TRANSGENIC TOMATO (*Solanum lycopersicum* Mill.) REGENERATION BY COMPARING DIFFERENT TRANSFORMATION TECHNIQUES

M.S. THESIS

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE MASTER OF SCIENCE IN BIOTECHNOLOGY

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Dedicated

to

my beloved mother

Declaration

I hereby declare that the research work representing the results reported in this thesis entitled "Transgenic tomato (*Solanum lycopersicum* Mill.) regeneration by comparing different transformation techniques**"** submitted by this thesis has been carried out by myself under supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and any part of this thesis has not been submitted to any other institution for any degree or diploma.

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List of Abbreviations

A.	*Agrobacterium*
BAP	6-benzylaminopurine
CaMV35S	Cauliflower mosaic virus 35 S
Cm	Centimeter
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic acid
G	Gram
GUS	β glucuronidase
H	hour
HKT1	High affinity potassium (K^+) transporter
hpt	Hygromycin phosphotransferase
IAA	Indole-3-acetis acid
Kb	Kilo bases
kg	Kilogram
Kin	kinetin
mg/l	Milli gram/Litre
mM	Milli mole
Ml	Milli Litre
MS	Murashige and Skoog, 1962
NaOH	Sodium Hydroxide
NAA	α-Naphthalene acetic acid

ND^+	NADPH (Nicotinamide adenine dinucleotide phosphate)
Nos	Nopaline synthetase
npt II	Neomycin phosphotransferase
OD	Optical Density
pH	-log H^+
T-DNA	Transfer DNA
Ti-plasmid	Tumor inducing plasmid
X-Gluc	5-Bromo-4-Chloro-3-indoyl-β-D-glucuronide
YEP	Yeast Extract Peptone Media
µl	Micro litre
µM	Micro mole
%	Percentage

Abstract

Production of saline-tolerant high-yielding crop has become most important in the current global scenario. Biotechnological approaches are extensively employed for introducing foreign genes into high-yielding genotypes. However, the success of genetic manipulation of a plant depends on the transformation as well as regeneration frequencies of the explants. This study presents a comparison of tissue-culture based and non-tissue-culture based transformation protocol for tomato (*Solanum lycopersicum* Mill.) using the *Agrobacterium*-mediated transformation of two different explants by three different vectors. Whole seed and cotyledonary leaves were used as explants of tomato varieties, named, BARI tomato-2, BARI tomato-3, BINA tomato-2, BINA tomato-3 and Bahar. Efficient and reproducible regeneration protocol was evaluated. Regeneration media with 2 mg/l BAP supplementation was found best for maximum number of shoot formation for all five varieties tested. For transformation, *Agrobacterium tumefaciense* strain LBA4404 harboring recombinant vectors, *pBI121*, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* having kanamycin selectable marker gene were used. The parameters optimized here include optical density, infection time, co-cultivation period and pre-culturing of explants for all the varieties. In tissue-culture based transformation of cotyledonary leaves, transformation with bacterial solution (OD_{600} 0.6-0.8) with infection time 30 min followed by 24 hrs of co-cultivation showed higher regeneration response under selection for all the varieties except BINA tomato-3 which gave its best result at OD_{600} 1.0-1.2 with 48 hrs of co-cultivation period. Regeneration frequency of putative transformants appeared more than 35% under selection though the non-transformed plants gave more than 80% without selection (control experiment). This elaborate culture procedure resulted in low transformation frequency leading to necrosis of the regenerants, which urged to switch to the non-tissue-culture based transformation. During *in planta* transformation of whole seeds, OD_{600} 1.1-1.4 with 30 minutes of incubation period and 24 hours of co-cultivation period was optimum for achieving more than 90% transformation efficiency in transient GUS expression for all the varieties. After optimization of *in planta* protocol by *pBI121*, the other vectors were employed for transformation. The vector *pK7WG2_AtNHX1_1.6* has achieved maximum percentage of regeneration

frequency over the other two vectors throughout the parameter optimization. Wounding of seeds resulted in reduction of germination percentage of infected seeds for all the tested varieties. The pre-culture period of 24 hours prior to infection were considered effective for regeneration of both explants. The process of transformant detection was carried out in three distinct ways, such as, antibiotic selection, GUS assay and leaf disc senescence assay. Kanamycin at a concentration of 50 mg/l found to be efficient for this purpose. In addition to bioassay, micropropagation of putative transformed leaves observed to regenerate during antibiotic selection confirms stable transformation. The percentage of transformation efficiency diverges with tomato genotype, explant, infecting vector and method of transformation. The *in planta* protocol ensured generation of putative transgenic plants with considerable ease in a short time and is not genotype-dependent. The putative transformed plants obtained from *in planta* transformation observed to tolerate upto 100 mM of salt. Successful transformation of tomato will be greatly aided by genotype-specific determination of crucial parameters on improving *in vitro* regeneration after transformation, followed by growth into a whole plant transformant through acclimatization to net house conditions. Further, the study offers independence to select suitable tomato variety, explant, vector and method of transformation to generate transgenic salt tolerant tomato.

Introduction

1.1 Preface of tomato plant

Tomato (*Solanum lycopersicum* Mill.) is a valuable species for studying plant biology because it allows studying the integration of the tools and concepts of genetics, molecular biology and genetic engineering for studying and manipulating all of these processes. Indeed, this vegetable is one of the most investigated crops both at genetic and genomic level not only because of its economic importance but also it is one of the best characterized plant systems. Tomato has diploid genetics, a small genome size, has a short generation time, and is easily reproduced by seed and vegetative propagation. All these characteristics make it amenable to genetic analysis. Tomato is susceptible to *Agrobacterium* infection, and is therefore amenable to current plant transformation techniques.

Origin: The species originated in the South American Andes and its use as a food originated in Mexico, and spread throughout the world following the Spanish colonization of the Americas. There are around 7,500 tomato varieties grown for various purposes (https://en.wikipedia.org/wiki/Tomato; date: 15.1.2015).

Plant description: The tomato is grown worldwide for its edible fruits, with thousands of cultivars having been selected with varying fruit types, and for optimum growth in differing growing conditions.

***Root*:** Tomato plants form different root systems depending on the method of propagation. Seed-planted tomatoes develop a taproot system, consisting of a strong central root that shoots deep into the soil and smaller lateral roots that grow out of the main root, near the top of the root. Tomatoes grown from cuttings develop a fibrous root system, comprised of a series of small, stringy roots that form a horizontal mat near the soil line (http://www-plb.ucdavis.edu/labs/rost/tomato/tomhome.html; date: 15.1.2015).

Stem: The stem forms a terminal bud at the tip and lateral branches that form a spiral pattern of nodes along the length of the stem. The stem, branches and foliage are comprised of vascular bundles that carry moisture and nutrients to the plant and growing fruit. As the tomato plant ages, the stem becomes woody and fibrous but maintains the vascular system to continue feeding the plant. Pruning changes the structure of the plant by causing an increase in lateral branch production (http://www-plb.ucdavis.edu/labs/rost/tomato/tomhome.html; date: 15.1.2015).

Flower**:** Flowers form on the branches in simple or complex patterns depending upon pruning activity. Many factors affect the number of blooms produced, including temperature, nutrients and moisture levels. Tomato flowers, pale to bright yellow in color, are typically less than one inch in diameter. Classified as perfect flowers, tomato blossoms have both male and female organs, five long petals, and the ovary that will eventually form the fruit. (https://en.wikipedia.org/wiki/Tomato; date: 15.1.2015). In order for fruit to form on the plants, pollination must be initiated, manually or through the aid of insects and wind.

Fruit**:** After successful pollination, tomato flowers fade and fall away to expose the swelling ovary. Fruits start out small, firm and bright green in color. They maintain the green coloring until the fruit has formed its final shape and size, at which point the color of the flesh and skin start to change until it reaches its final hue, indicating ripeness (https://en.wikipedia.org/wiki/Tomato; date: 15.1.2015).

Cultivated tomatoes vary in size, from tomberries, about 5 mm in diameter, through cherry tomatoes, about the same 1–2 cm (0.4–0.8 in) size as the wild tomato, up to beefsteak tomatoes 10 cm (4 in) or more in diameter. The most widely grown commercial tomatoes tend to be in the 5–6 cm (2.0–2.4 in) diameter range. Most cultivars produce red fruit, but a number of cultivars with yellow, orange, pink, purple, green, black, or white fruit are also available. Multicolored and striped fruit can also be quite striking.

Tomatoes grown for canning and sauces are often elongated, 7–9 cm (3–4 in) long and 4–5 cm (1.6–2.0 in) diameter; they are known as plum tomatoes, and have lower water content (http://allaboutgrowingtomatoes.blogspot.com/p/tomato-plant-details.html; date: 15.1.2015).

Nutrition: Tomatoes are consumed generously throughout the world. They contain the carotene lycopene, one of the most outstanding natural antioxidants and some unusual phytonutrients (http://www.whfoods.com/; date: 15.1.2015). In some studies, lycopene, especially in cooked tomatoes, has been found to help prevent cancer. Lycopene has also been shown to improve the skin's ability to protect against harmful UV rays.

A study done by researchers at Manchester and Newcastle universities revealed that tomato can protect against sunburn and help keeping the skin looking youthful (http://www.whfoods.com/; date: 15.1.2015). Natural genetic variation in tomatoes and their wild relatives has given a genetic plethora of genes that produce lycopene, carotene, anthocyanin, and other antioxidants. Tomato varieties are available with double the normal vitamin C (Doublerich), 40 times normal vitamin A (97L97), high levels of anthocyanin (resulting in blue tomatoes), and two to four times the normal amount of lycopene (numerous available cultivars with the high crimson gene) (FAOSTAT, 2014).

Genome sequencing: The researchers from the International Tomato Genome Sequencing Consortium, started sequencing of tomato genome in 2003, and created a database of genomic sequences and information on the tomato and related plants (Mueller *et al.*, 2005). A prerelease version of the genome was made available in December 2009. The genomes of its mitochondria and chloroplasts were also sequenced as part of the project. The complete genome for the cultivar Heinz 1706 was published on 31 May, 2012 in *Nature* (Sato, *et al*., 2012). Since many other fruits, like strawberries, apples, melons, and bananas share the same characteristics and genes, researchers stated the published genome could help to improve food quality, food security and reduce costs of all of these fruits.

1.2 Tomato as a model plant for genetic engineering

Manipulation of the plant genome by introducing foreign genes has become a core tool in plant biology. Targets include enhancement in productivity by increasing resistance to abiotic and biotic stresses as well as fundamental studies such as identification and characterization of key regulatory genes. Within the *Solanaceae*,

tomato is a broadly used model system for its simple diploid genetics, the short generation time, the routine protocols for production of transgenic plants, and its exceptional widely available genetic and genomic resources.
It has a relatively small genome (0.7-1.0 pg), well developed classical and molecular genetic maps and a complete genomic library in yeast artificial chromosomes. The first resistant gene (Pto) that elicits a hypersensitive response to disease resistance was cloned in tomato (Martin *et al*., 1993). The natural ability of *Agrobacterium tumefaciens* in infecting only dicotyledonous plants because of the signaling acetosyringone phenolic compound released from the wounds of the plant cells has paved the way for the researchers in attaining more understanding and a precise manner of working upon this process.

Agrobacterium mediated transformation has remarkable advantages over other transformation methods which include preferential integration of defined T-DNA into transcriptionally active regions of the chromosome. The transgenic plants are generally fertile and the foreign genes are often transmitted to progeny in a Mendelian manner (Paramesh and Fakrudin, 2010)**.**

Since the first report of *Agrobacterium*-mediated tomato transformation (McCormick *et al.*, 1986), there have been many reports of tomato being engineered for a variety of purposes, including characterization of gene function, production of insect- and disease-resistant plants, herbicide tolerance, improved fruit quality, delay in fruit ripening, production of foreign proteins and improved transformation protocol (Lin *et al*., 2004; Park *et al.*, 2003).

1.3 An overview of tomato production

About 163.9 million tonnes of tomato were produced in the world in 2013. China, the largest producer, accounted for about one quarter of the global output, followed by India and the United States. For one variety, plum or processing tomatoes, California accounts for 90% of U.S. production and 35% of world production. In 2012, tomato production was valued at 58 billion dollars and tomatoes were the eighth most valuable agricultural product worldwide. (FAOSTAT, 2014)

In Bangladesh, the area of cultivation is about 23,813 hectares with the production of about 190,213 metric tons, having an average yield 6.7 metric tons per hectare (BBS, 2010). The average yield of our country is quite low as compared to other leading tomato producing countries (BAKB, 2010). Farmers usually grow 50 Indian and Bangladeshi varieties available in the market (BARI, 2010).

1.4 Tomato production constraints

Being a tropical plant, tomato is well adapted to almost all climatic regions of the world. The production of tomato is influenced by several biotic and abiotic factors. There are several common diseases of tomato crops viz., bacterial wilt caused by *Pseudomonas solanacearum* and bacterial scab, which is caused by *Xanthomonas campestris*. Fungal diseases have resulted in decreased trend of its yield/acre, powdery mildew caused by *Leveillula taurica*. Other main diseases are early blight, leaf spot, leaf mold and wilts etc. In U.S.A. around 50% of tomato fields faced pest attack per year and yield lost has been recorded upto 40% in severely infected fields while the other countries face production loss upto 35% (http://pmep.cce.cornell.edu/fqpa/crop-profiles/tomato.html; date: 15.1.2015). Moreover changes in insect's biotype and disease resistance are becoming a continuing threat to increased production (Chaudhry *et al*., 2010).

While biotic stress responses are well investigated in plants, the responses to abiotic stress are more complex and not clearly understood (Pandey *et al*., 2011). Abiotic stress is a broad term, which includes multiple stresses, such as, high temperature, chilling, excessive light, drought, water logging, wounding, and exposure to ozone, UV-B irradiation, osmotic shock, and salinity. These stresses reduce agricultural production world-wide by more than 50% of average yield in most major crops (Chaudhry *et al*., 2010). Tomato is highly sensitive to salinity stress, which inhibits seed germination during early stages of plant growth (Lin *et al*., 1995), whereas in later stages it affects reproductive development. Fruit set also decreases due to poor pollen germination (Pandey *et al*., 2011). Salt stress is reflected in growth reduction, tissue necrosis, wilting, leaf curling, leaf abscission, decreased photosynthesis, respiratory changes, loss of cellular integrity, and potentially death of tomato plant (Lin *et al*., 1995).

As saline soils and saline waters are common around the world, great effort has been devoted to develop salinity tolerant genotypes. Therefore, crop improvement activities have been focused towards the development of tomato varieties with high yield and stress tolerance. Although there are comparatively salt-tolerant relatives of the cultivated tomato, it has proved difficult to develop new cultivar by conventional breeding approaches. Conventional breeding programmes to improve the salt tolerance of elite genotypes using wild genotypes as donors are inefficient at the selection stages (Cuartero *et al.,* 2006). This could be overcome through *in vitro* regeneration of tomato, followed by transformation with desired trait, such as, salt tolerance.

1.5 Salinity: A threat towards crop production

Accumulation of salts in irrigated soil is a primary factor depressing yield in crop production. The detrimental effects of salt on plants are a consequence of osmotic stress and the toxicity of excess sodium ions to many critical biochemical processes.

Based on the capacity to grow on a salinity environment, plants can be classified into glycophyte and halophyte. Most plant species are glycophytes, which are salt sensitive. In contrast, halophytes are able to grow in habitats excessively rich in salts, such as, salt marshes, sea coasts, and saline or alkaline semi-deserts and steppes. Most of the eatable plants are glycophytes and susceptible to salinity, in the other hand, nearly all of the plants in reproductive stage are not tolerant to salinity (Zhang *et al.,* 2001). Based on information from FAO, more than 400 million hectares of the world land, including most parts of the continents has the salinity problem. Growing salinization phenomena in agricultural fields decreases the proper land for cultivation, even maybe up to 50% by the year 2050 (FAOSTAT, 2014).

The salt tolerance in halophytes involves a range of adaptations, including ion compartmentalization, osmolyte production, germination responses, osmotic adjustment, succulence, selective transport and uptake of ions, enzyme responses, salt excretion, and genetic control (Zhang *et al.*, 2001). The molecular basis of the stress tolerance of halophyte, however, is still far from clear.

Present engineering strategies for salinity tolerance rely on the transfer of one or more genes that are either involved in the signaling pathways or that encode enzymes required for the functional and structural protectants, such as, osmolytes and antioxidants or that encode the proteins that confer stress tolerance. However, considering the complex metabolic response of the plants under stress, there is still need to know about candidate genes function in conferring salinity tolerance (Saxena *et al.*, 2011).

Engineering of salinity tolerance in agronomically important crop plants is required to increase their productivity by enabling them to grow in saline soils, which are otherwise left uncultivated.

1.6 Plants in saline condition

Plants as the first chain of production are ordinarily exposed to different environmental stresses, including drought, salinity, high and low temperatures that reduce crop yield, which decrease the universal food production approximately 70%. One of the most important stresses, especially in arid regions is salinity. Salinity could decrease the quantity and quality of plants yield. The meaning of salinity is having of electrical conductivity (ECe) 4 dS m-1 to up at 25˚C (Bahmani *et al.*, 2015).

Cations and anions with most important role in salinity are sodium (Na^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}), chloride (Cl^-), sulfate (SO_4^{2-}) and bicarbonate (HCO_3^-), meanwhile the most prevalent of them are firstly Na (in almost plants) and secondly Cl (especially in trees like citrus). Therefore, in salinity researches on plants NaCl is used as the salinizing salt. Plants can sense salt stress by ionic (Na^+) and osmotic signals. Excess Na is sensed either by the transmembrane protein on the plasma membrane (Membrane bound histidine kinases) or within the cell by Na sensitive enzymes or membrane proteins (Bahmani *et al.*, 2015).

Salinity has several deleterious effects on plants, such as:

- Osmotic stress (resulted in loss of cell turgor),
- Ion toxicity (mainly due to the Na^+ and Cl^- and SO_4^{2-}),
- Mineral deficiency (specially Potassium, Iron and Zinc),
- Ion imbalance,
- Oxidative stress,
- Stomatal blockade (resulted in reduction of carbon dioxide availability),
- Photosynthesis prohibition,
- Cell division prohibition,
- Increase of sensitivity to diseases and a combination of these.

The physiology of plant responses to salinity and their relation to salinity resistance have been much researched and frequently reviewed for many crop species, including bean, tomato, onion, pepper, corn, potato etc. They are sensitive to salinity, resulting in reduction in crop productivity (Ashraf, 2009).

1.7 Molecular mechanisms of plant salinity tolerance

Understanding the cellular basis of salt stress tolerance mechanisms is necessary for breeding and genetic engineering of salt tolerance in crops. Tolerance mechanisms mainly are applicable to practical manipulations.
Tolerance mechanisms in plant can be categorized as:

- Salt Overly Sensitive 1 (SOS1) pathway
- Sodium influx and Na^+/K^+ balance
- Sodium efflux
- Sodium compartmentalization

Salt Overly Sensitive 1 (SOS1) pathway

Understanding of plant responses to excessive Na^+ concentration has been progressed since the discovery of the Salt Overly Sensitive 1 (*SOS1*) pathway in *Arabidopsis* (Shi *et al.*, 2000). The prominent genes working in this pathway include SOS1, a plasma membrane Na^+/H^+ antiporter (Shi *et al.*, 2000), *SOS2*, a serine/threonine protein kinase (Liu *et al*., 2000), *SOS3*, a Ca^{2+} sensor (Liu *et al*., 2000), High-affinity K^+ Transporter (*HKT1*), a transporter that facilitate K^+ or Na^+ uptake into the cell and *NHX1*, a Na^+/H^+ antiporter localized on vacuolar membrane for Na^+ compartmentation in the vacuole. Upon salt stress, plants up-regulated the SOS1 transcript level that resulted in lower Na^+ accumulation in root, xylem stream and shoot cells.

Sodium influx and Na^+/K^+ balance

Under normal physiological conditions, plants maintain a high potassium and sodium (K^+/Na^+) ratio in the cytosol (Khan, 2011). Accumulation of high Na^+ concentration in the extracellular spaces generates a very high electrical membrane potential difference, which facilitate a passive movement of Na^+ ions into the cytosol. This passive movement of Na^+ ions into root cells is facilitated by ion transporters or channels. These different transporters function in parallel, mediating Na^+ uptake into the roots and are dependent upon species and growth conditions.

Several members of the High-affinity K^+ Transporter (*HKT1*) family have been identified that play a significant role in the Na^+ influx (Khan, 2011). The tissue specific activity of *HKT1* has recently been shown to correlate with Na^+ movement from root to shoot and the subsequent salt tolerance. Transgenic *Arabidopsis* expressing the *HKT1* gene in the mature root stele cells showed a drastic decrease of Na^+ accumulation in the shoot by 37 to 64% (Khan, 2011). This decrease in shoot Na^+ content was mediated by the increased Na^+ influx into the root stele cells, which in turn, decreased the flow of Na^+ from root to shoot and increased salt tolerance.

Sodium efflux

Sodium efflux from root cells prevents accumulation of toxic levels of Na^+ in the cytosol and transport of Na^+ to the shoot. In the presence of high concentration of Na inside and outside of the cell, the electrochemical gradient that is generated across

the membrane makes an active transport of Na^+ out of the cell (Zhang and Blumwald, 2001). For this purpose, specific Na^+-ATPases are present in the plasma membrane. The only channels through which Na^+ is excluded of the cell are Na^+/H^+ antiporters. These Na^+/H^+ antiporters play a crucial role in maintaining cellular sodium level, cytoplasmic pH and cell turgor (Zhang and Blumwald, 2001).

Sodium compartmentalization

Besides excluding excess Na from cytosol, the vacuolar compartmentalization of Na^+ is another very important mechanism to cope with salinity stress (Apse and Blumwald, 2002). Excess Na^+ in the cytosol is taken into the vacuole by cation/H^+ antiporters localized on vacuolar membranes. These antiporters are driven by electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes such as H^+-ATPase and H^+-PPase (Apse and Blumwald, 2002).

1.8 Improvement of salt tolerance by genetic engineering

Several studies have suggested that plants naturally exhibit various physiological and biochemical mechanisms to respond and adapt to stresses through production of signal molecules, such as, ABA, Ca^{2+}, jasmonic acid (JA), ethylene, and salicylic acid (SA), which function as signal transducers or messengers of environmental stresses and trigger defense responses. Elimination of salts from the root zone of plants by traditional methods, like, irrigation correction, leaching, cultivation change, especial kind of fertilizer application, or reduction in harmful effect of salinity using some especial bacteria and mycorrhyzal fungi (like *Ectomycorrhizas*, *Arbuscular Mycorrhizas*), plant treatment (by salicylic acid, brassinosteroids, abscisic acid) may be the first solutions for salinity (Bahmani *et al*., 2015). However, few cultural and breeding approaches have been reported in tomato for improvement of plant tolerance to abiotic stresses, such as, drought, salinity, and cold.

