IN PLANTA TRANSFORMATION OF THREE BANGLADESHI TOMATO VARIETIES WITH A VACUOLAR ANTIPORTER

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DECLARATION

I, hereby solemnly declare that the research work embodying the results reported in this thesis entitled "***In Planta* Transformation of Three Bangladeshi Tomato Varieties with a Vacuolar Antiporter**" submitted by the undersigned has been carried out under the supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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List of Abbreviations

A.	*Agrobacterium*
CaMV35S	Cauliflower mosaic virus 35 S
Cm	Centimeter
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GUS	β glucuronidase
HKT1	High affinity potassium (K^+) transporter
Hpt	Hygromycin phosphotransferase
mg/l	Milli gram/Litre
mM	Milli mole
ml	Milli Litre
MS	Murashige and Skoog, 1962
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
NHX	Sodium proton antiporter
Npt II	Neomycin phosphotransferase
OD	Optical Density
pH	-log H^+
YEP	Yeast Extract Peptone Media
µl	Micro litre
µM	Micro mole
%	Percentage

// Abstract

Salinity is one of the major obstructions in Bangladesh towards achieving maximum crop production. Being a consequence of climate change, soil salinity has rapidly increased over the years without any remedy in sight. Along with other major crops, tomato, an economically important and nutritionally wholesome crop, is being affected adversely. On top of that, demand of tomato in the country is on the rise. Hence, research on developing salinity tolerant tomato has been given considerable priority. Although a large number of studies have been carried out for salinity tolerance in rice, studies on tomatoes are not that prominent. In this study, an attempt was made to transform tomato seeds of three varieties: BINA Tomato 2, BINA Tomato 3 and Bahar. The gene of interest was *AtNHX1* (under the constitutive promoter CaMV35S), a vacuolar antiporter from *Arabidopsis thaliana* which has been shown to possess significant potential to confer tolerance against salinity. For transformation, *in planta* method was chosen to attain a sizeable percentage of putatively transformed seedlings after selection. In this method, seeds were infected with *Agrobacterium* strain LBA 4404 carrying plasmid *pK7WG2_AtNHX1_1.6* for 15 and 30 minutes in an optimized bacterial density of 0.8-1.2 at OD_{600}. The various morphological parameters of infected and non-infected plants were documented both for *in vitro* stage and mature stage in natural environment. Though some striking features of the putatively transformed plants in the early stages such as: higher time requirement for germination, lower height and root strength were observed, no significant differences were seen in overall survival percentages between control and putatively transformed plants of BINA Tomato 3 and Bahar. In mature stage, putatively transformed plants of all the varieties showed similar height to controls but higher total average leaf surface area (at a larger extent in BINA Tomato 3 and Bahar). Though time periods needed for flowering and fruiting were lower in controls (in most cases), the fruiting percentages were considerably higher in putatively transformed plants. Finally, in leaf bioassay of one putatively transformed plant, tolerance to 100 mM salt for 12 days was observed. Though it is a long way to commercial release, this study presents the potential of *in planta* method and *AtNHX1* to attain a salinity tolerant tomato variety without any marked morphological or reproductive disadvantages.

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Chapter 1

Introduction

Introduction

Tomato, scientifically named *Solanum lycopersicum* Mill., is one of the most popular vegetables used in a wide range of ways as a part of daily diet all around the world. Tomato belongs to the nightshade family, Solaneceae. The tasty, red fruit can be used in/with salads, side dishes, curries, sauces and fries. Moreover, the plant has been widely used as a model for breeding and genetic research (Kimura and Sinha, 2008).

