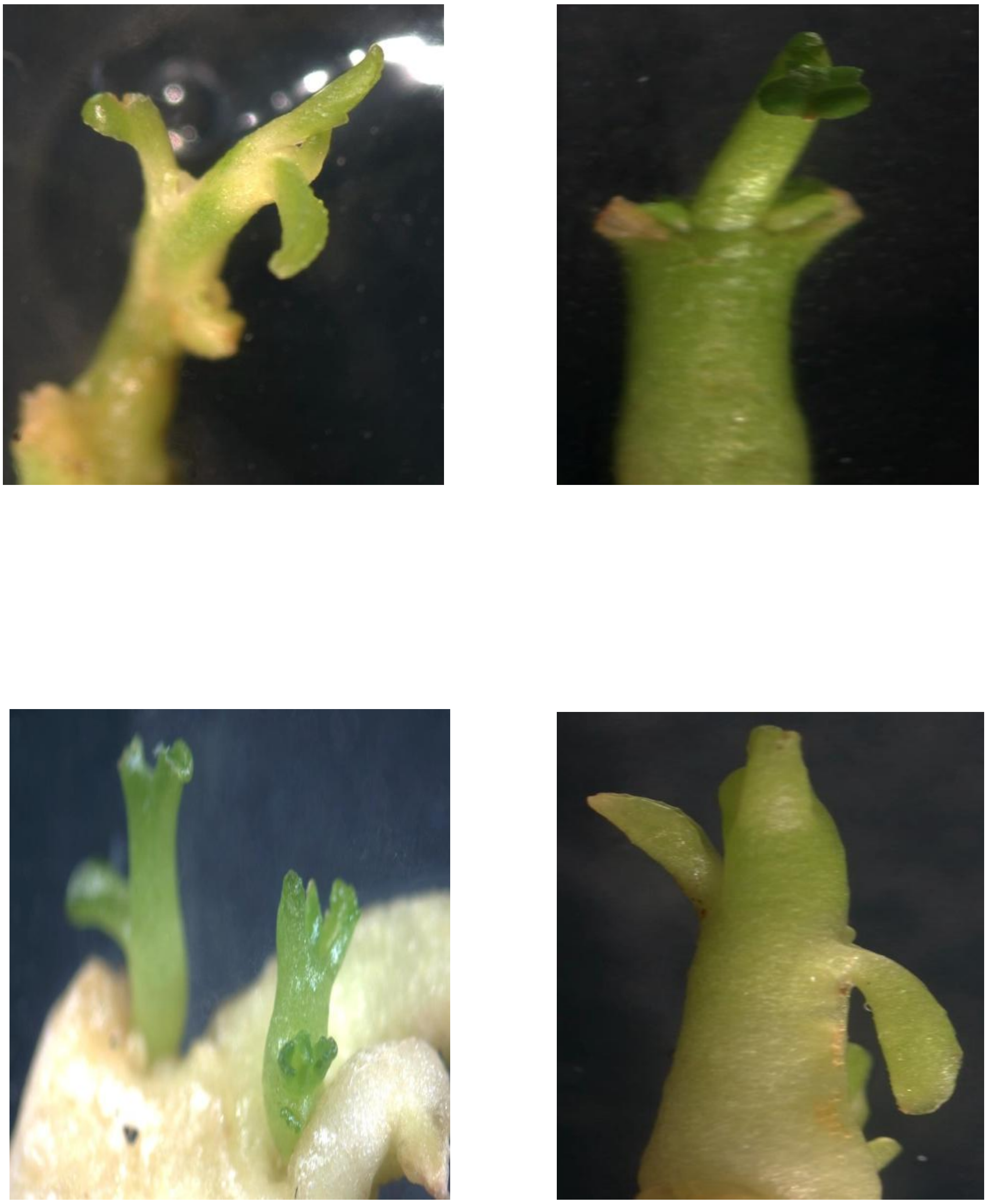

Plate 14: Regenerated shoots (50X magnification) in 3rd selection after 3 weeks of culture on RMII medium containing kanamycin (50mg/L) and timentin (150mg/L)