Genetic engineering approaches for developing abiotic stress tolerant tomatoes are considered to be an attractive alternative to conventional breeding. Manipulating the production of such enzymes or compounds through transgenic approaches has resulted in the development of plants with enhanced abiotic stress tolerance in

several plant species including tomato. Presently, genetic engineering of tomatoes expressing several genes encoding ion transporters, osmoprotectants, and antioxidant enzymes has resulted in notable success in enhancing abiotic stress tolerance.

To sustain against the harmful effects of salinity stress, plants have evolved several biochemical, morphological and molecular mechanisms with their signal transduction. After the first recognition of the tonoplast Na^+/H^+ antiporter in barley root tips many Na^+/H^+ antiporter genes have been identified in plants, such as, *Arabidopsis thaliana*, *Oryza sativa*, *Atriplex gmelini*, *Beta vulgaris*, *Gossypium hirsutum* (Bahmani *et al*., 2015). The overexpression of Na^+/H^+ antiporter genes could increase tolerance under saline conditions in transgenic *Arabidopsis*, *Brassica*, tomato, rice and wheat. These works demonstrated that Na^+/H^+ antiporter genes were crucial to plant salt-tolerance, and they could be used in crop improvement by genetic transformation technique (Bahmani *et al*., 2015).

In tomato, reports on enhancing salt tolerance with transgenic approaches have been very insignificant (Zhang and Blumwald, 2001). Only one report of transgenic tomato plant present where over-expressing *AtNHX*1, a single-gene controlling vacuolar Na^+/H^+ antiporter gene from *Arabidopsis thaliana* has been incorporated (Zhang and Blumwald, 2001). This transgenic tomato plant can grow upto 200mM by encoding a protein that exports excess to vacuole before it damages the tissues. According to this report, very high concentrations of Na^+ and Cl^- were accumulated in the leaves of transgenic tomato plants where salinity conditions were elevated. The ability of the transgenic plants was enhanced due to overproduction of the vacuolar Na^+/H^- antiporter protein. Therefore, it seized Na^+ in their vacuole while averting its toxic effects in the cell cytosol. In addition, there were only minimum increases in Na^+ and Cl^- concentrations in the fruit.

There are some other important genes in maintaining K^+ or Na^+ homeostasis in higher plants, and could be considered candidates for genetic manipulation. These are listed in Table 1.1.

1.9 Preface of Na^+/H^+ antiporters as candidate to salt tolerance

Salt tolerant plants have been characterized by their ability to cope with osmotic and ionic stresses caused by elevated sodium chloride (NaCl) concentrations. For homeostatic control of Na^+, plants have evolved a system of membrane channels and antiporters that facilitate the influx and efflux of sodium (Na^+) ions at the roots and establish a steady state rate of entry of Na^+ into the plant, compartmentation of Na^+ into the cell vacuoles and transfer to various plant tissues (Khan, 2011). To enhance the salt tolerance of salt sensitive plants, genetic engineering with sodium and hydrogen (Na^+/H^+) antiporters is one of the preferred methods in recent years.

The only channels through which Na^+ is excluded of the cell are Na^+/H^+ antiporters. In plants, various Na^+/H^+ antiporters have been classified into three families;

- CPA1 (NHX, eight members),
- NhaD (two members), and
- CPA2 (including CHX-twenty eight members and KEA-six members).

Among the eight members of the CPA1 family, two members (*AtNHX7*/SOS1 and *AtNHX8*) have been identified as localized on the plasma membrane. *AtNHX7*/SOS1 is a Na^+/H^+ antiporter, while *AtNHX8* has been characterized as Li^+/H^+ antiporter. The other six members (*AtNHX1* to 6) are vacuolar/endosomal antiporters (Khan, 2011).

Various vacuolar antiporters, such as, *AtNHX1* to 6 have been cloned from *Arabidopsis*. Since then, such types of vacuolar antiporters have been identified in a wide range of plant species. Among the six different Na^+/H^+ antiporters isolated from *Arabidopsis*, only *AtNHX1*, *AtNHX2* and *AtNHX5* were reported as functional Na^+/H^+ antiporters and were functionally characterized (Khan, 2011).

In several studies, the role of vacuolar antiporters in Na^+/K^+ homeostasis, pH regulation and overall plant salt stress tolerance has been explained. It was reported that during exposure to saline condition, various crop plants up-regulate the expression of genes encoding NHX like antiporters, which in turn, play important role in the salt tolerance of these plants. The expression of NHX genes was up-regulated by salinity in cotton (Wu *et al*., 2004), rice (Fukuda *et al*., 1999) and wheat (Saqib *et al*., 2005).

The *Arabidopsis* vacuolar membrane Na^+/H^+ antiporters have been introduced in several crop plants to enhance their salt tolerance. Introduction of *AtNHX1* in *Brassica* conferred salt stress tolerance and transgenic plants showed 2.3% high plant fresh weight and 2.34% high grain yield under (10 mM) NaCl (Zhang and Blumwald, 2001). Transgenic cotton that expressed *Arabidopsis AtNHX1* showed enhanced salt stress tolerance (He *et al.*, 2005).

Tomato plants over expressing vacuolar *AtNHX1* showed improved growth, flower and seed production under high salt concentration (200 mM NaCl) (Zhang and Blumwald, 2001). Transformation of rice "Binnatoa" with *OsNHX1* and *PgNHX1* conferred salt tolerance and transgenic plants showed higher shoot and root growth (Seraj *et al.*, 2010). Transformation of wheat and maize with *AtNHX1* showed tolerance to salt stress (Supartana *et al.*, 2006).

Table 1.1 Cloned genes with likely relevance for controlling Na^+ or K^+ uptake the plant, and which are candidates for overexpression studies

Type of transporter	Gene family	Candidate genes for salt tolerance	Probable function in higher plants
K^+ transporter	*HKT* family	*HKT1*	K^+ starvation induces *HKT1* (high-affinity K^+ transporter) expression in plants, indicating that it functions in high-affinity K^+ uptake, but it also transports Na^+.
Cation antiporter	*CHX* family	*CHX10, 15*	Cation hydrogen exchangers regulate K^+ uptake by vacuoles. They may carry Na^+, and their expression is down- regulated under salt stress.
Na^+ antiporter	*NHX* family	*NHX1*	*AtNHX1* (Na^+/H^+ exchanger) is an Na^+/H^+ antiporter expressed in roots and leaves, and selectively transports Na^+ into the vacuole, as well as K^+ in nonsaline conditions.
		NHX2–5	*AtNHX2–5* are expressed in specific cell types, transport Na^+ or K^+ into the vacuole, and have a likely role in K^+ or pH regulation.
		SOS1	*SOS1 (AtNHX7)* is a Na^+/H^+ antiporter on the plasma membrane. *SOS1* would efflux Na^+ from cells and may be important in Na^+ extrusion from roots into the external medium.
Proton pump	AHA P-type H ± ATPase	*AHA2*	H^+ transport across plasma membrane.
Proton pump	H ± PPase	*AVP1*	H^+ transport across tonoplast

Type of transporter	Gene family	Candidate genes for salt tolerance	Probable function in higher plants
K^+ channel	Shaker type (single pore, tetramer) inward channel	*AKT1, AKT2, KAT1*	*AKT1* (*Arabidopsis* K^+ transporter) is an inward rectifying K^+ channel expressed in roots. *AKT2* and *KAT1* are related. These are expressed in leaf phloem tissue and guard cells but may function in other cell types in other species.
K^+ channel	Shaker type, outward channel	*SKOR*	*SKOR*(stellar K^+ outward rectifier) is important in maintaining K^+ homeostasis in both roots and shoots. *SKOR* is probably located on the plasma membrane.
K^+ channel	*KCO* family (two pore channel)	*KCO1*	*KCO1* (K^+ channel outward) rectifier is expressed in leaf cells, probably on the tonoplast.
Nonselective cation channel	*CNGC* and *GLR* families	*CNGC1–20, GLR1–20*	Some members of the *CNGCs* (cyclic nucleotide-gated channels) and *GLRs* (glutamate receptors) families are predicted to have a similar permeability to Na^+ and K^+, and to be regulated by $Ca2^+$.
K^+ antiporter	K/H antiporter	*KEA or CPA (CHA) family*	*KEA (K^+ exchange antiporter)* is present in the plant genome, K^+ antiporters may be important in K^+ homeostasis by loading K^+ into vacuoles. It is possible that these could carry Na^+, just as Na^+/H^+ antiporters can carry K^+.
K^+ transporter	*KUP/HAK/KT* family	*HAK1–10, KUP1–4*	There are many variants of K^+ transporters, in bacteria, fungi and higher plants, and they are very important in control of K^+ homeostasis.

1.10 *In vitro* regeneration of tomato

In vitro regeneration of cultivated tomato has been a subject of research because of the commercial value of the crop and its amenability for further improvement via genetic manipulation. Tissue culture is an important tool of biotechnology, which can be used to improve productivity of crop via rapid availability of superior planting stock (Bhatia *et al.,* 2004).

Distinct feature of tomato includes high degree of self pollination and unavailability of suitable germplasm to breed a new variety. Moreover, it is a pre-dominantly inbreeding species and its genetic variation tends to decrease. These features hamper to improve tomato characters through conventional breeding program. Besides, this method takes long time, extending over several years involving crossing and selection of desirable traits. Thus, *in vitro* regeneration technique helps to provide unique possibilities for overcoming the barriers of incompatibility between remote species and it facilitates rapid introduction of new varieties (Praveen, 2011).

Mass propagation of tomato has been attempted through the use of various types of explants viz. cotyledon, hypocotyl, pedicel, peduncle, leaf, stem sections and inflorescence for organogenesis. Gubis *et al.* (2004) studied the effect of different growth regulators and plant regeneration of tomato explants, where tomato regeneration response has been found to depend largely on genotype, explants, and plant growth regulator used in culture medium. Plant growth regulators affect morphogenic tomato cultures (Bhatia *et al.,* 2004). For tomato regeneration, a wide variety of plant growth regulators have been used with varying concentrations. Many cytokinin and auxin combinations could induce shoot proliferation in tomato from different source of explants. Jatoi *et al.* (2001) found that 6-benzylaminopurine (BAP) with indole-3-acetic acid (IAA) are the best for callus induction from shoot tips. Chaudhury *et al.* (2010) found that callus formation from hypocotyl is best in 2 mg/l IAA with 2 mg/l BAP or 2 mg/l NAA with 4 mg/l Kin.

Maximum percentage of shoot formation was found on the MS medium supplemented with 2 mg/l IAA with 5 mg/l BAP or 2 mg/l BAP with 4 mg/l Kin. They reported that hypocotyl is the best explant source for callus formation and regeneration. Half strength of MS was found to be the best rooting medium. Jabeen *et al.* (2005) found that the regeneration capacity was strongly influenced by the cultivar and explant type. The explant types of shoot tip were found to be the best explant source for direct shoot formation (80% shoot primordial were regenerated) while hypocotyl was found to be the best explant source for shoot formation through callogenesis (64.5% shoot primordial were regenerated). The medium supplemented with 2 mg/l BAP was most effective in the induction of adventitious shoots for both the hypocotyls and cotyledons.

To develop transgenic crops with useful traits efficient *in vitro* plant regeneration protocol is necessary. In case of tomato, a number of tissue culture approaches has been done to regenerate plants through *in vitro* culture systems. Researchers of Dhaka University and BRAC University are working to establish a reproducible regeneration protocol of locally grown tomatoes (Sarker *et al.,* 2009; Chowdhury *et al.,* 2010; and Ferdous, 2012). However, establishment of standard regeneration protocol with farmer popular tomato varieties of Bangladesh is still under process.

1.11 *In vitro* transformation of tomato

Genetic engineering approaches for developing abiotic stress tolerant tomatoes are considered to be an attractive alternative to conventional breeding. Manipulating the production of such enzymes or compounds through transgenic approaches has resulted in the development of plants with enhanced abiotic stress tolerance in several plant species including tomato. Toward the improvement of tomato through genetic engineering, reliable regeneration and transformation procedures are essential. Over the past two decades a number of techniques have been employed for the introduction of foreign DNA into plant cells of monocotyledon and dicotyledonous plants.

For tomato, genetic transformation via *Agrobacterium* is certainly an important tool to facilitate genetic improvement along with other methods such as particle bombardment and *Agro*-infiltration. Since the first report of tomato transformation by McCormick *et al.* (1991), numerous publications on the transformation of various tomato genotypes have been reported (summarized in Table 1.2).

1.12 Factors influencing efficient transformation in tomato

Transformation efficiency in tomato is influenced by many factors including cultivar (Hamza and Chupeau, 1993), explant type (McCormick *et al*., 1991), explant age (Hamza and Chupeau, 1993), phytohormones (McCormick *et al*., 1991), bacterial contamination (Ling *et al*., 1998) and *Agrobacterium* virulence gene inducers (Stachel *et al.*, 1986). Regeneration and transformation studies have primarily been focused on tomato species *S. lycopersicum* (Hasan *et al.*, 2008), studies have also been reported for other tomato species such as *S. pennellii* (van Eck *et al.*, 1995), *S. peruvianum* (Hamza and Chupeau, 1993), and *L. chilense* (Agharbaoui *et al.*, 1995).

A range of explants have been used for developing transgenic plants in tomato including leaves (Agharbaoui *et al*., 1995). Among them, cotyledonary leaves have been frequently used because of the availability of seeds, reproducibility of sterilization and germination conditions, and the possibility of controlling the developmental stage.

1.13 Constrains of *in vitro* transformation and plant regeneration

The big advantage of *Agrobacterium*-mediated method is an ability to insert almost any genes into the T-DNA region. There are two types of *Agrobacterium* transformation methods: *in vitro*, involving the cultivation of plant cells and tissues with the subsequent regeneration of the plant, and *in planta*, where these steps are missing.

The use of *Agrobacterium* transformation gene delivery system however requires the identification of competent to be transformed as well as the development of a tissue culture system (Ismail *et al*., 2005).

Most transient *in vitro* transformation methods have certain disadvantages, most noteworthy is, its dependency to tissue culture for regeneration. The lack of reproducibility of regeneration protocols, highly problematic rooting and subsequent transplantation of *in vitro* regenerated shoots are major limiting factors for obtaining complete transgenic plants and their progeny (Feldman and Marks, 1987). Tissue culture is labor intensive and can be difficult to master. Even under optimal transformation and regeneration conditions tissue culture can result in somaclonal variations, morphological abnormalities, changes in chromosomal number and loss of fertility (Chumakov, 2011).

In addition to the lower transformation efficiency, equipment dependency and requirement of auxiliary material for transformation has also been reported (Kedong *et al*., 2014). Some other major drawbacks of the *Agrobacterium*-mediated transformation are the recalcitrance of plant and its difficult-to-regenerate nature (Rohini and Rao, 2000). In some cases, *in vitro* regeneration from callus is limited to non-indigenous cultivars or closely related genotypes. Elaborate culture procedures, relatively long time period required for regeneration and high seed to seed variation in response, collectively pose serious technical difficulties and restrict progress in plant biotechnology (Rohini and Rao, 2000). Hence this necessitates development of easy, reliable and efficient transformation protocols for tomato transformation for improvement, particularly in the Bangladeshi cultivars which are adapted to local conditions.

To tackle the problems pertaining to regeneration of shoots from *in vitro* transformation, alternate methods to minimize or eliminate the steps of regeneration are being standardized. These are called the *in planta* transformation protocols.

Research with *Arabidopsis* has benefited from the development of high throughput transformation methods that avoid plant tissue culture (Feldmann and Marks, 1987). *In planta* transformation methods have also been standardized for rice, buckwheat kenaf and mulberry (Rohini and Rao, 2000; Supartana *et al*., 2005).

Table 1.2 Summary of researches that achieved successful *Agrobacterium*-mediated genetic transformation of tomato

Cultivar(s)/ accession	*Agrobacterium* strains	Vectors and marker gene(s) used	Tissue type	Transformation efficiency/ result	References
S.lycopersicum cultivars and F1 hybrids	*A. tumefaciens* strain GV3111SE and A208 (host C58C1)	Ti plasmids *pTi6S3SE* and *pTiT37SE (ASE)* containing *nptII* gene	Leaves, cotyledons & hypocotyls	Transgenic plantlets showed kanamycin resistance	McCormick *et al.*, 1986
S. lycopersicum UC82B, Monalbo, Castone, Ferline and *S. peruvianum* CMV sel. INRA	ND^+ (Nicotinamid adenine dinucleotide phosphate dehydrogenase)	Binary vector *p35SGUSINT* containing *nptII* and *GUS* genes	Cotyledons	Transformation frequency, 8% (Monalbo) and 14% (UC82B)	Hamza and Chupeau, 1993
S. lycopersicum Moneymaker	*A. tumefaciens* strain LBA4404	Binary vector *pBI21* containing *nptII* and *GUS* genes	Cotyledons and hypocotyls	Transformation frequency, 10.6%	Frary and Earle, 1996
S. lycopersicum Moneymaker	*A. tumefaciens* strain LBA4404	Transforma-tion vector *SLJ 44024* containing *nptII* gene	Cotyledons	Superiority of ticarcillin for removing *Agrobacterium* contamination and enhancing transformation	Ling *et al.*, 1998
S. lycopersicum Micro-Tom, Red Cherry, Rubion, Piedmont, and E6203	*A. tumefaciens* strain LBA4404	Binary vector *pBI21* containing *nptII* and *GUS* genes	Leaves, cotyledons and hypocotyls	Transformation efficiency, >20%	Park *et al.*, 2003
S. lycopersicum UC82B	*A. tumefaciens* strain LBA4404	Binary vector *pBIN19* containing *nptII* gene	Cotyledons	Transformation frequency, 12.5%	Cortina and Culianez-Macia, 2004
S. lycopersicum Lichun	*A. tumefaciens* strain LBA4404	Binary vector *pTOK233* with *nptII* and *GUS* genes	Cotyledons , hypocotyls	Transgenic shoots showed resistance to kanamycin	Wu *et al.*, 2006

Cultivar(s)/ accession	*Agrobacterium* strains	Vectors and marker gene(s) used	Tissue type	Transformation efficiency/ result	References
S. lycopersicum Micro-Tom	*A. tumefaciens* strain EHA105	Binary vectors contained *nptII* gene	Cotyledons	Maximum transformation frequency, 20.87%	Qiu *et al.*, 2007
S. lycopersicum	*A. tumefaciens* strain EHA 105	*pROKIIGUSINTAP1* carrying *nptII* and *GUS* genes	Fresh, healthy & mature fruits	Transformation frequency ranged from 54 to 68.0%	Hasan *et al*., 2008
S. lycopersicum Zhongshu No. 4	*A. tumefaciens* strain LBA4404	Binary vector *pBI21* with *nptII* and *GUS* genes	Cotyledons	Transformation frequency, 44.7%	Gao *et al.*, 2009
S. lycopersicum Pusa Ruby, Sioux, Arka Vikas	*A. tumefaciens* strain AGL1	Binary vector *pCTBE2L pRINASE2L, pCTBE2L pCTBE2L*	Cotyledons	Transformation frequency ranged from 41.4%	Sharma *et al.*, 2009
S. lycopersicum Roma and Rio-grande	*A. tumefaciens* strain EHA101	Binary vector *pTCL5* containing *hpt* and GUS gene	Hypocotyls & leaf disks	Transformation efficiency, 24% (Riogrande) and 8% (Roma)	Chaudhry and Rashid, 2010
S. lycopersicum Mill. Pusa Ruby, Pusa Uphar, and DT-39	*A. tumefaciens* strain GV3101	Binary vector pBI101 containing *nptII* gene	Cotyledons	Transformation frequency, >37%	Kaur and Bansal, 2010

1.14 *In planta* transformation of seeds

Development of a method to obtain transformants, which is independent of the problems inherent to tissue culture of plants i.e. tomato, would represent a major accomplishment. *In planta* transformation is an alternative method which does not involve *in vitro* culture of plant, thereby reducing time, labor cost and most importantly avoiding somaclonal variation encountered during *in vitro* regeneration.

Feldman and Marks (1987) were the first to report the success of utilizing dry seeds as material for *in planta* transformation and produced transgenic plants rapidly without an intermediate callus phase. Phenotypically normal, these fertile transgenic plant contained functional transgenes which were inherited in a Mendelian fashion (Ismail *et al*., 2005). Transgenic plants were also successfully generated utilizing dry seeds for transformation in pea, peanut and soy bean (Ismail *et al*., 2005).

In planta transformation has been used successfully in monocotyledonous plants like, rice, wheat and maize as well as dicotyledonous plants like, *Arabidopsis*, apple, pear, peach, strawberry, tomato, pigeon pea, radish, peanut, citrus castor, and cotton. The transformation frequency obtained in rice and wheat by using *in planta* transformation was much higher than that of previously reported methods of transformation (Supartana *et al*., 2005).

In planta transformation was carried out by infecting germinating seed of radish, wheat, rice, cotton and masterd seed; via pollen tube pathway in soybean (Chee *et al.,* 1998), floral dip of *Arabidopsis* (Ye *et al.,* 1999), floral buds of *Arabidopsis* (Bent, 2000), shoot apical nodes of alfalfa, epicotyle segments of citrus (Ahmad and Mirza, 2005), mature embryo of rice (Kojima *et al*., 2000), through fruit injection of tomato (Yasmeen *et al*., 2009), wheat and radish; pistal dip of maize and cotton (Abdellatef, 2007).

The seeds transformation by *in planta* methods includes the seeds incubation with *Agrobacterium* cells and growing plants under natural conditions until harvesting seeds of the next generation, which are then placed on the medium with a selecting agent (Pang *et al*., 1999). The efficiency of *Arabidopsis* transformation under these conditions was moderate.

Gradually, techniques increasing the efficiency of seed transformation were developed and the variety of transformed plants was increased. For example, the efficiency can be increased if seeds are treated with ultrasound in the presence of aluminium oxide during *Agrobacterium* incubation or if inoculation is carried out with vacuum assistance (Chumakov, 2011). Apart from these techniques, researchers use mechanical damaging of seeds during transformation, for example, damaging corn seeds with a scalpel before incubation with an *Agrobacterium* suspension (Chumakov, 2011) or puncturing two holes in the surface of a wheat or rice seed in the expected area of germination and subsequently submerging the seeds in an inoculation medium with *Agrobacterium* (Chumakov, 2011).

1.15 Research aims

To provide a solution to the constrain "salinity", that limits the production and quality of tomato; it is imperative that there is a need to widen the genetic base of tomato by using transgenic technology for incorporating the trait salt-tolerance. Aimed to this, the present study was carried out to achieve the following objectives:

a. To assess *in vitro* regeneration protocol for five Bangladeshi tomato varieties.
b. To test the sensitivity of bacteriostatic antibiotics on tomato tissues.
c. To compare transformation efficiency of different tomato varieties by direct and indirect approaches of transformation.
d. To compare transformation efficiency of different explants.
e. To compare transformation efficiency of different vectors.
f. To determine factors that influence transformation efficiency for *in vitro and in planta* transformation.
g. To regenerate putative transgenic tomato plants using *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* by *Agrobacterium* mediated genetic transformation.
h. Bioassay of putative transgenic tomato plants.