The tomato plant is a dicot plant and has a perennial (4-10 months) life cycle. It's a diploid organism with a total of 24 chromosomes and a genome size of 900 Mega base pairs (The Tomato Genome Consortium, 2012). It's a decumbent plant meaning that the stem might run parallel to the ground but will have an upright stem tip. The terminal bud is the deciding tissue for its height. Tomato stem is pubescent. The fine hairs proximal to the ground may turn into roots when buried or under wound stress. Roots of tomato from seeds are usually taproots (Thomas, 1996). Along with the previous information Thomas (1996) published in the web portal under University of California Davis that tomato leaves are compound leaves. Both Raceme and Cyme inflorescence is seen in tomato and the number of flowers depend on other environmental factors. The fertilization section of the portal reports both self and cross pollination of tomato plants. This also backs the assumption that fertilization of tomato needs pollinating agents. The tomato fruit carries all the characteristics of berries and is fleshy because of its enlarged pericarp (Thomas, 1996).

1.1 A brief history and the present status of tomato

Tomato has its origin in the South American Andes valleys (*Solanum lycopersicum* Garden tomato, 2014). The tastefully delicious and visually attractive fruit soon made its way to the European market by the Spanish explorers. Long before that, the Aztecs had domesticated large-red tomato ("Ethno botany of tomato", 1996). Interestingly enough, the Europeans did not immediately accept tomato as an edible fruit. It was rather an ornamental option (Filippone, 2014). They, specially the British, thought erroneously of tomato as being poisonous. However, during late nineteenth century tomato was adopted into most kitchens in Europe ("A brief history lesson about the tomato," 2005). The latest statistics (2014) from FAO (FAOSTAT, 2016) shows 170 million tons of tomato production worldwide. This ranks tomato only second vegetable

to potato in terms of production quantity. Such trend of increased tomato production is notable since 1961 (FAOSTAT, 2016.), indicating their demand with growing population. In 1998, the production crossed 100 million tons mark (FAOSTAT, 2016) and consistently kept increasing.

1.2 Health benefits of tomato

The reasons behind popularity of tomato is not merely its taste, color and versatility. Health benefits of tomato have been well established. First of all, the vitamin C, A, B2, folate and K^+ contents of tomato are substantially rich ("Food Composition Databases Show Foods -- Tomatoes, red, ripe, raw, year round average", 2017). On top of that, the data chart shows absolutely no trace of cholesterol, LDL, trans-fats and only minute amount of lipid and calories (18 kcal). Such a combination makes tomato a nutritional choice for children, patients with diabetes and heart diseases (Kobylecki, 2015).

Lycopene, a carotenoid found in tomato apart from vitamin A, beta carotene and other pigments is the source of some important health benefits.

By scavenging free oxygen species, antioxidant lycopene reduces cell damage and other related oxidative stresses. In fact, among other antioxidants, carotenoid lycopene is the most effective one acting on singlet oxygen entity (Mascio, 1989).
Lycopene has been suggested for cardiovascular disease prevention even though the mechanisms are still understood only in *in vivo* stage (Mordente, 2011). Likewise lycopene is also statistically proven to decrease the risk of any stroke and ischemic stroke in men (Karppi *et al.*, 2012).
Sahni *et al.* (2009) showed that higher blood lycopene concentration is related to lower chances of hip fracture. Another study by Mackinnon (2010) concluded that lycopene supplementation reduces bone resorption in postmenopausal women.
Finally, the anticancer quality of tomato is largely accredited to lycopene. Giovannucci (1999) reviewed that tomato intake and higher plasma lycopene levels are positively associated with lower risk of cancer. In the review, a wide range of cancer types showed similar result with none showing the opposite. Recent studies also concluded that α-tomatine from tomato works against prostate cancer (Lee *et al.*, 2011) and lung cancer (Shieh *et al.*, 2011).

Apart from all these, consumption of a single cup of tomato fulfills 9% of daily dietary fiber need (Szalay, 2016). The popular news agency Huffington Post (Oliver, 2013)

published all the skin care methods that can be used to fight acne, blackheads, dry scalp and aging using tomato.