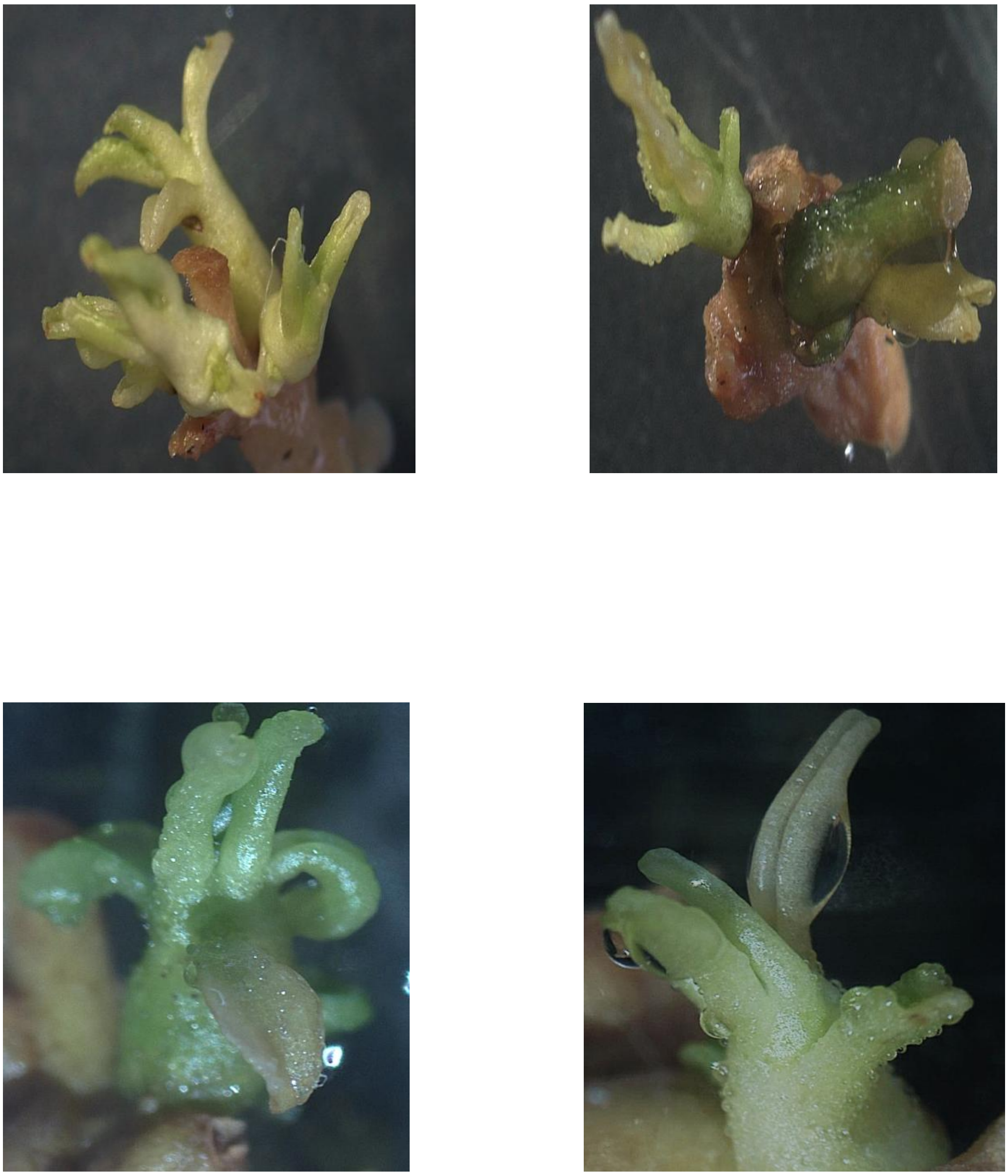

Plate 15: Putative transformed shoots (50X magnification) on shooting medium RMG containing kanamycin (50mg/L) and timentin (150mg/L) after 5 weeks of culture