Materials and Methods

2.1 Plant material

Seeds of five varieties of tomato (*Solanum lycopersicum* Mill, Family: Solanaceae) were used in tissue culture and transformation study. Among these five varieties, two varieties were collected from Bangladesh Agricultural Research Institute (BARI) named as BARI tomato 2, BARI tomato 3 and the remaining three varieties were collected from Bangladesh Institute of Nuclear Agriculture (BINA) named as BINA tomato 2, BINA tomato 3 and Bahar. A brief description of the mentioned tomato varieties are represented in Table 2.1**.** Whole seed and cotyledonary leaves collected from 8-12 days old seedling of these five varieties were used as explants source to perform transformation.

2.2 *Agrobacterium* strain and plasmid vectors

Agrobacterium tumefaciense strain LBA4404 with three plasmids constructs, *pBI121*, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* were used for transformation.

pBI121: The total size of *pBI121* is 14.8 kb according to its construction map. The Ti plasmid contains a plant selectable marker gene *neomycin phosphotransferase II* (*npt II*) conferring resistance to kanamycin and a *uidA* gene encoding β-gluduronidase (GUS) reporter gene (1812 bp). These two genes were separately fused under the control of the nopaline synthase promoter (NOS pro) and CaMV 35S promoter (CaMV35S-pro) within the left and right border region (Fig. 2.1 A).

pK7WG2_OsNHX1_1.6: The Na^+/H^+ antiporter gene (*OsNHX1_1.6*) cloned from rice was immobilized to gateway vector, *pK7WG2_OsNHX1_1.6* under the control of promoter p35S and terminator T35S. This final construct *pK7WG2_OsNHX1_1.6* (Fig. 2.1 B) was transformed into *A. tumefaciense* LBA4404 to be used in tomato transformation. It contains kanamycin resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria.

***pK7WG2_AtNHX1_1.6*:** The Na^+/H^+ antiporter gene (*AtNHX1_1.6*) cloned from Arabidopsis was immobilized to gateway vector, *pK7WG2_AtNHX1_1.6* under the control of promoter p35S and terminator T35S. This final construct *pK7WG2_AtNHX1_1.6* (Fig. 2.1 C) was transformed into *A. tumefaciense* LBA4404 to be used in tomato transformation. It contains kanamycin resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria.

2.3 Media used in different phases of the study

2.3.1 Media used for tissue culture

In this study, Murashige and Skoog (MS) medium (1962) along with suitable concentration of different growth hormones were used for different types of tests, such as:

2.3.2 Media used for seed germination and seedling development

MS basal medium solidified with agar or phytagel were used for seed germination. No hormone was added in this media.

2.3.3 Media used for shoot regeneration

For shoot regeneration, solid MS medium supplemented with BAP (2mg/l) was used.

2.3.4 Media used for root induction

The basal medium for rooting was half strength MS medium. The medium was supplemented by 0.1 mg/l IAA. For solidification, 0.6% (w/v) phytagel (Sigma) was used in root formation media.

2.3.5 Media used for transformation

2.3.6 Media used for *Agrobacterium* culture

YEP (Yeast Peptone Media) media with appropriate concentrations of antibiotics were used for bacterial culture. Liquid YEP medium was used for growing *Agrobacterium tumefaciense* strain LBA4404. This bacterial suspension was used as working culture for infection. YEP medium solidified with agar was used to maintain bacterial pure culture.

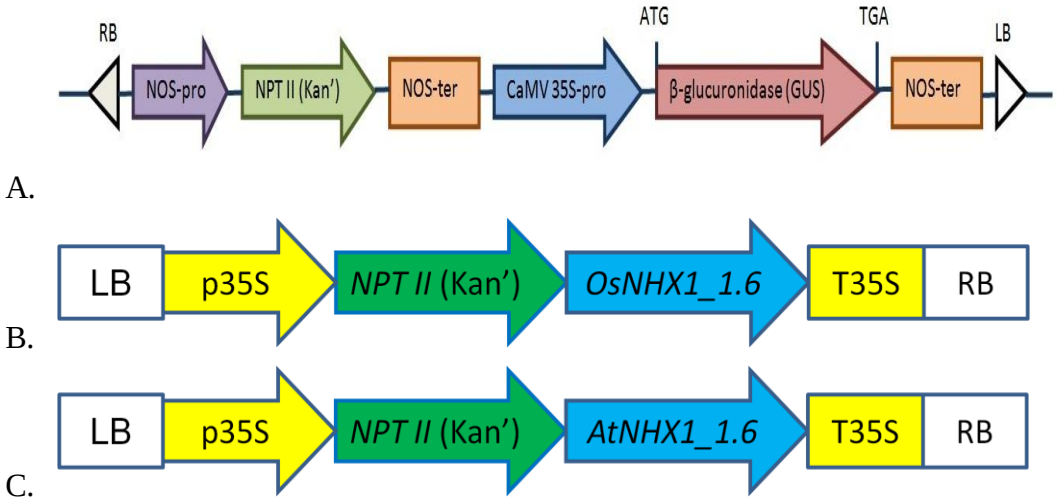

Fig. 2.1 Schematic diagram of A. the T-DNA region of the binary *pBI121*, B. Constructed vector *pK7WG2_OsNHX1_1.6*, C. Constructed vector *pK7WG2_AtNHX1_1.6*

Table 2.1 List of tomato varieties used

Tomato Variety	Crop Duration (days)	Year of Release	Yield (ton/ha)	Identifying Characteristics	Fruit Size & Color	Sowing Time
BINA tomato 2	60-70	1997	38-45	Summer Variety. Fruits are round and average fruit weight is 55 g. Vitamin C content is 18.0 mg/100.	Round red	May-June
BINA tomato 3	60-65	1997	48	Heat resistant, but have less fruit production, average fruit weight is 82 g, Not good to taste	Oval-Red	May- June
BARI tomato 2	120-130	1986	85-90	High yielder and tolerant to BW, Fruits are round and red in color, Average fruit weight 85-90 g, Good shelf life	Round red	September-October
BARI tomato 3	120-130	1996	85-90	Fruits are fleshy, semi-globe and red in color, Number of fruits/plant is about 28-30 and average fruit weight is about 85-90 g.	Semi-globe, red	September-October
Bahar	90-120	1992	65-75	Plants are determinate in habit, Fruits are large, fleshy and tastier, contain less number of seeds. Average fruit weight is 110 g.	Round	October-November

Ref: www.bina.gov.bd/; date: 10.10.2014; www.bari.gov.bd/; date: 10.10.2014; Chowdhury (2009); Dutta *et al.*, (2004)

2.3.7 Media used for co-cultivation

Shoot regeneration media without antibiotics were used as co-cultivation medium.

2.3.8 Media used for selection

Three antibiotics (streptomycin and spectinomycin for *Agrobacterium*, kanamycin for all the plasmid *pBI121, pK7WG2_OsNHX1_1.6* and *pK7WG2_AtNHX1_1.6*) were used for *Agrobacterium* culture. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic against *Agrobacterium*. Appropriate concentration of kanamycin was used as selectable agents in the regeneration media.

2.4 Methodology

2.5 Preparation of stock solutions for MS media

In the present study, Murashige and Skoog (MS) medium (1962) was used for tomato tissue culture. Different components were required for the preparation of stock solution in MS media (Table 2.2).

2.5.1 Preparation of stock solution-macro nutrients (10X)

All the components of Macro-stock measured in 10X amount (Table. 2.2) and dissolved in half of the total volume of distilled water. One component was added after complete dissolve of the previously added one. Then desired volume (1000 ml) was made by adding distilled water. The solution was poured into a clean container and tagged. Finally the solution was autoclaved (Model: WAC-47, Korea) and stored in a refrigerator at 4ºC for several weeks.

2.5.2 Preparation of stock solution-micro nutrients (100X)

Components (mentioned in Table 2.2) were added one by one and stirred till dissolved. Final volume (1000 ml) was made by adding distilled water. After autoclaving it was stored at 4ºC for some weeks.

2.5.3 Preparation of stock solution-iron EDTA (100X)

$FeSO_4.7H_2O$ (27.8 mg/l) was added in distilled water and stirred in hot plate till dissolved. After that $Na_2EDTA.2H_2O$ (37.3 mg/l) was added and again stirred in hot

plate till dissolved. The solution was made 1liter and preserved at 4ºC in amber bottle as it is light sensitive.

2.5.4 Preparation of stock solution-organic nutrients (100X)

The components of organic nutrients were added one by one and stirred in magnetic stirrer (no hot plate) before adding next. The final volume was made 1000 ml and it was stored at 4ºC.

2.5.5 Preparation of stock solution- growth hormones

2.5.6 Preparation of stock solution-BAP (1mg/10ml)

The BAP (Sigma) stock solution was prepared by dissolving 10 mg of BAP in 1 ml to 2 ml of 1 N NaOH and made up to 100 ml by additional distilled water. The stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

2.5.7 Preparation of stock solution-IAA (1mg/10ml)

The IAA stock solution was prepared by dissolving 10 mg of IAA (Sigma) in 1 ml to 2 ml of 1 N NaOH and made up to 100 ml by additional distilled water. Finally it was filtered and labeled and was stored below 4°C for several months.

2.6 Preparation of MS medium

Murashige and Skoog (1962) medium (MS) was used as basal tissue culture medium for tomato regeneration. All components were added to a volumetric flask and the volume was made up to 1000 ml with ddH_2O (Table. 2.3). The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl as needed. For solid medium agar (Sigma) was added in 0.8% (w/v) ratio. The media was divided into conical flasks in required amount then it was autoclaved (Model: WAC-47, Korea). Media were stored in culture room at 25ºC for few days.

2.7 Sterilization of media

A certain volume of medium was prepared, heated to dissolve agar or phytagel and then dispensed into conical flasks. These flasks were tagged, sealed by non-absorbent cotton plugs, covered by aluminium foil. These media containing flasks

were then autoclaved (ALP-32, Japan) at 15 lb/sq inch at 121°C temperature for 15 minutes.

2.8 Preparation of germination media

For seed germination and seedling development, solid MS medium without any hormone supplementation was used.

2.9 Preparation of shoot regeneration media

For shoot regeneration, solid MS medium supplemented with BAP (2mg/l) was used.

2.10 Preparation of media for subculture

Regenerated explants needed to be subculture in every 4-6 weeks but here this was done in 2-3 weeks depending on the expansion of explants. Media with same hormonal supplementation was used to subculture the regenerated explants.

2.11 Preparation of root induction media

The basal medium for rooting was half strength MS medium. The medium was supplemented by 0.1 mg/l IAA. For solidification, 0.6% (w/v) phytagel (Sigma) was used in root formation media.

Table 2.2 Composition of stock solutions in MS media

Macronutrients	mg/l	Micronutrients	mg/l
KNO_3	1900	KI	0.83
$NH_4 NO_3$	1650	H_3BO_3	6.2
$MgSO_4 \cdot 2H_2O$	370	$MnSO_4 \cdot 4H_2O$	22.3
$CaCl_2 \cdot 2H_2O$	440	$ZnSO_4 \cdot 7H_2O$	8.6
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
		$CuSO_4 \cdot 5H_2O$	0.025
		$CoCl_2 \cdot 6H_2O$	0.025
Fe-EDTA	**mg/l**	**Organic**	**mg/l**
$FeSO_4 \cdot 7H_2O$	27.8	Nicotinic acid	0.5
$Na_2EDTA.2H_2O$	37.3	Pyridoxin HCI	0.5
		Thaimin HCl	0.1
		Glycin	2.0

Table 2.3 Different components for preparation 1 litre of MS media

Components (stock concentration)	Amount (for 1000 ml)
Macronutrients (10x)	100 ml
Micronutrients (100x)	10 ml
Fe- EDTA (100x)	10 ml
Organic nutrients (100x)	10 ml
Sucrose	30 g
Myo-inositol	0.1 g

2.12 Precaution to maintain an aseptic condition

To maintain aseptic condition, all inoculation was carried out under the Laminar Air Flow Hood (SCV-AI, Singapore). UV light of laminar hood was put "ON" for 20-30 minutes. Then the laminar hood was cleaned with 70% ethanol spray and hands were sterilized with antimicrobial hand wash (Hexisol®, ACI Ltd.). The instruments (forceps, scalpel, Petri-dish etc.) were sterilized by using a spirit lamp to prevent air borne bacteria and immersed into absolute alcohol during manipulation. The flask and Petri-dish covers were flamed twice, once after opening and again before closing them. Then the flasks were covered with para-films for two times. All pipettes were disposed and reused after autoclaved. Antibiotics were added to the media under laminar air flow hood, when required. All contaminants and old bacterial culture were discarded after autoclaving to maintain bio-safety procedure.

2.13 Axenic culture

2.14 Sterilization of seed

The work was carried out under the environment of laminar air flow hood. At first the tomato seeds were subjected to surface sterilization by immersing them in 70% ethanol for 5 min. Then it was decanted and followed by addition of about 20 ml of 30% Clorox with two drops of Tween-20 to the seeds. Seeds were then shaked by rotatory shaker for 5 minutes. After that seeds were rinsed well with autoclaved distilled water for three times or more, to remove any trace of sterilant. Finally the seeds were kept in a rotatory shaker (Model: WIS-20, Korea) at 28°C with 120 rpm for overnight to remove the gelatinous layer around the seeds. After overnight shaking, sterilized seeds were directly transferred on to germination media.

2.15 Germination of seeds

Seeds were allowed to germinate at 25±2°C with 16h photoperiod. Time required for seed germination and seedling development was recorded.

2.16 Culturing of leaf explants and regeneration of shoots

Cotyledonary leaves from 8 to 12 days old seedlings were collected as explants and cut into small pieces which were placed to the medium for regeneration. The explants were placed in abaxial orientation with spacing of 1.5 cm between them.

Results were recorded according to the presence of shoot after 45-60 days of inoculation. Regularly cultures were monitored for contamination and also response of regeneration.

2.17 Transferring of explants to fresh media

Regenerated explants were subcultured into fresh media containing the same hormonal supplement for further proliferation and development. Subculture was performed regularly at an interval of 2-4 weeks for maintenance. Cultures were routinely examined for different morphogenic development and data were recorded soon after 10-12 days of inoculation.

2.18 Transferring of explants to fresh media for root induction

Well developed shoots around 3-4 cm long, were placed individually in the root induction medium to obtain sufficient root formation. Data were recorded after 10-15 days of placement in the root induction media.

2.19 Procedure of plant acclimatization in natural environment

Acclimatization is required to achieve adaptation of the regenerated plantlets to the natural environment. Following steps were taken in the process,

a. The regenerated plantlets were carefully removed from the rooting media using forceps when the roots were 6 to 8 cm long. The phytagel attached to their root part was gently washed with running tap water. It was done to make sure that the entire phytagel was removed completely to avoid any contamination and to ensure nutrients uptake by roots from soil.
b. The soil was autoclaved before pouring into pots where the plants were transferred. Perforated plastic bags were taken to cover the potted plantlets. The inside of the bags were sprayed with water to maintain the humidity and to prevent moisture shock. Plantlets were kept inside the culture room for 15 to 20 days. During these days the moisture inside the bags were maintained constantly. Liquid MS medium was given to the plants as water supplement.
c. After 20 days the bags were removed and the plantlets were kept for next 15 days inside culture room.

d. About four weeks after transplantation, plants were then kept in a shade place outside the culture room each day for 2 hours for 1 week.
e. On the eighth week, the plants were exposed to direct sunlight for 2 hours a day. This treatment was continued for 2 more weeks. Lastly the plants were placed in natural environment.
f. At this stage leaves were dark green than it was before and stem had secondary thickness. Finally the plants were transferred to pots containing soil and peat (3:1) in net house.

2.20 Analysis of reproductive response of the regenerated plantlets

Following acclimatization of regenerated plantlets survivability, flowering and fruiting response in natural environment were assessed by fruit weight, number of fruits per plant etc. Seeds were collected and germination was tested.

2.21 Viability test of seeds

Seeds were sterilized and incubated overnight on a shaker incubator. The seeds were dried on sterilized filter paper and transferred to solidified germination media in 25±2°C with 16h photoperiod. Time required for germination initiation and regeneration percentage was recorded.

2.22 Antibiotics used in transformation of tomato

Four antibiotics were used in this study. For *Agrobacterium* culture, three antibiotics (streptomycin and spectinomycin (Duchefa Bioc) for *Agrobacterium* strain LBA4404 and kanamycin for plasmid *pBI121*, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6*) were used. Kanamycin (Duchefa Bioc) was also used as selectable agent to screen out the non-transformed explants. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic against *Agrobacterium*.

2.23 Preparation of stock solution-antibiotics (25 mg/ml)

Stock solutions of kanamycin sulfate, streptomycin, spectinomycin and cefotaxime were prepared. 1 g of each was dissolved in 35 ml of ddH_2O and the volume was made up to 40 ml with ddH_2O. The solutions were sterilized by syringe filtration, poured in autoclaved eppendorf and finally stored at -20ºC.

2.24 Preparation of culture media for *Agrobacterium tumefaciense*

Yeast Extract Peptone (YEP) medium was prepared to culture *Agrobacterium tumefaciense* strain LBA4404 (Table 2.4). The pH of the medium was set at 7.0-7.2 and the volume was made up to 1 litre. Then agar 0.6% (w/v) was added to prepare solid media. After cooling down the autoclaved media, antibiotic were added. *Agrobacterium tumefaciens* containing each of the vectors, kanamycin was added at 200 mg/l, 100 mg/l streptomycin and 200 mg/l spectinomycin were added to each 100 ml YEP media.

2.25 Preparation of co-cultivation media

MS medium with BAP was used as co-cultivation media. Hormonal concentration that was found best for tissue culture of tomato varieties was added to this media. No antibiotics were added here.

2.26 Preparation of media for kanamycin or cefotaxime sensitivity test

Regeneration media with different concentrations of kanamycin or cefotaxime was used for plant sensitivity tests.

2.27 Preparation of selection media

For transformed shoot selection, cefotaxime along with kanamycin was used with regeneration media. The regeneration media contained best hormonal concentration found in plant tissue culture experiment.

2.28 Determination of baseline saline tolerance level of tomato seedlings

In the present study, the effect of salinity on germination of tomato seeds was investigated as it is the first stage towards salinity tolerant transgenic tomato production. Therefore, five local varieties, namely, BARI tomato 2, BARI tomato 3, BINA tomato 2, BINA tomato 3 and Bahar were selected for the study.

To represent various salinity conditions, different NaCl concentrations ranging from 5-200 mM, which in turn stands for 0.5-20 dS/m were chosen for the study (Table 2.5). MS media was prepared with different amount (5 mM, 10 mM, 20 mM, 50 mM and 100 mM) of NaCl in each 100 ml of media. Seeds were placed in these media and the result was recorded after a week to get their germination response in different salt concentrations.

Table 2.4 Components for YEP medium preparation

Components	**Amount (g) for 1 liter media**
Yeast extract	10.0
Peptone	10.0
Sodium Chloride (NaCl)	5.0

Table 2.5 NaCl concentrations and amount in 100 ml media used in salinity test

NaCl concentration in MS media		**Amount of NaCl added in MS media**
5 mM	0.5 dS/m	0.02925 g
10 mM	1 dS/m	0.0585 g
20 mM	2 dS/m	0.117 g
50 mM	5 dS/m	0.2925 g
100 mM	10 dS/m	0.5852 g
200 mM	20 dS/m	1.1704 g

2.29 Transformation of tomato

In the present study, *Agrobacterium*-mediated transformation of five tomato varieties was carried out by two different method of transformation with two different explants.

2.30 Procedure of transformation of cotyledonary leaf explants

Day 1: YEP solid media was prepared with required antibiotics (kanamycin for *Agrobacterium* strain with *pBI121* and both streptomycin and spectinomycin for *Agrobacterium* strain with other three plasmid vectors for *Agrobacterium* stock maintenance.

Day 2: A single colony of engineered *Agrobacterium tumefaciense* was streaked on an antibiotic containing YEP media plate with a sterilized inoculation loop. The Petri-dishes were sealed with Para-film and kept upside down at 28°C for 72 hours and after that stored at 4°C to control overgrowth of bacteria. The subculture of bacteria containing plate was done in fresh media in every month to maintain the stock.

Day 3: Liquid YEP medium was prepared for liquid culture of bacteria. MS media with hormonal supplements was prepared for preculturing of explants, co-cultivation and transferring explants after infection.

Day 4: Single colony was picked from *Agrobacterium* culture to inoculate with an inoculation loop in 50 ml of antibiotic containing liquid YEP media and the liquid culture was kept in a shaker (180 rpm) at 28°C for overnight. Cotyledonary leave explants were cut and placed in regeneration media for pre-culture.

Day 5: Optical Density (OD_{600}) of the overnight grown culture was measured by spectrophotometer at 600 nm, where autoclaved fresh liquid YEP media was used as blank. The zero time absorbance of culture density was obtained from the blank.

The Petri-dish with filter paper is soaked with liquid MS media and then it was used to cut explants. Explants were dipped in bacterial suspension for 15 to 30 minutes infection and then placed on co-cultivation media and kept there for next 24 to 72 hours (co-cultivation period).

Day 6: The Petri-plates were checked for bacterial overgrowth.

Day 7: If there is any bacterial overgrowth shown on explants, then those explants were washed with cefotaxime and transferred to cefotaxime containing MS media. After 2 weeks, explants were placed on kanamycin containing regeneration media to allow the transformed explants to grow.

Then selected healthy shoots were transferred to the rooting media. Non-infected explants were placed on regeneration media for comparative studies of regeneration between transformed and non transformed plants.

2.31 Procedure of *in planta* transformation of whole seed explants

In planta is an indirect transformation technique which is mediated by *Agrobacterium tumefaciens* and tissue culture independent.

2.31.1 Pricking of seeds

This portion of experiment was based on the protocol of Eimert *et al.* (1992). Seeds of tomato were pricked with a sterile 25G needle at the embryonic region for one to three times.

Prior to pricking the needle was dipped in *A. tumefaciense* solutions with individual vectors. The incisions for tomato consisted of targeting the embryonic region of the tomato seed, as indicated by a raised region on the testa (Fig. 2.2). The raised region is termed as plumule where the cotyledons first emerge. The needle tip penetrated the seed no more than 1-2mm deep (Fig. 2.3).

2.31.2 *In planta* transformation of whole seed

Day 1: Single colony was picked from *Agrobacterium* culture to inoculate with an inoculation loop in 50 ml of liquid YEP media containing appropriate antibiotic and the liquid culture was kept in a shaker (180 rpm) at 28°C for overnight.