1.3 The economic importance of tomato in Bangladesh

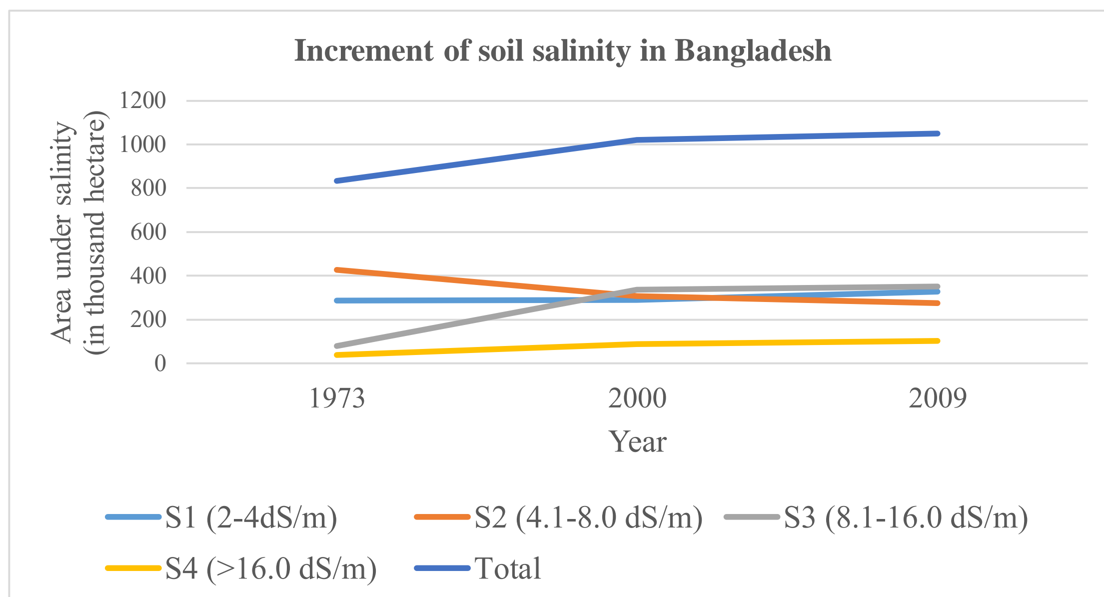
In 2013, the total area of land used to produce tomato was 65 thousand acres. That rose to 76 thousand acres in 2015 which is about 8% of total area under vegetable production. Simultaneously, the total yield rose gradually to 414 thousand metric tons from (2013) 251 m. tons. This accounts for over 11% of all vegetable production in 2015. Within these three years the yield per acre also increased almost 1.5 times (5471 kg/acre in 2015). Using the wholesale price reported in (Year book of agricultural statistics, 2015), it was found that (approximately) BDT 1827 crore was the total worth of tomato produced nationally in 2015. However, BDT 27.6 crore was spent to import tomato in the same year. Such high amount of imported tomato has not been seen before. This clearly indicates to the vastness of the market.

To analyze the impact of salinity on tomato yield, the total productions from districts affected by salinity was calculated. It turned out that 64 thousand metric tons (15.5% of total production) of tomato production took place in the salinity intruded districts. This firmly establishes salinity as a major obstacle to meet the national demand (Year book of agricultural statistics, 2015).

1.4 Soil salinity and its extent in Bangladesh

Soil salinity is a global problem (FAO Soil Portal, 2016). With climate change and rising sea levels different areas around the world has shown increased soil salinity. The prevailing situation costs US$ 27.3 billion globally every year (Qadir *et al.*, 2014). In Bangladesh the effect is nothing less than highly detrimental. According to the latest available data 1.05 million hectare of land is affected with soil salinity (SRDI, 2010). Implying from the records, it can be seen that around 170 thousand hectare of land has been newly intruded with salinity in 30 years between 1973 and 2000 (Hossain, 2010). The increment rate of (approx.) 30%, till 2000 declined to just 3.5% (SRDI, 2010) in the next decade. Moreover, SRDI report also showed S4 level of salinity (very high salinity, over 16dS/m) in Barishal, Bagerhat, Potuakhali and Noakhali in 2000. Such a level was not reported previously. Moreover, northern districts Faridpur, Madaripur,