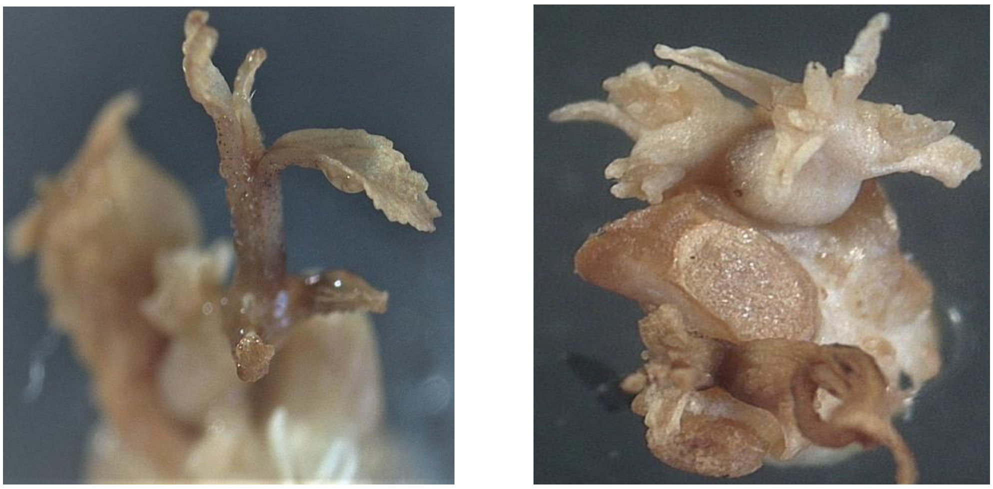

Plate 16: Non transformed shoots (50X magnification) in shooting medium RMG containing kanamycin (50mg/L) and timentin (150mg/L)

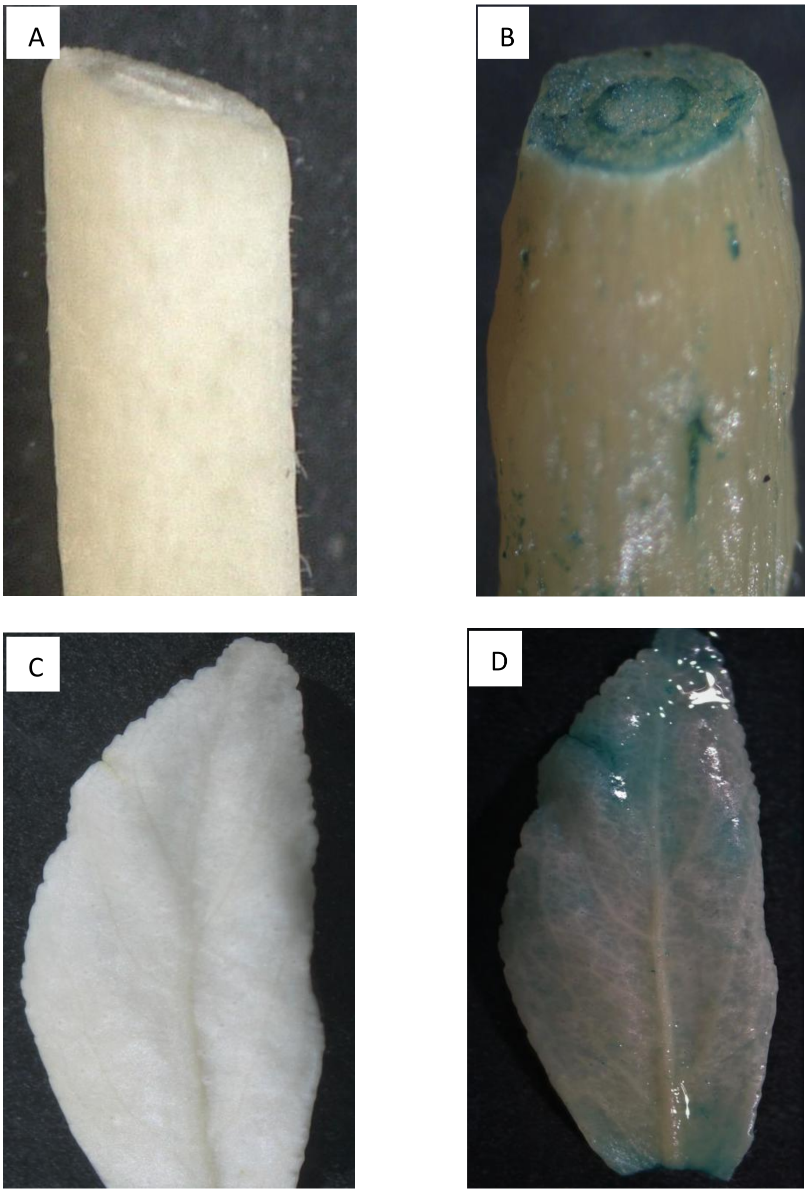

Plate 17: GUS histochemical assay. Putative transformed shoots showing expression of GUS in the phloem region (A) Control shoot, (B) Transformed hypocotyl shoot, (C) Control leaves and (D) Transformed leaves