Day 2: Optical density (OD_{600}) of the overnight grown culture was measured by spectrophotometer, where autoclaved fresh liquid YEP media was used as blank. The zero time absorbance of culture density was obtained from the blank.

Whole seed explants both pricked and non-pricked were dipped in bacterial suspension for 15 to 30 minutes infection and then placed on co-cultivation media and kept there for next 24 to 72 hours (co-cultivation period).

Day 3: The Petri-plates were checked for bacterial overgrowth.

Day 4: If there is any bacterial overgrowth shown on seed explants, then those seed explants were washed with cefotaxime and transferred to cefotaxime containing germination (MS) media.

Non-infected seed explants were placed on germination media for comparative studies of regeneration between transformed and non transformed plants. The culture room was maintained at 25-28°C under a 16h photoperiod with fluorescent light. In each experiment, 50 seeds were infected and the experiments were repeated multiple times.

One month old putative transgenic plantlets were transferred to pots containing autoclaved soil moistened with liquid MS medium or distilled water. The plantlets were allowed to grow under growth room conditions for at least 45-60 days before they were transferred to the net house. The plants were allowed to mature and produced seeds for the regeneration of T_1 plants.

2.32 Analysis of regeneration expression of the transformed plantlets

The seedlings regenerated from putative transformed seeds were subjected to three different aspects (Fig. 2.4).

Firstly, the seeds infected with the vector *pBI121* were subjected to the method of Jefferson (1987) for the assessment of *uid*A gene expression in the tissues of primary transformants.

Secondly, putatively transformed shoots were selected through antibiotic selection; these seedlings were allowed to grow on natural environment. The plants were allowed to mature and produced seeds for the regeneration of T_1 plants. The plant material as leaves, shoots, shoot-tips were also needed for molecular analysis of the putative transformants.

Lastly, micropropagation (clonal propagation through tissue culture) of cotyledonary leaves from the putative transformed seedlings was tried. It was done for the multiplication of genetically identical copies of a cultivar by asexual manner. By this method, it is possible to produce plants in large numbers starting from a single individual.

2.33 Methodology of cefotaxime sensitivity test

To study the effect of cefotaxime on the regeneration of tomato cotyledonary leaf explants, regeneration media containing various concentrations (0, 50, 100, 150 and 200 mg/l) of cefotaxime were added to autoclaved regeneration media after cooling down inside laminar air flow hood and then it was divided into Petri-dishes. Ten explants were subjected to each concentration of cefotaxime. The cotyledonary leave explants were placed in those media to check their regeneration response. The result was recorded after 30 days of inoculation of cotyledonary leaf explants in BINA tomato 2, BINA tomato 3, BARI tomato 2 and Bahar.

2.34 Methodology of kanamycin sensitivity test

To study the effect of kanamycin on the growth of tomato cotyledonary explants, regeneration media containing various concentrations (0, 7.5, 12.5, 25, 50, 100, 150 and 200 mg/l) of kanamycin were added to autoclaved regeneration media after cooling down inside laminar air flow hood and then it was divided into Petri-dishes. Ten explants were subjected to each concentration of kanamycin. The cotyledonary leave explants were placed in those media to check their regeneration response. The result was recorded after 25-30 days of inoculation of cotyledonary leaf explants in BINA tomato 2, BINA tomato 3, BARI tomato 2, BARI tomato 14 and Bahar.

2.35 Bioassay

2.35.1 Preparation of histochemical reagent (X gluc) solution

A clean pyrex tube was taken to take 10 mg of X-Gluc (β- glucuronide, cyclohexylaminonium salt, $C_{14}H_{13}BrClNO_7$. $C_6H_{13}N$, 1mg/ml) which was dissolve in 100 µl of dimethyl formamide (DMF). Volume was made up to 10 ml with 50 mM phosphate buffer, pH was adjusted to 7.0. X-Gluc solution was stored in dark container at -20°C.

2.35.2 Histochemical GUS assay

Tissue segments from the putative transformed seedlings were immersed in fixation solution in sterile eppendorf tubes and incubated for overnight. Then the solution was discarded and washed the tissue at least three times with 50 mM phosphate buffer, pH 7.0. Enough X-Gluc solution was added to cover the tissue pieces in eppendorf tubes. Incubated at 37°C overnight and allow the blue color to develop. X-Gluc solution was discarded and 70% ethanol was added and again incubated at 37°C for 48 hours for degreening. Slides of transformed explants were prepared for observing under microscope.

The plantlet, which was used to collect the plant tissue, is then transferred to pots for acclimatization to natural environments.

2.36 Leaf disc assay for tolerance against salinity stress

Leaf disk assay was carried out to evaluate the sensitivity of the transformed and untransformed tomato plants to sodium chloride (NaCl) stress as described by Fan *et al.*,(1996). Fully developed healthy leaves of wild-type and transgenic plants (of similar age, about 50 days old) were washed with double distilled water. Leaf disks (~1 cm diameter) were excised and floated on 100 mL of NaCl solution (5 mM, 10 mM, 20 mM, 50 mM and 100 mM) and 0 mM NaCl (sterile distilled water) was used as an experimental control for 14 days (Table. 2.4). The same were kept under the standard photoperiod at 25°C. The effect of salt treatment on leaf disks was observed by monitoring phenotypic changes, as browning, bleaching and freshness of leaves.

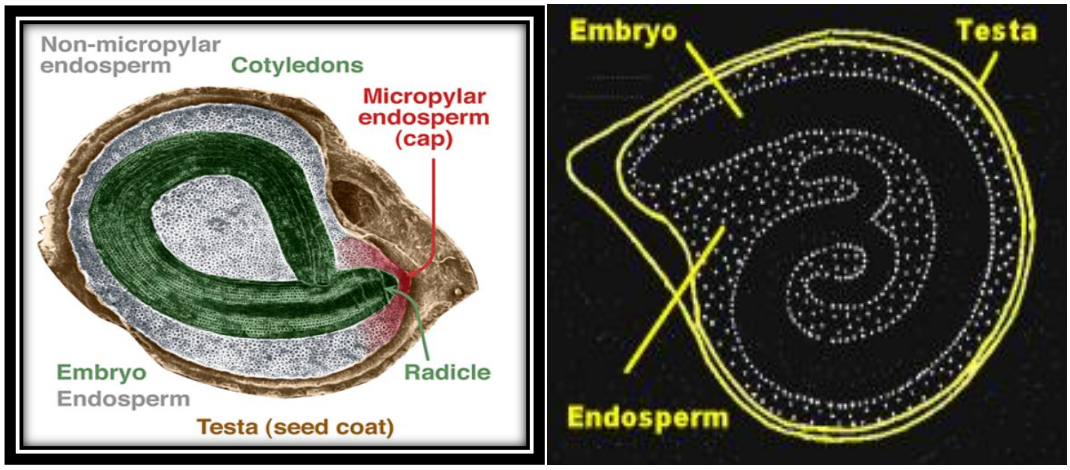

Fig. 2.2 Diagram of pricking sites on *Solanum lycopersicon* Mill.

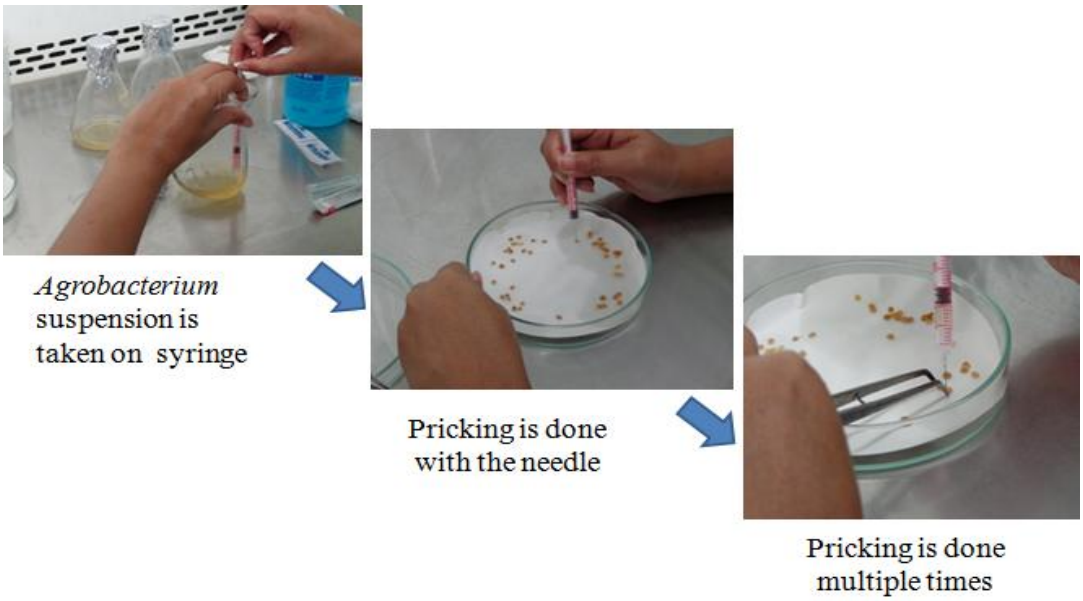

Fig. 2.3 Process of pricking on whole seed of tomato

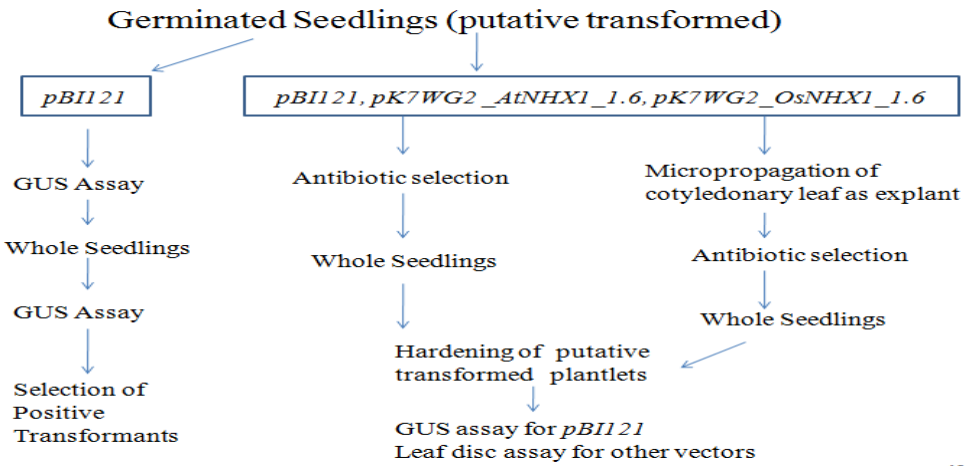

Fig. 2.4 Steps followed after *in planta* transformation

Results

3.1 Tissue culture of cotyledonary leaves

Current study was conducted in three phases. The work started with a regeneration assessment study, followed by *Agrobacterium* mediated transformation of cotyledonary leave explants and ended at establishment of a reproducible *in planta* transformation protocol. Five farmer popular varieties, named as BARI tomato 2, BARI tomato 3, BINA tomato 2, BINA tomato 3 and Bahar with three *Agrobacterium* vectors were used for transformation experiment.

In vitro regeneration skills are fundamental tools for present plant improvement programs and the plant regeneration protocol is a pre-requisite for the plant genetic transformation studies. A standard tomato regeneration protocol that has been used by several researchers for tissue culture of a number of tomato varieties of Bangladesh have been followed in this piece of work to assess the regeneration response of BINA tomato 2, while the other varieties have already been subjected to the regeneration protocol. All the varieties have been subjected to *Agrobacterium* mediated transformation and their results are going to be discussed here.

3.1.1 Regeneration of tomato varieties

The objective of this section was to test out the response of selected tomato varieties to the established protocol and review of regeneration response. Here, BINA tomato 2 variety was subjected to test its capability for *in vitro* regeneration for the first time along with other varieties that have been assessed earlier. Successful regeneration was regeneration in all the tested varieties. In case of BARI tomato 3, relatively lower regeneration (82%) was observed. Among the five varieties, maximum regeneration was observed in BARI tomato 2 (96%) and BINA tomato 2 (96%) (Table 3.1).

During this study, regenerated shoots were transferred to root induction medium for rhizogenesis. Lowest root induction (80%) was found in Bahar. But, both BARI tomato 2 and BINA tomato 2 gave good response (Table 3.2). Morphologically tap root was found in all the varieties with a slight difference in root length among the varieties.

Table 3.1 Regeneration of tomato varieties in MS media containing 2mg/l BAP

Tomato varieties	Time required for regeneration initiation (days)	Mean no. of shoots ± SD	Time required for shoot development (days)	Time required for root development (days)
BARI tomato 2	18	9.6±0.51	15	15
BARI tomato 3	18	8.2±0.31	17	16
BINA tomato 3	15	9.1±0.21	15	16
Bahar	16	9.4±0.25	17	16
BINA tomato 2	**18**	**9.6±0.35**	**15**	**15**

Values were obtained from triplicate trials.

Data were taken 50 days after inoculation.

Table 3.2 Rhizogenesis of tomato varieties in root induction media containing 2 mg/l BAP with 0.1 mg/l IAA

Tomato varieties	Time required for root induction (days)	Percentage of rhizogenesis response	Average root length (cm)	Types of root	Survival rate of plants in natural environment (%)
BARI tomato 2	15	90	8-10	Tap roots, long	88
BARI tomato 3	16	85	6-8	Tap roots	84
BINA tomato 3	16	85	6-8	Tap roots	82
Bahar	16	80	4-6	Tap roots	76
BINA tomato 2	**15**	**90**	**8-10**	**Tap roots, long**	**90**

Values were obtained from triplicate trials.

Fig 3.1 Regeneration response of various tomato varieties on MS media containing 2 mg/l BAP for shoot initiation and rhizogenesis on MS media containing 0.1 mg/l IAA in BARI tomato 2 (A, B); BARI tomato 3 (C, D); BINA tomato 2 (E, F); BINA tomato 3 (G, H); Bahar (I, J) (Photographs of shooting and rooting taken 45 and 60 days after inoculation, respectively).

3.1.2 Acclimatization and survival of regenerated plantlets to natural environment

Mature plantlets were transplanted to the soil in small pots and covered with pierced poly bag for adaptation process (Fig 3.2). During hardening stage (Table 3.3), maximum success was obtained in BINA tomato 3 (80%) and the lowest in Bahar (40%). In the natural environment, survival rate of the varieties was found similar when they were relocated to larger pots and shifted to net house (Fig 3.3).

Transplanted plants flowered and set fruit in natural environment. Transplantation took place in October-November and all of them flowered within 3-4 weeks. All the plants set fruits 15 to 20 days after flowering (Fig 3.3) and 4 to 5 weeks were needed for maturation (Fig 3.3). Fruits were obtained in December-January. Records on time requirement for reproductive cycle are presented in Table 3.4. Maximum number of fruits with highest number of seeds was produced by BINA tomato 3 (Table 3.4).

3.1.3 Viability test of seed collected from matured fruits produced by regenerated plants

During seed viability test, all varieties showed good germination responses. BINA tomato 2 gave the highest germination rate (95%) while Bahar showed the lowest (80%) (Table 3.5). No significant difference was observed within parent seed stock and seeds from regenerated plants.

3.2 Transformation of cotyledonary leaves

In this study effect of optical density (OD_{600}) and incubation period on transformation efficiency was checked with *Agrobacterium* vector *pBI121*, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* in five tomato varieties.

Table 3.3 Regenerated plantlets of tomato in soil environment

Tomato Varieties	Percentage of survival during acclimatization	No. of plants survived in soil	Percentage of success in natural environment
BARI tomato 2	70	7	70
BARI tomato 3	70	7	70
BINA tomato 2	70	7	70
BINA tomato 3	80	8	80
Bahar	40	4	40

10 plantlets of each variety were transferred to soil for this experiment.

Table 3.4 Analysis of reproductive responses of regenerated plantlets

Tomato varieties	Time to get flowering (days)	Time to get fruits (days)	No. of fruits set on plants	Days for maturation of fruits	Average fruits weight (gm)	Average number of seeds per fruit
BARI tomato 2	107±15.28	20±5.52	4	35±5.03	16	90
BARI tomato 3	95±5.01	17±2.65	2	35±4.72	14	70
BINA tomato 2	104±15	16±2.08	5	39±5.29	18	85
BINA tomato 3	112±17.56	15±2.08	10	29±5.13	35	80
Bahar	117±25.17	21±5.03	2	34±4.58	12	65

Fig 3.2 Acclimatization of plantlets. BARI tomato 2 (A); BINA tomato 2 (B) and BINA tomato 3 (C).

Fig 3.3 Flowering and fruit setting in regenerated plants. Flower of BARI tomato 2 (A), BINA tomato 2 (B), BINA tomato 3 (C); Fruit formation in BARI tomato 2 (D), BARI tomato 3 (E), BINA tomato 2 (F), BINA tomato 3 (G) and Bahar (H); Ripened fruit of BINA tomato 2 (I).

Table 3.5 Viability response of regenerated seed through germination in five varieties of tomato

Tomato variety	Mean no. of germinated seeds ±SD		Percentage of germinated seeds		Days required for germination	
	Parent seed	Testing seed	Parent seed	Testing seed	Parent seed	Testing seed
BARI tomato 2	44±1.3	43±2.1	86	85	5-6	4-5
BARI tomato 3	42±1.5	41±2.3	84	82	4-5	5-6
BINA tomato 2	45±2.3	48±1.3	93	95	5-6	4-5
BINA tomato 3	44±1.9	45±1.5	92	90	4-6	6-7
Bahar	43±1.7	40±1.7	85	80	8-9	8-10

Average values are from three trials. 50 seeds were tested in each trial. Data were collected within two week of inoculation.

3.2.1 Transformation parameter analysis

3.2.2 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency

For *Agrobacterium* vector *pBI121*, OD_{600} 0.6-0.8 confers better transformation with incubation period of 30 minutes, in BARI tomato 2, BARI tomato 3, and BINA tomato 2. Maximum regeneration on selection was found at OD_{600} 0.6-0.8 in BINA tomato 2 (48%), BARI tomato 3 (47%), and BARI tomato 2 (44%). BINA tomato 3 expressed 52% regeneration and Bahar gave 36% regeneration on selection at OD_{600} 1.0-1.2 with 15 minute incubation time respectively (Table 3.6).

When, all the five tomato varieties were infected with *pK7WG2_AtNHX1_1.6*, OD_{600} 0.6-0.8 with 30 minutes incubation period, BARI tomato 3, BINA tomato 2 and Bahar (42-47%) showed similar regeneration frequency. However, slight higher regeneration was found at OD_{600} 1.0-1.2 in BINA tomato 3 (48%), with 15 minute incubation time (Table 3.7) whereas BARI tomato 2 showed same response (48%) at OD_{600} 0.6-0.8 with 15 minutes incubation period.

Maximum regeneration after transformation with the vector *pK7WG2_OsNHX1_1.6* at OD_{600} 0.6-0.8 with incubation period of 15 minutes was showed in BARI tomato 2, BINA tomato 2 and Bahar (Table 3.8). Maximum regeneration on selection was found at OD_{600} 1.0-1.2 in BINA tomato 3 with 15 minute incubation time (Table 3.8) whereas BARI tomato 3 experienced better response at OD_{600} 0.6-0.8 with 30 minutes incubation period. Therefore, OD_{600} and infection time found to have varied response in the tested varieties.

Table 3.6 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency with *Agrobacterium* vector *pBI121*

Tomato Varieties	Optical density (600 nm)	Incubation period (min)	Regeneration efficiency (%) on selection media
BARI tomato 2	**0.6-0.8**	15	37
		30	**44**
	1.0-1.2	15	39
		30	42
BARI tomato 3	**0.6-0.8**	15	33
		30	**47**
	1.0-1.2	15	35
		30	42
BINA tomato 2	**0.6-0.8**	15	33
		30	**48**
	1.0-1.2	15	35
		30	42
BINA tomato 3	0.6-0.8	15	35
		30	38
	1.0-1.2	**15**	**52**
		30	40
Bahar	**0.6-0.8**	**15**	**36**
		30	28
	1.0-1.2	15	25
		30	22

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 50mg/l kanamycin along with 100mg/l cefotaxime.

Table 3.7 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6*

Tomato varieties	Optical Density (600 nm)	Incubation period (min)	Regeneration efficiency (%) on selection media
BARI tomato 2	**0.6-0.8**	**15**	**45**
		30	44
	1.0-1.2	15	38
		30	42
BARI tomato 3	**0.6-0.8**	15	37
		30	**44**
	1.0-1.2	15	39
		30	42
BINA tomato 2	**0.6-0.8**	15	33
		30	**47**
	1.0-1.2	15	35
		30	42
BINA tomato 3	0.6-0.8	15	35
		30	42
	1.0-1.2	**15**	**48**
		30	33
Bahar	**0.6-0.8**	15	32
		30	**42**
	1.0-1.2	15	38
		30	35

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 50mg/l kanamycin along with 100mg/l cefotaxime.

Table 3.8 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency with *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*

Tomato varieties	Optical density (600 nm)	Incubation period (min)	Regeneration efficiency (%) on selection media
BARI tomato 2	**0.6-0.8**	**15**	**35**
		30	32
	1.0-1.2	15	27
		30	31
BARI tomato 3	**0.6-0.8**	15	33
		30	**45**
	1.0-1.2	15	38
		30	35
BINA tomato 2	**0.6-0.8**	**15**	**52**
		30	40
	1.0-1.2	15	38
		30	42
BINA tomato 3	0.6-0.8	15	35
		30	38
	1.0-1.2	**15**	**52**
		30	40
Bahar	**0.6-0.8**	**15**	**36**
		30	28
	1.0-1.2	15	25
		30	22

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 50mg/l kanamycin along with 100mg/l cefotaxime.

3.2.3 Influence of 24 hours of co-cultivation period on regeneration efficiency

The time of co-cultivation has immense influence in transformation efficiency and following regeneration capability. In this study, co-cultivation time of 24 hours found to be the best for BARI tomato 3, BINA tomato 2 and Bahar while 48 hours found to be best for BARI tomato 2 and BINA tomato 3. But the varieties showed a varied range of regeneration with different vectors. Co-cultivation time of 24 hours gave better regeneration in case of, BINA tomato 2 (47%) and Bahar (42%) with the vector *pK7WG2_AtNHX1_1.6* whereas in BINA tomato 3, best regeneration (52%) came from 48 hours of co-cultivation with the vector *pK7WG2_OsNHX1_1.6* (Table 3.9).

Some incidence showed that extended co-cultivation period (about 48 hours) was found to encourage overgrowth of bacteria on the infected explants and also explants undergo adverse physical condition showing browning at the cut ends. As a result, these explants failed to regenerate. Hence, 24 hours of co-cultivation was preferred as best for all the five varieties tested with three different vectors.

3.2.4 Effect of pre-culture on transformation efficiency

Pre-culture is the preparation of explants made prior to infection by *Agrobacterium*. The non per-cultured and pre-cultured explants were subjected for transformation experiment. The pre-cultured explants were noticed to regenerate faster and performed better than the non-pre-cultured explants on 50 mg/l kanamycin selection media without showing any variation in transformation efficiency. The difference in regeneration response between pre-cultured and non-pre-cultured explants varied from 1% to 6% on average (Table 3.10).

3.2.5 Determination of antibiotic concentrations for selection medium

Antibiotics are used in selection medium to prohibit regeneration of chimeric and non-transformed plantlets.

Table 3.9 Influence of co-cultivation period on transformation efficiency

Tomato varieties	Co-cultivation period (hours)	Regeneration efficiency (%) on 50 mg/l kanamycin selection media		
		pBI121	*pK7WG2_AtNHX1_1.6*	*pK7WG2_OsNHX1_1.6*
BARI tomato 2	24	43	38	36
	48	**44**	**45**	**40**
BARI tomato 3	**24**	44	**44**	**45**
	48	**50**	36	32
BINA tomato 2	**24**	**36**	**47**	**42**
	48	28	35	33
BINA tomato 3	24	33.5	37	35
	48	**43**	**48**	**52**
Bahar	**24**	**37.5**	**42**	**36**
	48	25	32	34

Values were obtained from three independent trials of the five tomato varieties with the specified vector. In each trial ten explants were inoculated. Experiment conditions were optical density of 0.6-0.8, incubation time 30 minutes. Data were taken 30 days after infection.

Table 3.10 Effect of pre-culture on transformation efficiency

Tomato varieties	Infected Explants	Days required for regeneration initiation			Regeneration efficiency on 50 mg/l kanamycin selection media (%)		
		A	B	C	A	B	C
BARI tomato 2	Non pre-cultured	20	22	23	40	42	46
	Pre-cultured	**16**	**20**	**19**	**41**	**44**	**48**
BARI tomato 3	Non pre-cultured	23	21	24	45	45	41
	Pre-cultured	**15**	**18**	**20**	**50**	**48**	**42**
BINA tomato 2	Non pre-cultured	19	23	22	46	43	44
	Pre-cultured	**14**	**18**	**17**	**48**	**45**	**46**
BINA tomato 3	Non pre-cultured	20	22	23	41	42	40
	Pre-cultured	**17**	**16**	**19**	**43**	**48**	**42**
Bahar	Non pre-cultured	25	24	23	35	41	43
	Pre-cultured	**18**	**17**	**20**	**38**	**44**	**45**

Values were obtained from three independent trials of the five varieties with the specified vector. In each trial ten explants were inoculated. Experiment conditions were optical density of 0.6-0.8, incubation time 30 minutes, co-cultivation time 24 hours. Data were taken within 30 days after infection.

The letter "A" notifies vector *pBI121*; "B" notifies vector *pK7WG2_AtNHX1_1.6* and "C" notifies vector *pK7WG2_OsNHX1_1.6.*

3.2.6 Kanamycin tolerance test for selection of transformed tissue

Sensitivity of explants towards different concentrations of kanamycin (0, 7.5, 12.5, 25 and 50 mg/l) was examined. It was observed that, in the control trial (without kanamycin), all the incubated explants survived while survival percentage of the explants dropped gradually with the raise of kanamycin concentrations in the regeneration media (Fig 3.4). The explants became albino at 7.5 mg/l kanamycin. In 12.5 mg/l of kanamycin they became brown which was followed by death of explants at 50 mg/l (Table 3.11).

Therefore, following transformation experiment 50 mg/l kanamycin was used in selection media. Explants which stayed alive at this concentration for at least one month would be considered as transformed.

3.2.7 Cefotaxime sensitivity test

Bacteriostatic antibiotic, cefotaxime was used in this study to control *Agrobacterium* growth after co-cultivation period. To analyze the effect of cefotaxime in the morphogenesis of plant tissue, cotyledonary leaf explants were subjected to five different concentrations, starting from 0 mg/l, which gradually increased to 50 mg/l, 100 mg/l, 150 mg/l and 200 mg/l (Fig 3.4).

Here, the minimum tolerance level of explants was examined in the specified concentrations of antibiotic. The explants regenerated in presence of cefotaxime upto 100 mg/l. In 150 mg/l of cefotaxime they become albino which was followed by necrosis of explants at 200 mg/l. So, 100 mg/l cefotaxime used in media as it was found to be the most favorable for morphogenesis (Table 3.12).

Table 3.11 Effect of various kanamycin concentrations on the regeneration of non-transformed control cotyledonary leaf explants of tomato

Kanamycin concentrations (mg/l)	Percentage of regeneration response	Percentage of survival	Visual appearance
0	70	90	Green, Healthy
7.5	**15**	**0.15**	**Albino**
12.5	08	0.08	Albino
25	02	0.02	Brown
50	0	0	Necrosis

Ten cotyledonary leaves were subjected in each trial. Values were obtained from three independent trials. These data were collected after 30 days of inoculation and shoot formation rate was recorded after 45 days of inoculation.

Table 3.12 Effect of various cefotaxime concentrations on the regeneration of tomato cotyledonary leaf explants

Cefotaxime concentration (mg/l)	Percentage of shoot formation	Percentage of survival	Visual appearance
0	80	90	Green, Healthy
50	65	80	Green, Healthy
100	75	85	Green, Healthy
150	**45**	**50**	**Albino**
200	35	40	Necrosis

Ten cotyledons were subjected in each trial. Values were obtained from three independent trials. These data were collected after 30 days of inoculation and shoot formation rate was recorded after 45 days of inoculation.

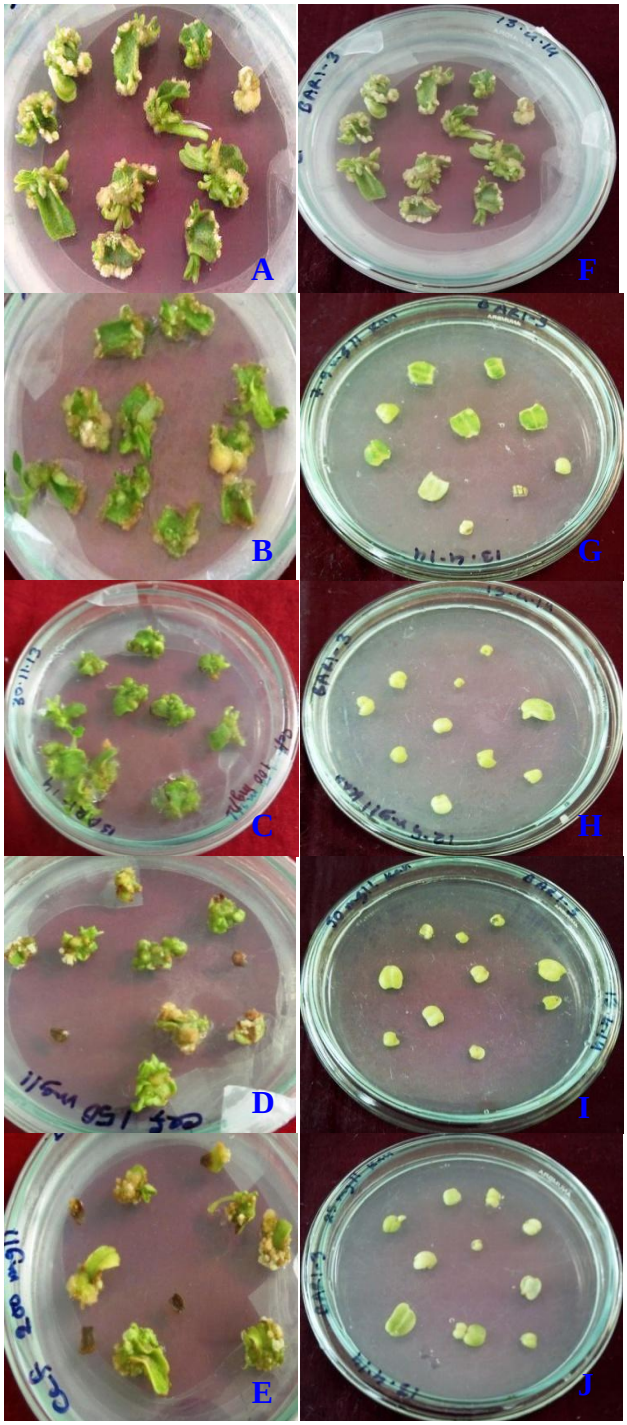

Fig 3.4 Effect of various concentrations of cefotaxime and kanamycin on tomato cotyledonary leaf explants of BARI tomato 3. In left column cefotaxime, A. Control (0 mg/l), B. 50 mg/l, C. 100 mg/l, D. 150 mg/l and E. 200 mg/l; in right column kanamycin, F. Control (0 mg/l), G. 7.5 mg/l, H. 12.5 mg/l, I. 25 mg/l and J. 50 mg/l (Photographs were taken 30 days after inoculation).

3.2.8 Transformation and selection of putative transformed shoots

Following genetic transformation of cotyledonary leaf explants, shoot organogenesis was observed through placing the explants in regeneration media supplemented with kanamycin. After co-cultivation, the treated explants were first placed on media containing 100mg/l cefotaxime to control *Agrobacterium* overgrowth with 50mg/l kanamycin, to get regeneration of putative transformed shoots. The survived explants were then transferred on media containing 100mg/l kanamycin within two to four weeks and the amount of kanamycin was maintained in "on-off" manner in subsequent sub-cultures. Transformation capability was calculated by the mean number of regenerated putative transformed shoots on selection. The control trial (leaf explants without infection) was found to have no regeneration by any means.

In selection media with 50mg/l kanamycin, all the regenerated shoot tips were observed green and healthy. This condition adversely changed when they were transferred to media containing 100mg/l kanamycin. Here, the regenerated shoot tips became brownish. To maintain their regeneration, "on-off strategy" was applied, where the explants were sub-cultured on media supplemented with and without kanamycin in gradual manner at one week interval. But, the explants regeneration drastically reduced at this stage, and no single explant survived after about eight weeks. The experiment was repeated three times, but necrosis was always found.

The explants transformed with the *Agrobacterium* vector *pBI121*, the highest mean number of putative tramsformed shoot found in BINA tomato 2 (3.23) and the lowest found in Bahar (2.20) (Table 3.13) on selection media containing 50mg/l kanamycin with 100mg/l cefotaxime when transformation was performed with the OD_{600} 0.6-0.8 and incubation period of 30 minutes with co-cultivation time of 24 hours for all cultivers (Fig 3.5 A-E).

The reduction of shoot regeneration was observed in selection media with 100 mg/l kanamycin while the other conditions were maintained as same. In this stage, the highest mean number of regenerated shoots found in BINA tomato 2 (0.80) and the lowest found in Bahar (0.20) compared to controled experiment (Fig 3.5 F-J).

In this study, the highest reduction of shoot regeneration observed in Bahar (91%) and lowest reduction observed in BINA tomato 2 (75%) experiment (Table 3.13).

In case of explants transformed with the *Agrobacterium* vector *pK7WG2_AtNHX1_1.6*, the highest mean number of putative tramsformed shoot found in BINA tomato 2 and the lowest found in Bahar and BARI tomato 2 (2.30) (Table 3.14) on selection media containing 50mg/l kanamycin with 100mg/l cefotaxime when transformation was performed with the OD_{600} 0.6-0.8 and incubation period of 30 minutes with co-cultivation time of 24 hours for all varieties (Fig 3.6 A-E)

The reduction of shoot regeneration was observed in selection media with 100 mg/l kanamycin while the other conditions were maintained same. In this stage, the highest mean number of regenerated survived shoots found in BARI tomato 2 and the lowest found in Bahar and BINA tomato 3 (Table 3.14) (Fig 3.6 F-J). In this study, the highest reduction of shoot regeneration observed in BINA tomato 3 compared to controled experiment (Table 3.14).

The explants transformed with the *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*, the highest mean number of putative tramsformed shoot found in BINA tomato 2 (2.95) and the lowest found in Bahar (2.30) (Table 3.15) on selection media containing 50mg/l kanamycin with 100mg/l cefotaxime when transformation was performed with the OD_{600} 0.6-0.8 and incubation period of 30 minutes with co-cultivation time of 24 hours for all varieties (Fig 3.7 A-E).

The reduction of shoot regeneration was observed in selection media with 100 mg/l kanamycin while the other conditions were maintained same. In this stage, the highest mean number of regenerated survived shoots found in BARI tomato 3 (0.60) and the lowest found in Bahar (0.30) (Fig 3.7 F-J). In this study, the highest reduction of shoot regeneration observed in BINA tomato 2 (88%) and BARI tomato 2 (88%) at the same time highest response was observed in BARI tomato 3 (75%) compared to controled experiment (Table 3.15).

As a result of the observation of consistent reduction in survival of putative transformed shoots with the three examining *Agrobacterium* vectors, this investigation was rejected for further trials. The study was switched to the method of *in planta* transformation with tomato matured dried whole seed as explant.

Table 3.13 Regenaration of putative transformed shoots under selection (with *pBI121*)

Tomato varieties	Mean number of shoots in selection (kan 50 mg/l)	Visual appearance of explants	Mean number of shoots in selection (kan 100mg/l)	Visual appearance of explants	Percentage of reduction in shoot number on selection (kan 100 mg/l)
BARI tomato 2	2.50±0.25	Green, healthy	0.50±0.15	Albino, brown	80
BARI tomato 3	2.30±0.35	Green, healthy	0.40±0.18	Albino, brown	82
BINA tomato 2	3.23±0.13	Green, healthy	0.80±0.09	Albino, brown	75
BINA tomato 3	2.80±0.04	Green, Healthy	0.30±0.04	Albino, brown	89
Bahar	2.20±0.18	Green, healthy	0.20±0.08	Albino, brown	91

Values were obtained from three independent trials of the five varieties with the specified vector. In each trial ten explants were inoculated after infection. Experiment conditions were optical density of 0.6-0.8, incubation time 30 minutes, co-cultivation time 24 hours.

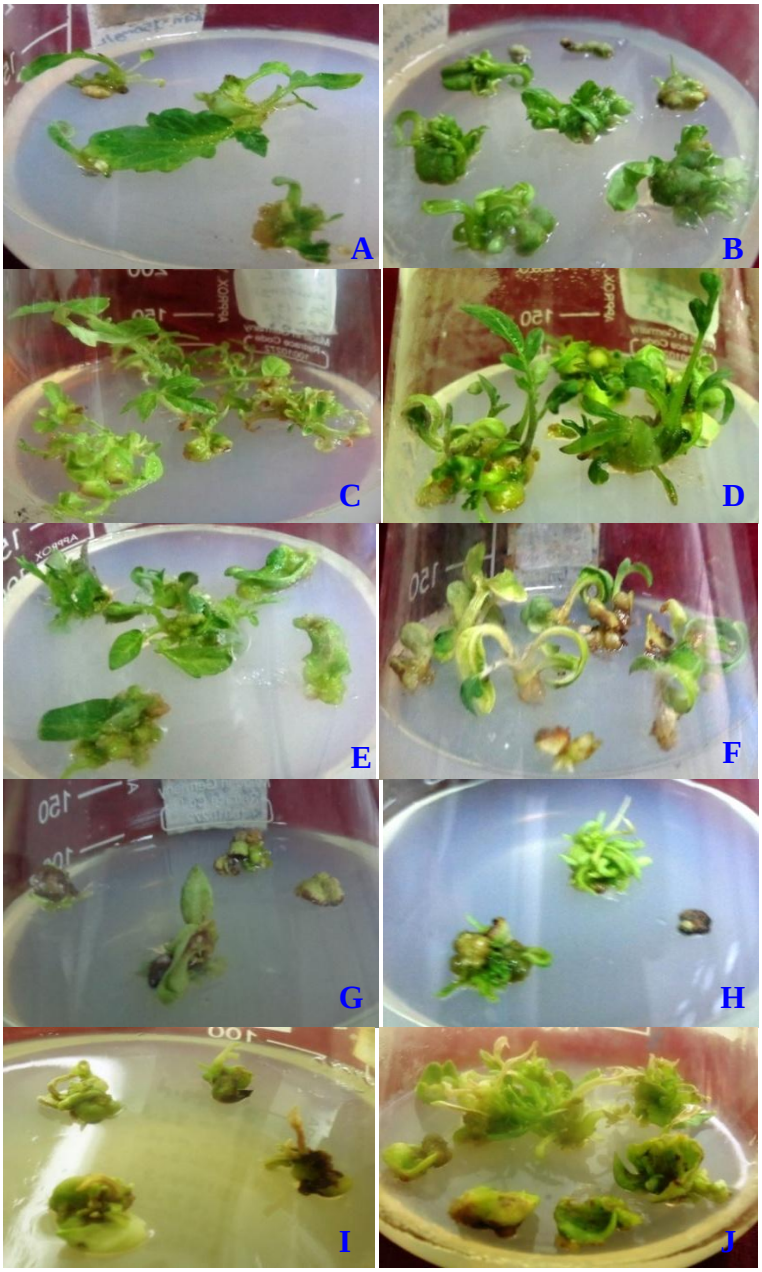

Fig 3.5 Regenaration response of putative transformed shoots under selection (with *pBI121*) on kanamycin selection media (50mg/l) has shown in A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. Regenaration response on kanamycin selection media (100 mg/l) has shown in F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3 and J. Bahar (Photographs were taken after 45 days following infection).

Table 3.14 Regenaration of putative transformed shoots under selection (with *pK7WG2_AtNHX1_1.6*)

Tomato varieties	Mean number of shoots in selection (kan 50 mg/l)	Visual appearance of explants	Mean number of shoots in selection (kan 100mg/l)	Visual appearance of explants	Percentage of reduction in shoot number on selection (kan 100 mg/l)
BARI tomato 2	2.30±0.07	Green, healthy	0.60±0.02	Albino, brown	74
BARI tomato 3	2.40±0.04	Green, healthy	0.40±0.03	Albino, brown	83
BINA tomato 2	3.20±0.13	Green, healthy	0.50±0.02	Albino, brown	84
BINA tomato3	2.50±0.04	Green, Healthy	0.30±0.04	Albino, brown	88
Bahar	2.30±0.04	Green, healthy	0.30±0.06	Albino, brown	87

Values were obtained from three independent trials of the five tomato varieties with the specified vector. In each trial ten explants were inoculated after infection. Experiment conditions were optical density of 0.6-0.8, incubation time 30 minutes, co-cultivation time 24 hours.

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Fig 3.6 Regenaration response putative transformed shoots under selection (with *pK7WG2_AtNHX1_1.6*) on kanamycin selection media (50 mg/l) has shown in A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. Regenaration response on kanamycin selection media (100 mg/l) has shown in F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3 and J. Bahar (Photographs were taken after 45 days following infectiontion).

Table 3.15 Regenaration of putative transformed shoots under selection (with *pK7WG2_OsNHX1_1.6*)

Tomato varieties	Mean number of shoots in selection (kan 50 mg/l)	Visual appearance of explants	Mean number of shoots in selection (kan 100mg/l)	Visual appearance of explants	Percentage of reduction in shoot number on selection (kan 100 mg/l)
BARI tomato 2	2.60±0.07	Green, healthy	0.30±0.04	Albino, brown	88
BARI tomato 3	2.40±0.04	Green, healthy	0.60±0.08	Albino, brown	75
BINA tomato 2	2.95±0.05	Green, healthy	0.35±0.03	Albino, brown	88
BINA tomato 3	2.70±0.06	Green, Healthy	0.50±0.02	Albino, brown	81
Bahar	2.30±0.03	Green, healthy	0.40±0.03	Albino, brown	82

Values were obtained from three independent trials of the five tomato varieties with the specified vector. In each trial ten explants were inoculated. Experiment conditions were optical density of 0.6-0.8, incubation time 30 minutes, co-cultivation time 24 hours.

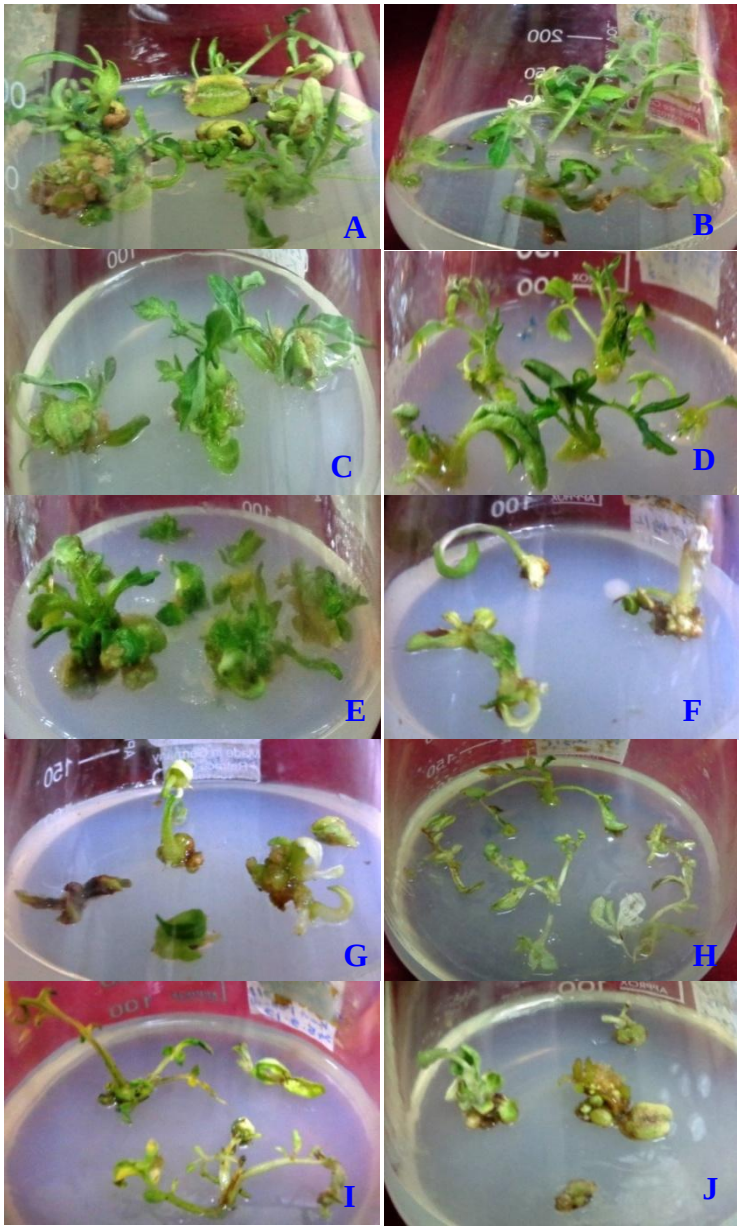

Fig 3.7 Regeneration response of putative transformed shoots under selection (with *pK7WG2_OsNHX1_1.6*) on kanamycin selection media (50mg/l) has shown in A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. Regenaration response on kanamycin selection media (100 mg/l) has shown in F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3 and J. Bahar (Photographs were taken after 45 days following infection).

3.3 *In planta* transformation

3.3.1 Optimization of factors affecting *in planta* transformation efficiency of five tomato varieties

The aim of this study expanded to the development of an easy, tissue culture independent and alternative *A. tumefaciens* mediated transformation method for tomato using seeds as explants. The variable factors involved in *in planta* transformation were evaluated. The three *Agrobacterium* vectors and the five tomato varieties were examined in this study.

3.3.2 Effect of pricking on seed germination efficiency after transformation with the *Agrobacterium* vector *pBI121* in five tomato varieties

The embryonic region of the tomato seed, indicated by a raised region on the testa was targeted for pricking of seeds. It was known, the raised region is the point at which the plumule first emerge. A set of 0 to 3-pricked seeds were allowed to germinate in MS media containing bacteriostatic antibiotic cefotaxime (100mg/l) after transformation with the *Agrobacterium* having vector *pBI121*(Fig. 3.8-3.9). Here, the incubation time of 30 minutes with the co-cultivation period of 24 hours, were maintained throughout this portion of work.

In this experiment, it was noticed that pricked seeds experienced lower rate of transient transformation with lower rate of germination. The survival rate of putative transformed seedlings varied notably between the 0 seeds with pricked seeds. In 0-pricked transformed seeds, 90% germination came from BARI tomato 2, BINA tomato 2, BINA tomato 3, followed by 86% in Bahar and 80% in BARI tomato 3 (Table 3.16). Besides this, maximum GUS positive explants came from BINA tomato 2 and Bahar (95%). The other varieties also gave GUS positive expression of about 90% in an average (Table 3.16).

The treatment of pricking should probably preferred due to the higher rate of GUS positive expression at the initial stages, but it was omitted during further experiment for the lower germination and frequency of GUS positive appearances.

Table 3.16 Effect of pricking on seed germination efficiency following transformation in five tomato varieties

Tomato varieties	No. of prick on seed	Mean no. of germinated seeds ±SD	Percentage of seed germination	GUS % of infected seed
BARI tomato 2	Non-pricked	**45±0.58**	**90**	**92**
	1	10±0.58	20	**45**
	2	10±0.68	20	**33**
	3	18±1.15	36	**40**
BARI tomato 3	Non-pricked	**40±1.52**	**80**	**90**
	1	8±1.15	15	**35**
	2	0	0	**0**
	3	15±1.52	30	**40**
BINA tomato 2	Non-pricked	**45±0.58**	**90**	**95**
	1	30±1.15	60	**40**
	2	16±1.52	32	**32**
	3	43±3.05	86	**20**
BINA tomato 3	Non-pricked	**45±2.08**	**90**	**88**
	1	20±1.13	40	**42**
	2	4±2.45	08	**30**
	3	13±0.57	26	**40**
Bahar	Non-pricked	**43±1.34**	**86**	**95**
	1	10±0.57	20	**25**
	2	18±1.13	36	**75**
	3	13±0.57	26	**40**

The experiment was run with three replicates, each with 50 seeds for each treatment.

Randomly selected 5 cotyledons were assayed for every GUS expression assessment. It was calculated from the percentage of the surviving explants.

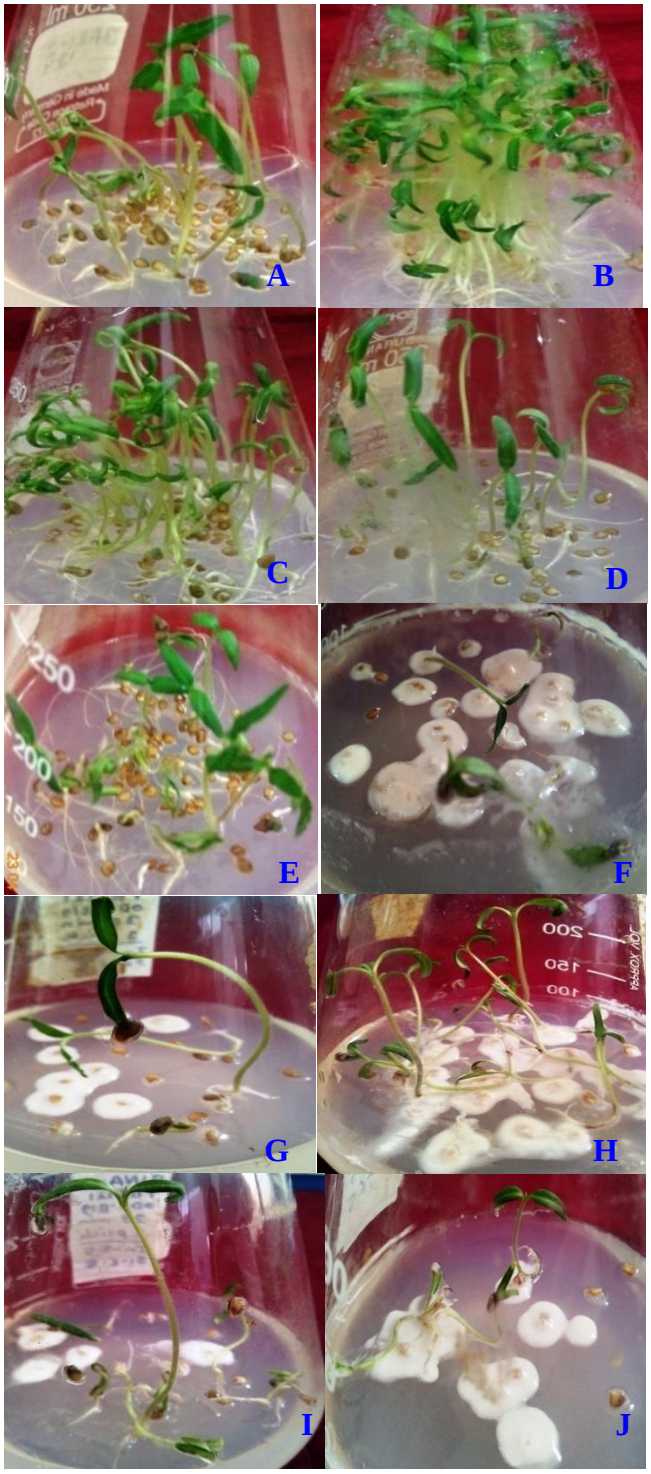

Fig 3.8 Germination of non-pricked seed after infection with *Agrobacterium* vector *pBI121* in five tomato varieties. A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. Germination of one-pricked seed after infection with *Agrobacterium* vector *pBI121* in five tomato varieties. F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3 and J. Bahar. All photos were taken on the 15th day of seed placement in germination media.

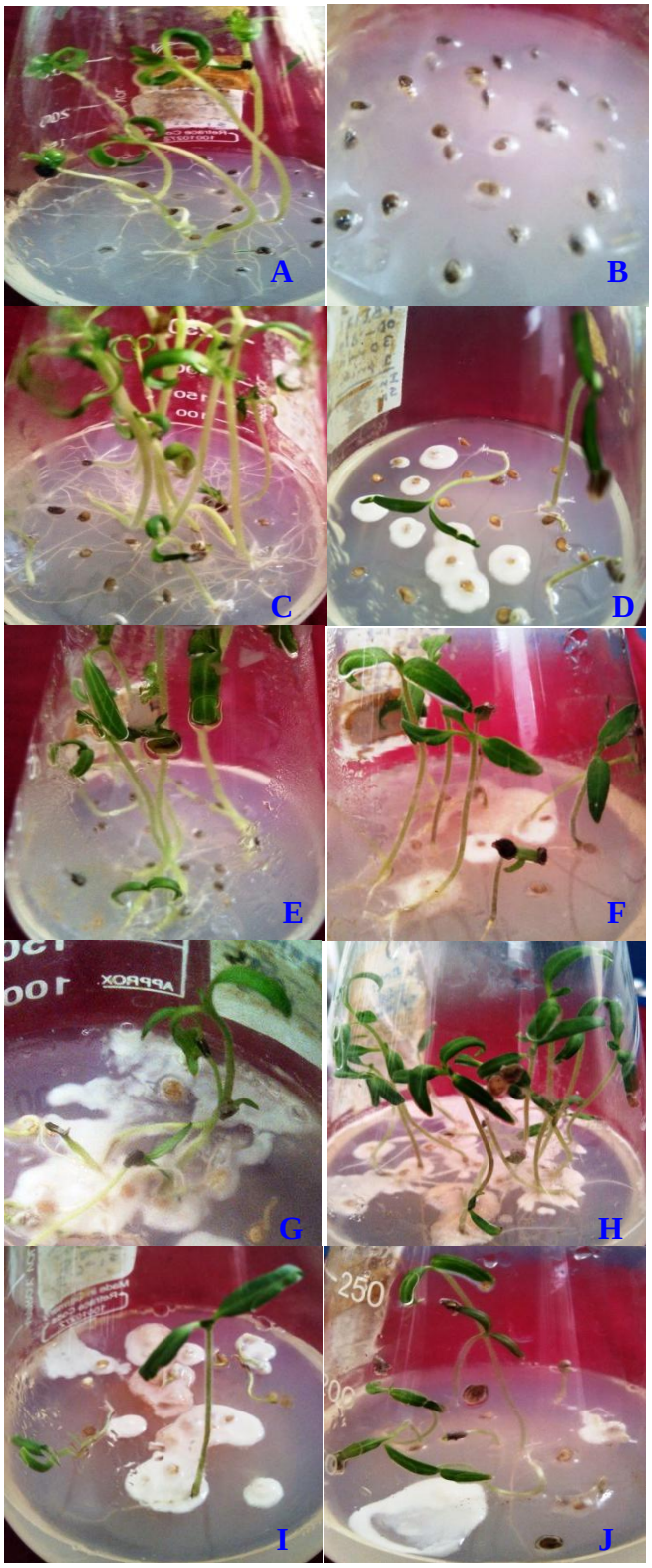

Fig 3.9 Germination of two-pricked seed after transformation with *Agrobacterium* vector *pBI121* in five tomato varieties. A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. Germination of three-pricked seed after transformation with *Agrobacterium* vector *pBI121* in five tomato varieties. A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar. All photos were taken on the 15th day of seed placement in germination media.

In summary, the non-pricked tomato seeds took 17-21 days for germination, after transformation by *Agrobacterium* vector *pBI121*. Here, relatively faster germination occurred in BINA tomato 2 (17 days), while maximum time consumed in Bahar (21days). Maximum GUS positive expression also came from BINA tomato 2 (95%) along with Bahar (95%). The rate followed by BARI tomato 2 (92%), BARI tomato 3 (93%) and BINA tomato 3 (88%). These values were much better than the multiple times pricked seeds transformation technique in this study (Table 3.16). Thus, no pricking of seeds was preferred for *Agrobacterium* vector *pBI121* mediated *in planta* transformation for all five tomato varieties throughout this study.

3.3.3 Effect of different bacterial culture density and incubation period on *in planta* transformation with *Agrobacterium* vector *pBI121, pK7WG2_AtNHX1_1.6, pK7WG2_OsNHX1_1.6* in five tomato varieties

For *Agrobacterium* vector *pBI121*, the five tomato varieties were subjected under a set of optical density ranged from 0.6-1.4, with two different incubation period 15 and 30 minutes. In all the varieties, OD_{600} 1.1-1.4 gave better transformation with incubation period of 30 minutes. Maximum GUS positive explants were found in BARI tomato 3 (95%), immediately followed by BINA tomato 2, BARI tomato 2, BINA tomato 3 (Table 3.17).

At the following stage, the five tomato varieties were subjected under the set of optical density ranged from 0.6-1.4, with a set of incubation period 15 and 30 minutes. All the varieties infected with *pK7WG2_AtNHX1_1.6,* OD_{600} 1.1-1.4 gave better transformation with incubation period of 30 minutes. Maximum germination percentage of putative transformed seeds was found in BINA tomato 2 (96%). Other varieties also showed similar response (Table 3.18).

When *pK7WG2_OsNHX1_1.6* was infected, OD_{600} 1.1-1.4 gave better transformation with incubation period of 30 minutes for all the varieties. Maximum germination percentage of putative transformed seeds was found at in BINA tomato 2 (94%), immediately followed by BINA tomato 3 and BARI tomato 3 (Table 3.19).

Table 3.17 Effect of different bacterial culture density and incubation period on *in planta* transformation with *Agrobacterium* vector *pBI121* in five tomato varieties

Tomato varieties	OD_{600}	Incubation period (min)	GUS % of transformed seed
BARI tomato 2	0.6-1.0	15	62
		30	77
	1.1-1.4	15	85
		30	**92**
BARI tomato 3	0.6-1.0	15	65
		30	78
	1.1-1.4	15	81
		30	**95**
BINA tomato 2	0.6-1.0	15	66
		30	72
	1.1-1.4	15	82
		30	**94**
BINA tomato 3	0.6-1.0	15	74
		30	80
	1.1-1.4	15	83
		30	**90**
Bahar	0.6-1.0	15	62
		30	66
	1.1-1.4	15	72
		30	**86**

Values were presented from three independent experiments, each with 50 seeds for each treatment. Randomly selected 5 cotyledons were assayed for every GUS expression assessment. It was calculated from the percentage of the surviving explants.

Table 3.18 Effect of different bacterial culture density and incubation period on *in planta* transformation with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6* in five tomato varieties

Tomato varieties	OD_{600}	Incubation period (min)	Germination efficiency (%)
BARI tomato 2	0.6-1.0	15	82
		30	86
	1.1-1.4	15	85
		30	**94**
BARI tomato 3	0.6-1.0	15	78
		30	81
	1.1-1.4	15	83
		30	**92**
BINA tomato 2	0.6-1.0	15	77
		30	85
	1.1-1.4	15	82
		30	**96**
BINA tomato 3	0.6-1.0	15	75
		30	79
	1.1-1.4	15	81
		30	**92**
Bahar	0.6-1.0	15	82
		30	89
	1.1-1.4	15	84
		30	**93**

Values were presented from three independent experiments, with 50 seeds for each treatment.

Table 3.19 Effect of different bacterial culture density and incubation period on *in planta* transformation with *Agrobacterium* vector *pK7WG2_OsNHX1_1.6* in five tomato varieties

Tomato varieties	OD_{600}	Incubation period (min)	Germination efficiency (%)
BARI tomato 2	0.6-1.0	15	77
		30	80
	1.1-1.4	15	82
		30	**86**
BARI tomato 3	0.6-1.0	15	80
		30	85
	1.1-1.4	15	84
		30	**90**
BINA tomato 2	0.6-1.0	15	83
		30	89
	1.1-1.4	15	84
		30	**94**
BINA tomato 3	0.6-1.0	15	82
		30	89
	1.1-1.4	15	84
		30	**93**
Bahar	0.6-1.0	15	62
		30	66
	1.1-1.4	15	72
		30	**86**

Values were presented from three independent experiments, with 50 seeds for each treatment.

3.3.4 Effect of co-cultivation period on *in planta* transformation efficiency

A noticeable difference was observed in various co-cultivation periods ranging from 24 to 72 hours in the germination efficiency and the time required for putative transformed seed germination. In all the varieties, transformed with the *Agrobacterium* vector *pBI121* with co-cultivation period of 72 hours gave faster germination of seeds. For example, in BARI tomato 2 it is 6d, BARI tomato 3 (9d), BINA tomato 2 (5d), BINA tomato 3 (10d) and Bahar (14d), when compared with the time required for seed germination in co-cultivation period of 24 hours. Here, the time for seed germination required were as follows; in BARI tomato 2 (15d), BARI tomato 3 (16d), BINA tomato 2 (14d), BINA tomato 3 (16d) and Bahar (14d) (Table 3.20).

Though 72 hours of co-cultivation period of seeds gave faster germination, the germinated seedlings gave a lower survival rate during hardening of putative transformed plants, in comparison with the survival rate with lower co-cultivation period. Moreover, the putative transformed seedlings with 24 h and 48 h co-cultivation period were healthier than the putative transformed seedlings with 72 h co-cultivation period.

3.3.5 Effect of co-cultivation period on germination efficiency with vector *pK7WG2_AtNHX1_1.6* in five tomato varieties

Likewise, all the varieties, transformed with the *Agrobacterium* vector *pK7WG2_AtNHX1_1.6* with co-cultivation period of 72 hours gave faster germination of seeds, when compared with the time requirement for germination following 24 hours of co-cultivation (Table 3.21).

3.3.6 Effect of co-cultivation period on germination efficiency with vector *pK7WG2_OsNHX1_1.6* in five tomato varieties

The tomato varieties, when transformed with the *Agrobacterium* vector *pK7WG2_OsNHX1_1.6* with co-cultivation period of 72 hours the germination time was less, compared with the time required for seed germination after co-cultivation time of 24 hours. Days needed range from 6-11 days for BARI tomato 2 (8d), BARI tomato 3 (6d), BINA tomato 2 (8d), BINA tomato 3 (10d) and Bahar (11d) (Table 3.22).

Table 3.20 Effect of co-cultivation period on germination efficiency with *Agrobacterium* vector *pBI121* in five tomato varieties

Tomato varieties	Co-cultivation period (hours)	Mean no. of germinated seeds ±SD	Day required for germination	Germination efficiency (%)
BARI tomato 2	**24**	**45±1.15**	**15**	**90**
	48	27±1.52	14	54
	72	44±0.58	06	88
BARI tomato 3	**24**	**42±1.42**	**16**	**84**
	48	22±1.13	12	44
	72	42±1.15	09	84
BINA tomato 2	**24**	**44±1.42**	**14**	**88**
	48	42±0.58	10	84
	72	40±1.34	05	80
BINA tomato 3	**24**	**46±1.08**	**16**	**92**
	48	35±2.16	12	70
	72	43±1.42	10	85
Bahar	**24**	**37**±1.14	**14**	**74**
	48	15±1.52	10	30
	72	32±0.57	14	64

Values were presented from three independent experiments, each with 50 seeds for each treatment.

Table 3.21 Effect of co-cultivation period on germination efficiency with vector *pK7WG2_AtNHX1_1.6* in five tomato varieties

Tomato varieties	Co-cultivation period (hours)	Mean no. of germinated seeds ±SD	Day required for germination	Germination efficiency (%)
BARI tomato 2	24	44±1.15	14	88
	48	40±1.52	11	80
	72	**45±1.21**	**06**	**90**
BARI tomato 3	**24**	**46±1.51**	**14**	**92**
	48	45±1.34	13	90
	72	45±0.57	11	90
BINA tomato 2	24	43±2.16	16	86
	48	46±2.44	09	92
	72	**45±1.47**	**06**	**90**
BINA tomato 3	24	43±1.05	16	86
	48	43±2.16	14	86
	72	**45±1.34**	**11**	**90**
Bahar	**24**	**45±2.52**	**15**	**90**
	48	08±2.47	12	16
	72	10±1.41	15	20

Values were presented from three independent experiments, each with 50 seeds for each treatment.

Table 3.22 Effect of co-cultivation period on germination efficiency with vector *pK7WG2_OsNHX1_1.6* in five tomato varieties

Tomato varieties	Co-cultivation period (hours)	Mean no. of germinated seeds ±SD	Day required for germination	Germination efficiency (%)
BARI tomato 2	**24**	**44±2.08**	**16**	**88**
	48	43±0.57	12	86
	72	40±1.15	08	80
BARI tomato 3	**24**	**47±2.52**	**15**	**94**
	48	45±1.13	10	90
	72	40±0.57	06	80
BINA tomato 2	24	40±1.53	17	80
	48	47±1.15	12	94
	72	**45±2.08**	**08**	**90**
BINA tomato 3	**24**	**42±1.42**	**17**	**84**
	48	43±1.14	12	86
	72	40±1.05	10	80
Bahar	**24**	**32±1.38**	**16**	**64**
	48	15±1.41	13	30
	72	30±1.15	11	60

Values were presented from three independent experiments, each with 50 seeds for each treatment.

3.3.7 Comparative analysis of the vectors' efficiency on effect of co-cultivation period

After the optimization of co-cultivation period, an analysis of individual vector's efficiency toward transformation was checked. As mentioned earlier, 72 hours of co-cultivation period, gave speedy germination from infected seeds. However, the putative transformed seedlings failed to stay alive in natural environment. All the varieties acted better with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6* than *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*, except BINA tomato 3 (Table 3.23). On the basis of varied survival rate; co-cultivation period of 24 hours was selected as the best co-cultivation time for transformation of tomato seeds by *in planta* techniques.

3.3.8 Acclimatization and development of regenerated plantlets to natural environment

Full-grown rooted plantlets were transplanted to the soil in small pots and covered with pierced poly bag for adaptation process (Fig. 3.10). During hardening maximum success was obtained in BINA tomato 2 (75%) while the lowest in Bahar (40%) (Table3.25). The survival rate of all varieties were found satisfactory when they were relocated to larger pots and shifted to net house (Fig. 3.10).

Transplanted plants flowered in natural environment. Plantlets transferred to the month of April-May flowered in 3-4 weeks. But they could not survive more than two months due to extreme heat and stormy weather during summer season. Thus, no seeds could be set.

Table 3.23 Comparison of vectors' efficiency on effect of co-cultivation period

Tomato varieties	Average time required for germination (day)	Co-cultivation period (hours)	Germination efficiency (%)		
			pBI121	*pK7WG2 _AtNHX1 _1.6*	*pK7WG2 _OsNHX1 _1.6*
BARI tomato 2	15	24	92	94	86
	14	48	54	80	86
	06	72	88	90	80
BARI tomato 3	16	24	95	92	90
	12	48	44	90	90
	09	72	84	90	80
BINA tomato 2	14	24	94	96	94
	10	48	84	92	94
	05	72	80	90	90
BINA tomato 3	16	24	90	91	93
	12	48	70	86	86
	10	72	85	90	80
Bahar	14	24	86	93	86
	10	48	30	16	30
	14	72	64	20	60

Values were presented from three independent experiments, each with 50 seeds for each treatment.

Table 3.24 Survival rate of regenerated tomato plantlets following *in planta* transformation

Tomato Varieties	Percentage of survival during acclimatization (%)	No. of plants transferred to the soil	Percentage of success in natural environment (%)
BARI tomato 2	70	14	35
BARI tomato 3	60	12	25
BINA tomato 2	75	15	35
BINA tomato 3	65	13	25
Bahar	40	08	20

20 plants of each variety were taken for acclimatization

Fig 3.10 Acclimatization and development of regenerated plantlets to natural environment transformed with *Agrobacterium* vector in all five varieties.
Mature plants infected with *pBI121*; A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3, E. Bahar; Mature plants infected with *pK7WG2_AtNHX1_1.6*; F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3, J. Bahar; Mature plants infected with *pK7WG2_OsNHX1_1.6*; K. BINA tomato 2, L. BINA tomato 3, M. Bahar; N. flower and fruit setting in BINA tomato 2; O. flowering in BINA tomato 2.

3.4 Micropropagation of seedlings germinated following *in planta* transformation

Micropropagation is the practice of rapidly multiplying stock plant material by plant tissue culture methods. It can provide a sufficient number of plantlets from a stock plant which may not produce seeds. To preserve the plant material of putative transformed seedlings from "*in planta*" seed transformation, micropropagation of these plantlets was tried. The leaf explant was collected from young cotyledonary leaves from the putative transformed seedlings, germinated after the *Agrobacterium* infection of tomato seeds with three different vectors.

The regeneration media was supplemented with bacteriostatic antibiotic cefotaxime (100mg/l) in initial phases. These work expanded through selection of regenerated explants in antibiotic (kanamycin) selection media, in which kanamycin concentration was kept 50mg/l in on-off manner at seven days interval (in average) throughout the study. The mean number of regenerated putative transgenic shoots on selection was selected to calculate transformation competence. The control trial (cotyledonary leaves from non-infected seedlings) was observed to have no regeneration by any means.

During micropropagation, the cotyledonary leaves from the seedlings transformed with the *Agrobacterium* vector *pBI121*, took 80 days in average to give a putative transformed plantlet under selection. In this phase, the highest mean number of putative transformed shoots came from BINA tomato 2 (3.33), which was followed by BINA tomato 3, BARI tomato 3, BARI tomato 2 and Bahar (Table 3.25). Here, as optimized before kanamycin concentration (50mg/l) for selection, incubation time (30 minutes), co-cultivation time (24 hours) was maintained for all the varieties (Fig. 3.11).

In the case of rhizogenesis from these regenerated shoots, better observation came from the shoots of *pK7WG2_AtNHX1_1.6* (Table 3.26), followed by *pK7WG2_OsNHX1_1.6* (Table 3.27) and *pBI121* (Table 3.25), in all the studied varieties.

Table 3.25 Micropropagation of seedlings germinated following *in planta* transformation with *Agrobacterium* vector *pBI121*

Tomato varieties	Time required for regeneration initiation (days)	Mean number of multiple shoots ±SD	Time required for shoot development (days)	Time required for root development after inoculation. (days)
BARI tomato 2	36	2.33±0.0901	53	62
BARI tomato 3	44	2.40±0.0808	61	66
BINA tomato 2	22	3.33±0.2010	58	70
BINA tomato 3	18	3.20±0.0351	39	56
Bahar	30	2.21±0.1743	45	61

Mean number of putative transformed shoots came from the average number of multiple shoots on selection. Mean value in each experiment is average of three sets of experiment with 50 leaf explants in each set. Data were collected after 60 days of inoculation of explants.

Table 3.26 Micropropagation of seedlings germinated following *in planta* transformation with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6*

Tomato varieties	Time required for regeneration initiation (days)	Mean number of multiple shoots ±SD	Time required for shoot development (days)	Time required for root development (days)
BARI tomato 2	22	2.66±0.0281	32	48
BARI tomato 3	18	2.28±0.0279	29	42
BINA tomato 2	26	2.33±0.1650	35	45
BINA tomato 3	18	2.25±0.1739	38	51
Bahar	24	2.02±0.1115	42	58

Mean number of putative transformed shoots came from the average number of multiple shoots on selection. Mean value in each experiment is average of three sets of experiment with 50 leaf explants in each set. Data were collected after 50 days of inoculation of explants.

Table 3.27 Micropropagation of seedlings germinated following *in planta* transformation with *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*

Tomato varieties	Time required for regeneration initiation (days)	Mean number of multiple shoots ±SD	Time required for shoot development (days)	Time required for root development after inoculation (days)
BARI tomato 2	19	2.40±0.0736	30	45
BARI tomato 3	13	2.55±0.1414	34	44
BINA tomato 2	19	2.72±0.0802	32	46
BINA tomato 3	18	2.62±0.1361	33	45
Bahar	22	2.31±0.01215	44	58

Mean number of putative transformed shoots came from the average number of multiple shoots on selection. Mean value in each experiment is average of three sets of experiment with 50 leaf explants in each set. Data were collected after 50 days of inoculation of explants.

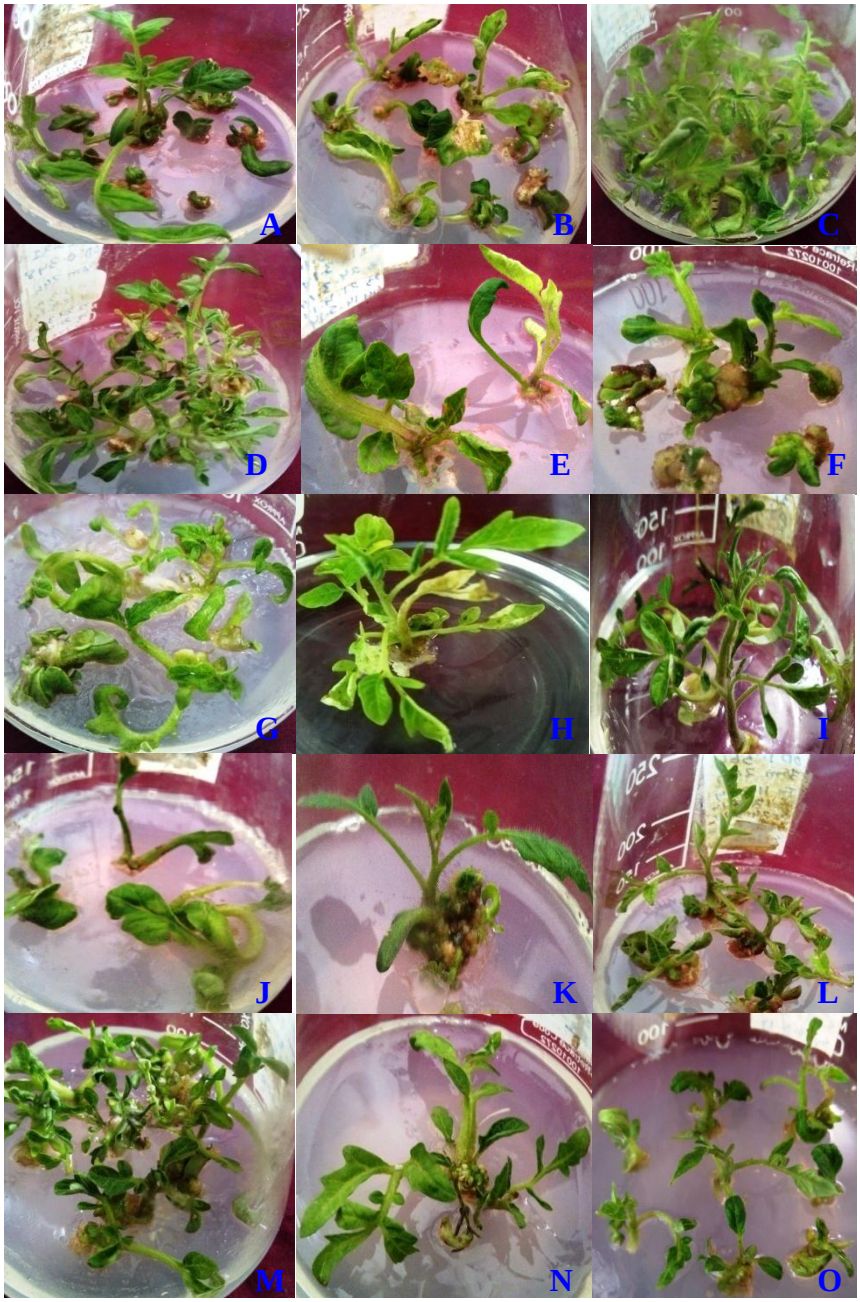

Fig 3.11 Regeneration of cotyledonary leave explants from putative transformed seedlings those were transformed with *Agrobacterium* vector *pBI121*; A. BARI tomato 2, B. BARI tomato 3, C. BINA tomato 2, D. BINA tomato 3 and E. Bahar (all photos were taken on the 80th day of seed placement in germination media); with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6;* F. BARI tomato 2, G. BARI tomato 3, H. BINA tomato 2, I. BINA tomato 3 and J. Bahar (all photos were taken on the 60th day of seed placement in germination media); with *Agrobacterium* vector *pK7WG2_OsNHX1_1*.6; K. BARI tomato 2, L. BARI tomato 3, M. BINA tomato 2, N. BINA tomato 3 and O. Bahar (all photos were taken on the 60th day of seed placement in germination media). Here, kanamycin concentration (50mg/l) for selection, incubation time (30 minutes), co-cultivation time (24 hours) was maintained firmly for all the varieties.

3.5 Salinity stress tolerance test of tomato leaf discs from transformed tomato varieties

To confirm transformation, salinity stress tolerance level of these tomato varieties need to be assessed. In this study, effect of salinity on tomato leaf discs has been analyzed within a time period of 14 day (Table 3.28).

The rate of bleaching increased with the increase of NaCl concentration in liquid MS media for control trials, whereas in transformed plants opposite scenario was observed. A reciprocal relation of time period with the rate of bleaching was also observed in control trials. In case of control trials, faster bleaching was viewed at 100 mM NaCl (within 3 days). The duration extended progressively at lower concentrations of NaCl but no regular pattern was observed, for example- at 20 mM NaCl 9 days was required to bleach the discs while at 50 mM NaCl 5 days was required to bleach the discs in control trials.

Leaf discs from transformed plants found to have a different scenario compared to control trials. The discs remain green at day 14 of the experiment up to 50 mM NaCl. Bleaching of discs started at 100mM NaCl at day 14. The leaf discs remain typical visually within this duration, no sign of bleaching was observed. The rate of bleaching did not increase with the increase of NaCl concentration in liquid MS media. Transformed plants were observed to have differing scenario. There was no relation of time period with the rate of bleaching observed in these trials. In case of control trials, faster bleaching was viewed at 100 mM NaCl within 3 days while leaf discs from transgenic plants were viewed to start bleaching at day 14. Lower concentrations of NaCl were found to create no variation here, which shows the capacity to tolerate salt level upto 50 mM.

Table 3.28 Effect of salinity on tomato leaf discs of transformed seedlings

Salt concentrations (mM)	Visual appearance of control plant	Time needed to bleach	Visual appearance of transformed plant	Time needed to bleach
0 mM	Normal	Not bleached	Normal, Green	Not bleached
5 mM	Bleached	14 days	Normal, Green	14 days
10 mM	Bleached	12 days	Normal, Green	14 days
20 mM	Bleached	9 days	Normal, Green	14 days
50 mM	Bleached	5 days	Normal, Green	14 days
100 mM	Bleached	3 days	Started to bleach	14 days

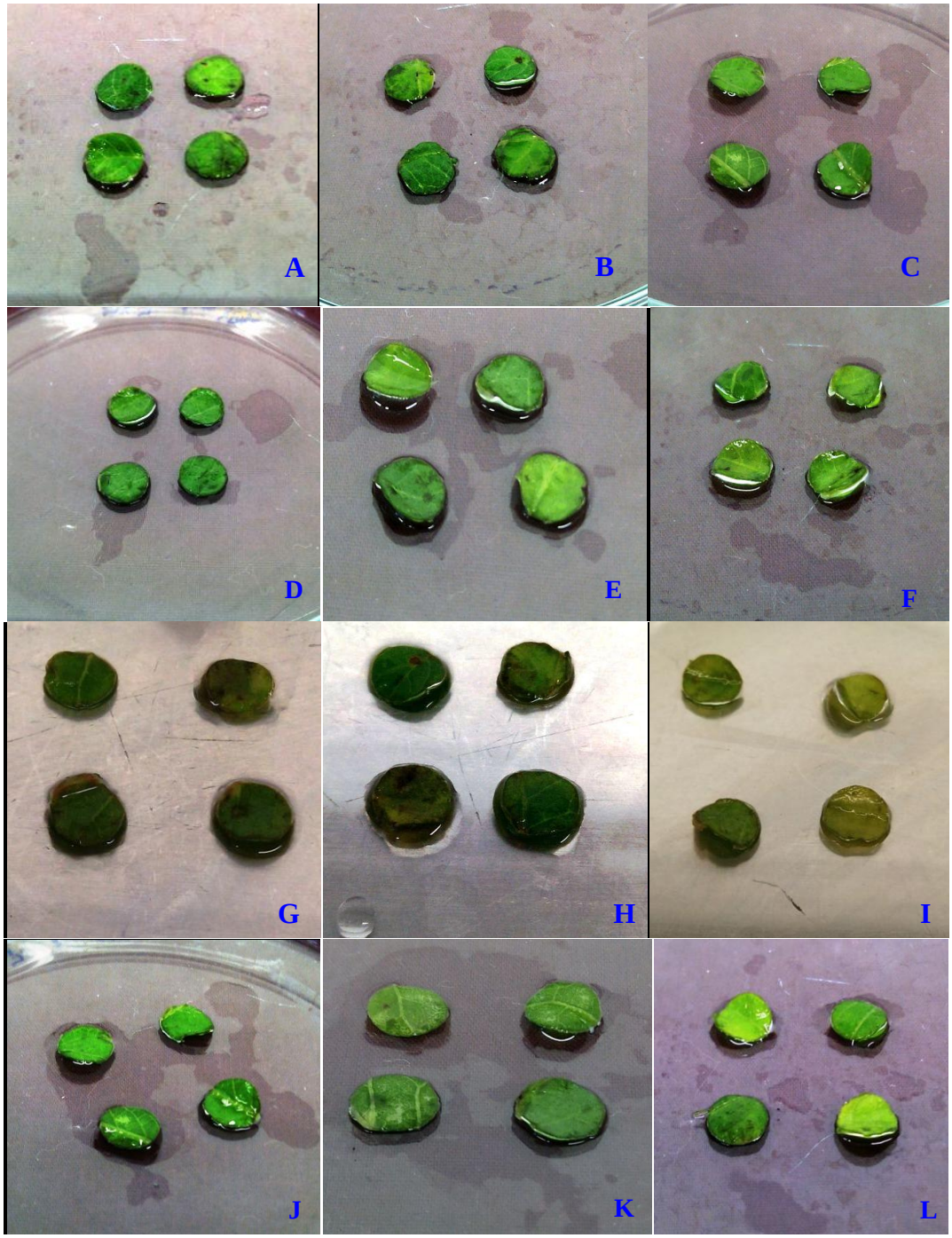

Fig 3.12 Effect of salinity on tomato leaf discs after 14 days; A. non-transformed at 0 mM NaCl, B. non-transformed at 5 mM NaCl, C. non-transformed at 10 mM NaCl, D. transformed at 0 mM NaCl, E. transformed at 5 mM NaCl, F. transformed at 10 mM NaCl, G. non-transformed at 20 mM NaCl, H. non-transformed at 50 mM NaCl, I. non-transformed at 100 mM NaCl, J. transformed at 20 mM NaCl, K. transformed at 50 mM NaCl and L. transformed at 100 mM NaCl.

3.6 Comparative analysis of facts observed in two different transformation methods

Agrobacterium-mediated transformation is both tissue-culture based and non-tissue culture based. In the present study, both the approaches were utilized. A comparison of both the transformation procedures in tomato indicated variations in transformation efficiencies and regeneration of transformants. This was analyzed by measuring different parameters, like; shoot regeneration, yield of transformed shoots and plantlet development etc. In this study, transformation of cotyledonary leaves found to be time consuming and required a large number of explants resulting few transformed lines. Thus, resulted low transformation frequency. In compare to that, a non-tissue culture based *in planta* transformation technique found to give putative transformed seedlings in short time with a higher percentage of transformants requiring less number of explants to obtain transformed plantlets. Therefore, the later remove all the drawback of tissue-culture based transformation method.

Different parameters of tissue-culture based and *in planta* transformations were compared to find out the most suitable transformation method for tomato. Initially, variations were observed during optimization of different transformation parameters. Optimization of OD_{600} found to be varied among varieties, and also for different vectors for both type of transformation technologies. However, infection time and co-cultivation time found to be same for both the techniques. During selection, *in planta* transformation gave above 90% regeneration compared to 25% from leaf transformation. Moreover, cotyledonary leaf failed to root from the putative transformed shoots. Ultimately, these shoots became necrotic after 30 days. On the other hand, seeds after *in planta* transformation germinated, gave shoots, roots and set flower and fruit. Leaf disc collected from these plants also showed salt tolerance upto 100 mM.

To sum up, *in planta* transformation stands over the tissue culture based transformation method for tomato.

3.16 Comparative analysis of facts observed in two different transformation methods

Categories	Tissue-culture based transformation	*In planta* transformation
Explant	Cotyledonary leaves	Whole seed
OD_{600}	0.6-0.8	1.2-1.4
Incubation time	30 min	30 min
Co-cultivation period	24 hours	24 hours
Effect of pre-culturing	Positive approach during transformation	Positive approach during transformation
Number of regenerated shoot	2.30±0.07	3.33±0.2
Time required for shoot development	65 days	35 days
Highest regeneration efficiency in selection (%)	25-35	92-95
Rhizogenesis	Do not appear	Took 45 days to appear
Survival in soil	No survival	Healthy, set flower and fruit
Time required to get transformed plant	No plantlet regenerated	65-70 days
Bioassay	Antibiotic selection 50 mg/l kanamycin	GUS assay, antibiotic selection, leaf disc senescence assay
Salt tolerance	Not tested	100 mM NaCl
Responsive varieties	BINA tomato 2, BINA tomato 3	BINA tomato 2, BINA tomato 3

Micropropagation was done with leaves regenerated from *in planta* transformed seed
Shoot regeneration was dated from inoculation of leaves regenerated from *in planta* transformed seed

Discussion

4.1 Phases of the study

This investigation was carried out in four phases. At first, established protocol of *in vitro* regeneration was revisited for five tomato varieties, namely, BARI tomato 2, BARI tomato 3, BINA tomato 2, BINA tomato 3 and Bahar. In the second phase, transformation experiment was performed on five varieties using cotyledonary leaves as explants with *pBI121* (containing *nptII* marker gene and *uidA* gene) to re-confirm the optimum conditions for transformation. Then, transformation was attempted with other vectors named as *pK7WG2_AtNHX1_1.6*, (containing *AtNHX1*, Na^+/H^+ antiporter gene, cloned from arabidopsis), and *pK7WG2_OsNHX1_1.6* construct (containing *OsNHX1*, Na^+/H^+ antiporter gene, cloned from rice) to obtain transgenic salt tolerant tomato varieties on the basis of optimum conditions determined at initial steps performed with *pBI121*.In the third phase, *in planta* transformation technology was developed; where direct infection was carried out using whole seed as explant, with all the three vectors. Factors affecting this *in planta* transformation were determined. At final phase, analysis of these putative transgenic plants was done through bioassay to confirm transgene expression through antibiotic selection and leaf disc senescence assay.

4.2 Evaluation and assessment of established *in vitro* regeneration protocol

Efficient plantlet regeneration in tomato has been reported from the cotyledonary leaf (Padmanabhan and Paddock, 1974; Behky, 1976; Chaudhry *et al.*, 2010). Several *in vitro* investigations have been conducted on this explant of tomato. They explore the callogenic and regeneration potential to establish a reproducible protocol for shoot induction. This regeneration response found to be highly dependable on genotype, explant and plant growth regulators used in the culture medium (Bhatia *et al.*, 2004; Ishag and Osman, 2009; Chaudhry *et al.*, 2010; Praveen, 2011). Inspired from these findings, present study was conducted to verify regeneration efficiency of the chosen varieties through previously optimized tissue culture protocol.

4.2.1 Shoot regeneration potential

In this study, shoot regeneration was observed to be 96-98% depending on genotype among the five varieties. This observation has been found to be similar with the regeneration potential (90%) reported by Janani and Girija (2013).

Here, only BAP at a concentration of 2 mg/l was found to give shoot proliferation in all five varieties. Ferdous (2012) reported to have maximum number of shoots on the same media supplementation for Bangladeshi varieties, e.g. BARI tomato 2, BARI tomato 3 and BINA tomato 3. Similar observation was also found in other reports as well (Sheeja and Mondal, 2004; Ntui and Iioka, 2009; Mohamed and Ismail, 2010). There were other reports of using BAP with IBA, NAA, GA_3 and Zeatin to have maximum number of shoots (Chaudhry *et al.*, 2010).

4.2.2 Rhizogenesis

The beneficial effect of using half strength MS medium for rooting of *in vitro* induced shoots has already been reported for tomato (Devi, 2008; Chaudhry *et al.*, 2010). All three auxins (IAA, IBA and NAA) reported to cause positive response and resulted towards formation of healthy, well-developed roots. Half strength MS medium supplemented with 0.2 mg/l IAA was reported to be best for rooting by Devi (2008). Half strength of MS medium has been reported as the best rooting medium with addition of IAA (1.0 mg/l) for the induction of longer roots by Sheeja and Mondal (2004).

Similarly, in the present study, typical tap root was found in all the varieties. Moreover, IAA (0.1 mg/l) containing half strength MS media was found to be best for rooting.

4.2.3 Response at the natural environment

According to the report by Hazarika (2003), the transplantation stage is considered to be a major bottleneck in the micropropagation of many plants. Plantlets or shoots that have grown *in vitro* have been continuously been exposed to a unique micro-environment that has been selected to provide minimal stress and optimum conditions for plant multiplication. These contribute to a culture-induced phenotype

that cannot survive the environmental conditions when placed in a greenhouse or field (Hazarika, 2003).

In the present study, all rooted tissue cultured plants flowered and set fruits but they differed slightly with the statistical data mentioned in BARI and BINA websites. The varieties studied here observed to differ in time required to set flower and fruits with the total number of flower and fruits obtained. The reason behind this divergence may be the acclimatization procedure and seasonal variation along with the variation of genotype, which was also reported by Ajlouni (1996), Ashrafuzzaman (2009), Haque and Islam (2010).

Viability test was done with the seeds of these mature fruits which showed high viability. It was seen that the germination response of these seeds was more or less the same as the seeds from naturally grown plants used as parent stock. This demonstrates that the regenerated plantlet raise through this protocol are comparable to their natural parent stock.

4.3 Transformation of cotyledonary leaves

Conditions that influence tomato transformation include the choice and age of explants (Chi, 1987; Sun and Watanabe, 2006), the duration of pre-culture (Paramesh and Fakrudin, 2010), the strain and concentration of the *Agrobacterium tumefaciens* culture used for co-cultivation (Islam, 2007), the length of co-cultivation and medium used (Fillati and Rose, 1987; Hamza and Chupeau, 1993; Park *et al.,* 2003; Cortina, 2004), the orientation (adaxial side up vs abaxial side up) of cotyledon explants on culture media. However, it is important to note that the conditions that result in an efficient transformation system for one genotype, do not always decode into an efficient system for other genotypes (Frary, 1997).

The selected five tomato varieties were tested with *Agrobacterium* strain LBA4404 containing *pBI121*, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* for the determination of factors, like, bacterial concentration, inoculation period, co-cultivation period and antibiotic concentrations for selection.

4.3.1 Optimization of optical density-incubation period

Transformation frequency was found to increase with the increase in optical density of the *Agrobacterium* suspension. Similar trend was reported by Sharma and his colleagues (2009) in three Indian tomato varieties, viz. Pusa Ruby, Sioux and Arka Vikas, and also by Sarker and Islam, (2009) in BARI tomato 2 and Pusa Ruby. Ferdous (2012) reported to have cent percent transformation efficiency at OD_{600} 0.68 in BARI Tomato 3, and BINA Tomato 3. Increase in incubation period beyond a critical time length resulted in decrease in transformation efficiency in all the tested varieties. Such tendency was also reported for BINA tomato 3 and Bahar by Chowdhury (2009). Gao *et al.* (2009) reported 15 min incubation to be effective for infection in tomato. On the other hand, exposure of cotyledonary explants to *Agrobacterium* inoculums of 0.8 $O.D_{600}$ for 30 mins are effective for transformation of tomato cultivar "Summer set" (Siddig *et al.,* 2009). Again, 30 minutes of infection time was also reported to be optimum for tomato varieties Pusa Ruby, Arka Vikas and Sioux, those were transformed with *Agrobacterium* strain, AGL1, carrying either *pCTBE2L* or *pRINASE2L* construct (Sharma *et al.,* 2009). However, Cortina (2004) in tomato cultivar UC82B; Sarker and Islam, (2009) in Pusa Ruby and BARI Tomato 3 preferred prolonged infection time to achieve the same result. In this study, OD_{600} 0.6 with 30 min incubation time was found to be effective for all the varieties with *pBI121* and *pK7WG2_AtNHX1_1.6* while with *pK7WG2_OsNHX1_1.6* better result was achieved in 15 minutes. Though this observation differs in some situations, but it indicates that there is a threshold range of a combination of bacterial concentration and time of infection required for efficient plant transformation.

4.3.2 Optimization of optical density-co-cultivation period

The *Agrobacterium* cell density used for the co-cultivation is equally important factor for the transformation experiments as the explants used for co-cultivation differs in the strength for overcoming the effect of immersion in *Agrobacterium* culture containing medium (Paramesh and Fakrudin, 2010). It was observed that co-cultivation time of 24 hours was found to be the best for BARI tomato 3, BINA tomato 2 and Bahar while 48 hours was found to be the best for BARI tomato 2 and BINA tomato 3.

Effectiveness of 48 hours co-cultivation period is supported by the reports of Patil *et al.,* (2002); Paramesh and Fakrudin (2010); Mythili *et al.,* (2011); Ferdous (2012). On the other hand, for BARI tomato 3 and BINA tomato 3 responded well with 24 hours of co-cultivation period which is similar to Micro-Tom tomatoes (Guo *et al.,* 2012). In this study it was also found that longer co-cultivation period delay growth of transformed explants due to overgrowth of bacteria which is supported by Christophe and Phan, (1999). Prolonged co-cultivation period (more than 72 h) was found to encourage overgrowth of bacteria on the infected explants and also necrosis occurred. Finally, these explants failed to regenerate which is in agreement with previous report by Islam and Chowdhury (2010)**.**

4.3.3 Pre-culturing of explants

According to Paramesh and Fakrudin (2010), the use of pre-cultured cotyledon leaf explants helped not only in easy handling of explants during transformation experiments, but also in reducing the *Agrobacterium* contamination and death before callusing due to insufficient strength of the explants to bear the antibiotic treatments. In this study, pre-culturing the explants for 24 hours before co-cultivation showed very good response compared to that of without pre-culture. However, not all the pre-cultured explants responded equally. It is important to select only those explants for co-cultivation which show expansion in response to the pre-culture medium. When placed with the adaxial side towards the medium, the maximum surface of the cotyledonary leaf explants makes contact with the medium. Therefore, this orientation was used in the present study. Similar orientation was also reported by Sharma *et al.* (2009).

4.4 Application of bacteriostatic antibiotic

High frequency transformation using *Agrobacterium* strains depends not only on the efficiency of the plant's *in vitro* regeneration system but also on the subsequent elimination of bacterial cells from infected tissues.

Antibiotics commonly used for elimination of *A. tumefaciens* after co-cultivation, are cefotaxime (Ismail *et al.,* 2005; Amugune and Anyango, 2011), carbenicillin (Meissner *et al.,* 1997; Mathews, 2003), clavamox (Park *et al.,* 2003), cefotaxime and ticarcillin/potassium clavulanate (Ling and Kriseleit, 1998), ticarcillin and combactum (Sarker and Islam, 2009), augmentin (Sun and Watanabe, 2006), timentin (Schroeder and Muller, 1993; Frary, 1996; Ling and Kriseleit, 1998; Clark and Nell, 1999; Opabode, 2006) etc.

4.4.1 Cefotaxime sensitivity

Cefotaxime, belonging to β–lactam group, have minimal toxicity on most plant tissues and thus have been widely accepted in *Agrobacterium*-mediated transformation (Pollock and Barfield, 1983; Okkels, 1988). There are reports that the addition of cefotaxime may exert an effect, either positive or negative on the growth and regeneration of explants following transformation. Sensitivity of plants to this antibiotic is species specific and depends on plant growth conditions (Lin and Assad, 1995; Joersbo, 1996; Chauvin *et al.,* 1999).

Amugune and Anyango (2011) stated that 250 mg/l cefotaxime was effective for shoot growth and selection of transformed plants in common bean while Rafael *et al.,* (2005) stated 300mg/l was effective for cucumber. The over growth of *Agrobacterium* were controlled with a concentration of 200 mg/l of cefotaxime in BARI tomato 2, BARI tomato 3, BINA tomato 3 varieties (Ferdous, 2012; Ahmad, 2012).

In the present study, 100 mg/l cefotaxime was used in selection media for tomato varieties which prevented bacterial overgrowth entirely without compromising the regeneration potential. Similar observation was also noticed in report by Farajollah and Hashemi (2006) for tomato varieties named KalG, Kal-early and Su2270. This study was supported by Mathias (1986), Borrelli and Di Fonzo (1992) in wheat and Mathias (1987) in barley, where they have found low concentration of cefotaxime enhanced shoot formation along with bacterial control.

However, a higher concentration (500 mg/l) of cefotaxime was used in many tomato varieties, such as, Pusa Ruby (Roy *et al.*, 2006; Mythili *et al.*, 2011), Micro-Tom tomato (Qiu *et al.*, 2007), Riogrande (Afroz *et al.*, 2009) and Money maker (Chaudhury *et al.*, 2010). This divergence is because of variation in antibiotic sensitivity of plants is dependent on species, genotype, physiology of plants and *Agrobacterium* strain (Lin and Assad, 1995; Joersbo, 1996; Chauvin *et al.*, 1999).

4.4.2 Kanamycin sensitivity

Kanamycin is one of the most frequently used selection markers for obtaining transgenic plants (Nap *et al.*, 1992; Anklam *et al.*, 2002; Sundar and Sakthivel, 2008). Neomycin phosphotransferase (*nptII*) gene is the sequence that encodes the enzyme having the ability to inactivate kanamycin (Suratman and Ughude, 2013). In the present study, kanamycin sensitivity was tested and finally 50 mg/l were used for selection.

In several studies, 50 mg/l of kanamycin was used for the selection medium of tomatoes, like, Pusa Ruby (Patil *et al.*, 2002), Shalimar (Janani *et al.*, 2013), tomato lines sp12 and sp5 (Thompson *et al.*, 2000; Riggs *et al.*, 2001). Higher concentration like 100 mg/l of kanamycin was used in tomato variety Arka Vikas (Mythili *et al.*, 2011) and tobacco (Lin *et al.*, 1995). In *Arabidopsis* 50 mg/l kanamycin was used by Li *et al.*, (2007). Urban and fellow researchers (1994) screened transgenic chrysanthemum 'Hekla and Polaris' by using the same amount of kanamycin, while Boase and Bradley (1998), Seo *et al.*, (2003) used 20 mg/l for chrysanthemum 'Peach Margaret' and 'Puma and Subangryeok'. In Bangladesh, 200 mg/l of kanamycin was used by Chowdhury (2009) in Bahar, BINA tomato 3 varieties. Similar concentration of kanamycin (200 mg/l) was reported by McCormick (1991), Ling and Kriseleit (1998), Cortina (2004) and Islam (2007) for positive selection of the transformed tomato tissue. Difference in the optimal concentration of kanamycin, vary with antibiotic type as well as plant species, age and explant type. This observation was supported by (Mathews, 1988; Dekeyser and Claes, 1989).

4.5 *In planta* transformation of tomato seed

In planta transformation procedures have been used successfully for various plant species, e.g., *Arabidopsis thaliana* (Bent, 2000), *Medicago truncatula* (Trieu *et al.*, 2000), apple, pear, peach, strawberry (Spolaore and Trainotti, 2001), and citrus (Ahmad and Mirza, 2005). However, for most plant species, except for *A. thaliana* and *M. truncatula*, only transient transformation has been successfully reported. It has been suggested that the target tissue for these *in planta* transformation procedures could be the gametophyte progenitor tissue, mature gametophyte, or fertilized embryos (Ye *et al.*, 1999; Desfeux and Clough, 2000).

This study was based on a process described by Feldman and Marks (1987). To obtain putative transformed tomato plants, seeds were subjected to *Agrobacterium* infection and allowed to germinate and grow into plant *ex vitro*, where they found to express the transgene. Feldman and Marks (1987) using *Arabidopsis*, Chee and Fober (1998) using soy bean, Rohini and Rao (2000) using peanuts and Ismail *et al.*, (2005) using chili, have demonstrated *in planta* methods of similar nature. In these reports, seeds of T_0 plants were grown in greenhouse, left to mature and seeds collected to produce T_1 regenerants and molecular analysis were carried out in the T_1 generation to study the integration and expression stability of transformed genes. Apart from these reports, Yasmeen *et al.* (2009) reported *in planta* transformation of tomato using fruit injection and floral dip.

In this study, transgene expression was confirmed by GUS histochemical analysis and antibiotic selection. Here, antibiotic selection was done in a different manner, which has not been reported earlier. Cotyledonary leaves from putative transformed germinated seedlings were subjected to micropropagation. These were allowed to regenerate in kanamycin selection media. The micropropagated shoots were placed for root induction and these plantlets were then transferred to natural environment. A swift confirmation of putative transgenic plantlets was provided by this approach. Still, there are no reports on successful *in planta* transformation followed by micropropagation in tomato or any other species.

Finally, leaf disc senescence assay was performed to confirm salt tolerance of the first generation plants, which indicates transgene expression as well.

4.5.1 Determination of baseline salinity tolerance level

Establishment of a baseline salinity tolerance level of untransformed tomato plants was performed through seeds, which were subjected to germinate on media containing different NaCl concentrations ranging from 5-100 mM (0.5-10 dS/m). In present study, germination rate of seedlings fall to 46.4% in presence of 20 mM (2.0 dS/m) NaCl compared to 81.6% in control experiment. Apart from germination rate, the time requirement for germination was also influenced by salinity. Similar report has been found where only a few genotypes were able to germinate at high salt concentration; additionally, it also increase the time for germination (Munns, 1993; Murphy, 2003; Shahid *et al.,* 2011). For example, tomato seeds needed 50% and 100% additional days to germinate at 80 and 190 mM NaCl, respectively, than in a medium without salt (Ayers, 1952). These reports indicated that salinity severely influence the plant physiology.

The tomato plant is very sensitive to salinity, although considerable differences between cultivars may be observed. In coastal saline belt of Bangladesh, 274000 hector areas is covered with salinity level of 4.1-8.0 dS/m (very slightly saline with some slightly saline). Siddiky *et al.* (2012) has reported to observe severe reduction in plant dry matter biomass production during the comparison of response of ten different Bangladeshi tomato varieties to salinity. At 4.1-8.0 dS/m these varieties showed adverse physiological conditions except for BARI tomato 2 which showed moderate tolerance at this level.

4.5.2 Marker gene selection

According to Guo and colleagues (2012), transformation frequencies of tomato are depended on various selection marker genes present within the engineered plasmid. For this reason, in the present study transformation experiments used plasmids containing kanamycin as selection marker. The enzymatic assay for *nptII* reported to have minimal effects on plant regeneration compared to others, like, *hptII* that encoding resistance to hygromycin (Li *et al.,* 2007; Guo *et al.,* 2012).

In planta Agrobacterium-mediated transformation of different crops usually includes GUS (*uidA*) as reporter gene and for selection *nptII* or *hptII* gene. Aim to use such markers was to develop an efficient transformation protocol with the aid of a set of known genes. This strategy was followed in several crops, such as, peanut (Rohini and Rao, 2000), safflower (Rohini and Rao, 2000), marula (Margaret *et al.*, 2004), chili (Ismail *et al.*, 2005), chick pea (Reddy *et al.*, 2007), cotton (Rohini and Rao, 2008), pigeon pea (Rohini and Rao, 2008), soybean (Zia *et al.*, 2010) and common bean (Amugune *et al.*, 2011) etc. Similar set of marker genes was also reported by Hasan *et al.* (2008) and Yasmeen *et al.* (2008) during infiltration of tomato fruit with a plasmid carrying *AP1* gene.

4.5.3 Seed as explants

Generation of transgenic plants following *in planta* transformation depends on the cells that can be efficiently regenerate to plantlets. Feldman and Marks (1987) were the first to report the success of utilizing dry seeds as explant for transformation and produced transgenic plants. Phenotypically normal, these fertile transgenic plant contained functional transgenes which were inherited in a Mendelian fashion.

Transgenic plants were also successfully generated utilizing dry seeds in soy bean (Chee and Fober, 1998), pea (Polowick and Quandt, 2000), and peanut (Rohini and Rao, 2000). Therefore, in this study also tomato seeds were used for this purpose.

4.5.4 Effect of pricking on *in planta* transformation

Several researchers have used mechanical damaging of seeds during *in planta* transformation. For example, damaging corn seeds with a scalpel before incubation with an *Agrobacterium* suspension (Suparthana *et al.*, 2005; Wang and Sun, 2007) or puncturing two holes on the surface of a wheat and rice seed at the expected area of germination (Rohini and Rao, 2000; Suparthana *et al.*, 2005).

Puncturing hypocotyls of a sprout and subsequently submerging the seeds in an inoculation medium containing *Agrobacterium* has also been reported (Kojima *et al.*, 2000). In all these reports wounding has been reported to promote transformation by enhancing the accessibility of *Agrobacterium* and thus, enhancing the plant cell competence towards transformation (Pan and Huang, 1996; Park and Pinson, 1996; Santarem *et al.*, 1998; Tang, 2002; Weber, *et al.*, 2003).

In this study pricking has been observed to reduce germination rate drastically. This was also observed in the report by Margaret (2004) where wounding did not significantly impact the number of transformants in marula (*Sclerocarya birrea* subsp. *caffra*). Similarly, Joubert *et al.,* (2002) reported wounding may have led to localized cell necrosis. Mondal and Bhattacharya (2001) reported wounding reduced the efficiency of transient transformation in tea (10% compared to 40% for control). In this study also the rate of transient transformation reduced to 80% when estimated through GUS histochemical assay. For this reason, transformation was achieved here by dipping the seeds in *Agrobacterium* solution without wounding. This piece of work agrees with Bent (2000) which says, plant could be transformed when dipped in *Agrobacterium* solution with no external measures.

4.5.5 Effect of *Agrobacterium* cell cultivation conditions and infection time on *in planta* transformation

Fresh bacterial cultures, obtained by cultivation in a liquid medium until the stationary phase (for 18-24 hours) at a temperature of 28°C, are used for transformation (Logemann *et al.,* 2006; Davis *et al.,* 2009). The uses of higher or lower density suspensions (OD_{600} from 0.15 to 1.75) practically affect the transformation efficiency (Clough, 1998). Zia *et al.,* (2010) reported that, T-DNA delivery time depends upon *Agrobacterium* strain, vector and explant used. One hour infection of soybean half seeds with *Agrobacterium* culture (OD_{600} 1.0) was found significant to get high number of transformants and also for survival of explants. While less time period (30 min) increased the survival of explants, but GUS expression was low.

In this study, OD_{600} of 1.0-1.2 with 30 min of incubation time gave maximum percentage of GUS assay and better survival of explants in selection medium. Zia *et al.,* (2010) has found 30 min infection time at OD_{600} 1.2 was best for the survival of explants (74%) on soybean. Similar observation was also found in wheat (Supartana *et al.,* 2006) and chickpea (Reddy *et al.,* 2007) while performing *in planta* transformation.

4.5.6 Effect of co-cultivation period on *in planta* transformation

The results of this study indicated that co-cultivation time was the main factor influencing transformation: too long a time resulted in multiplied bacteria and too short a time decreased transformation frequency, which also may be related to the genotypes of tomato and the use of different plant tissue (Guo *et al.,* 2012). In the present study, co-cultivation time span of one day (24 hours) was found appropriate in transient GUS expression (86-100%) for the tested varieties. This observation agrees with Rohini and Rao (2000) which stated that, co-cultivation period affects transformation efficiency as an increase in the co-cultivation period for more than a day drastically reduced the embryo survival rate in peanut. Contrary to this, two days of co-cultivation period was found ideal for chickpea (Reddy *et al.,* 2007) and wheat (Supartana *et al.,* 2006).

Following infection of tomato seeds by *in planta* means, infected seeds germinated faster with co-cultivation time of 72 hours. At hardening stage, the rate of survival of these seedlings reduced drastically in comparison with seedlings with 24 and 48 hours co-cultivation period. For this reason, co-cultivation time of 72 hours was not considered as appropriate co-cultivation time for *in planta* transformation. The reason behind this may be related to the tomato genotype and the use of different plant tissue as explants, different *Agrobacterium* strain and genes that have been transformed. Similar observation was also reported by Zia *et al.* (2010), where the short co-culture period was observed to have high survival rate of soybean explants but the transformation percentage was low.

Presence of the growth regulator BAP in both co-cultivation and shoot regeneration media greatly enhanced transformation and the survival rate of the *in planta* transformed explants. This result is in agreement with previous studies in which presence of growth regulators in co-cultivation medium has been shown to enhance the efficiency of *in planta* transformation in pea (Schroeder *et al.,*1993) and common bean (Zhang *et al.,* 1999; Amugune and Anyango, 2011).

4.5.7 Transformation analysis through GUS histochemical assay

In this present event, transformation of five tomato varieties with *Agrobacterium* strain containing *pBI121*, 90-95% efficiency was observed by transient GUS expression. Histochemical GUS assay revealed the presence of blue colored zones in a number of shoots and leaves that survived kanamycin selection.

Notably, a number of randomly selected shoots from initial selection medium showed positive GUS expression; however, all shoots that survived on the final selection pressure did not show positive GUS expression perhaps due to their reversion to non-transgenic state. A number of workers reported such trend in Carthamus (Ying and Dyer, 1992), in potato (Ottaviani and Smits, 1993) and also in tomato (Sarker and Islam, 2009).

Histochemical localization of GUS expression was carried out with the infected seedlings, which become fully developed plantlets grown in soil. It was done with the shoots obtained from micropropagation of leaves collected from infected seedlings. Noticeable blue color was observed in leaves from both type of plantlets and the other plant parts that indicate the stable integration of GUS gene following application of optimum concentration of kanamycin. Thus, the transgenic nature of the putatively transformed plants was confirmed through GUS histochemical assay and antibiotic selection which was followed by leaf disc senescence assay.

4.5.8 Salt tolerance analysis through leaf disc senescence assay

Leaf disk senescence assay of transformed and non-transformed plants was performed as a bioassay for the estimation of salt tolerance potential. Leaf disks from both non-transgenic and transgenic plants were suspended upto 100 mM NaCl salt solutions for 14 days to investigate the effect of Na^+ /H^+ *antiporter* gene expression on improving the tolerance to the toxic effects of NaCl. The leaf disks from non-transgenic plants showed extensive bleaching, which is a symptom of chlorosis, while the transgenic lines appear to have considerably less damage.

Present work shows that insertion and expression of the *Na^+ /H^+ antiporter* gene in tomato plants improved tolerance to salt stress. Here, the effect of salt treatment on leaf discs was observed by monitoring phenotypic changes. Wild-type plants display progressive chlorosis, reduced leaf area, and general growth inhibition when treated with high salt concentrations.

There are a number of instances where gene transfer have lead to the development of salt tolerant plants, as *Arabidopsis* (Cushman, 2000), tobacco (Reddy and Singla-Pareek, 2001), rice (Datta, 2002) and jute (Khan, *et al.*, 2013). Though there is no available report about the development of salt tolerant tomato by *in planta* methods, this study has compared the range of salt tolerance in putative transformed tomato with the range of salt tolerance in other plants. Saxena *et al.* (2011) has reported about production of transgenic *Brassica* plants with improved tolerance to salinity upto 400 mM after transfer of a single gene such as Na^+ /H^+ antiporter.

In line with the previous finding, transgenic indica rice cultivar BR5 was able to tolerate up to 200 mM NaCl stress for 2 weeks (Moriwaki and Yamamoto, 2008), transgenic jute can tolerate upto 150 mM for 14 days (Khan, *et al.*, 2013). Transgenic japonica rice plants were able to tolerate 100 mM NaCl stress while the transgenic indica rice cultivar BR5 was able to tolerate up to 200 mM NaCl stress for 2 weeks. A recent study has been found similar results for the transgenic indica rice cultivar Kasalath (Khan, *et al.*, 2013).

To improve salt tolerance in rice, the Na^+/H^+ antiporter gene, *OsNHX1*, was transformed to a Bangladeshi rice variety "Binnota", where the transgenic rice line showed healthy physiological status at 160 mM NaCl compared to the wild type (Seraj and Islam, 2009). This transgene was also transferred to BRRIdhan 28 (BR28) by crossing and the transgenic line observed to tolerate salt stress at 120 mM NaCl during seedling stage (Seraj *et al.*, 2015). Rice variety BR-49 and Binnota was transformed with transcription factor SNAC1 applying *in planta* method and salinity tolerance of the T_1 plants was analyzed by leaf disc senescence assay which showed tolerance upto 200 mM NaCl for 12 days (Seraj *et al.*, 2015). Leidi *et al.* (2010) has reported to resist salt shock upto 100 mM in transgenic tomato overexpressing *AtNHX1*.

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Appendix

Equation used to calculate regeneration frequency

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of shoot produced by explants}}{\text{Number of explants inoculated / Petri-dish}} \times 100$$

Equation used to calculate transformation frequency

$$\text{Transformation frequency (\%)} = \frac{\text{Number of shoot initiation by explants on selection media}}{\text{Number of explants inoculated / Petri-dish}} \times 100$$