Gopalgonj and Noakhali has been newly intruded with salinity (Hossain, 2010). The reasons behind fast salinity intrusion in Bangladesh has been discussed in details by



Graph 1 Soil salinity in Bangladesh over the last 35 years. (Source: SRDI, 2010)

Mahmuduzzam (2014). According to him, critical geographical location of the country, rise in sea level, low flow condition of the river by a barrage in the upstream neighboring country, faulty management of coastal polders, cyclone and storm surge, back water effect, precipitation and shrimp culture are the reasons. And clearly, these cannot be tackled within a short time span.

1.5 Effect of soil salinity on agricultural production

Such levels of salinity (Graph 1) threatens agriculture severely. Agricultural diversity has been reduced greatly in those areas (Gain *et al.*, 2014) in the recent years. A World Bank funded study predicted that 15.6% of production of high yielding varieties (HYV) rice will be hampered due to salinity. Studies which are already done in the coastal areas also depict a similar picture. Rabbani *et al.* (2013) reported that rice production in Satkhira has been reported to be lost entirely after due to soil salinity after cyclone "Aila" in 2009. A case study on Bangladesh (Rahman, 2003) estimated that a total of 196 thousand metric ton of production will be lost with baseline soil salinity. But the loss will amount to 658 thousand metric ton if climate change effects are at their worst. First hand researches (Uddin *et al.*, 2014; Warner and van der Geest, 2013; Rabbani *et al.*, 2013) has shown that people are now aware that agriculture is directly affected by

salinity. These reports also stated that the use of tolerant varieties is seen as one of the key tools to bolster agricultural output in those areas. Such use of tolerant varieties should be encouraged by policy makers.

1.6 Effect of salinity on plants

The impact of salinity on glycophytes needs no reiteration. Salinity affects these plants differently in seedling and mature stage of life cycle. Frequently reported effects of such stress on tomato plants are: lower germination percentage, delayed germination, stunted growth, reduced leaf surface area, and impaired reproductive capacity and higher mortality rates in all stages of life. These eventually lead to significant yield loss.

In one of the recent reviews (Deinlein *et al.*, 2014), it has been stated that two basic impacts from higher salt concentration are the root causes of all the above mentioned problems. Instantly, increased soil salt concentrations decrease the ability of a plant to take up water. Then over an extended period of time, usual metabolic pathways and photosynthetic abilities are altered negatively by higher Na^+ and Cl^- concentration in the cytosol. Osmotic stress may cause germination delay whereas Na^+ toxicity accounts for seedling mortality. Munns and Tester (2008) reviewed that ABA levels are increased upon salt stress and it inhibits GA production. Such hormonal signals keeps the shoot and root growth halted. Long period of salt stress will hence, result in stunted growth. In the same review, evidences were compiled about the unchanged rate of photosynthesis and reduced stomatal activity. The stunted growth created a dense but short (in area) leaf tissue. Therefore, the photosynthesis rate was unaltered but photosynthetic ability of unit chlorophyll was definitely reduced.

Under saline conditions K^+/Na^+ ratio is altered. Apart from being a major macronutrient, K^+ is a key factor in ion homeostasis and many enzyme's activity (Tuteja, 2007). Alteration of such balance may be accountable for low survival rates and reproductive inefficiencies.

Almost all the above mentioned problems are true for tomato. Tomato seed germination percentage, germination speed were found to be lowered by half in 3% NaCl mixed hogland's medium by Singh and his team (Singh *et al.,* 2011). Root and shoot Na^+/K^+ ratios increased almost the same amount. And Najla *et al.* (2009) showed how the

architecture of tomato plants are affected by salinity. They also depicted a 3D model of it. Considering production, Mizrahi back in 1982 reported tomato yield loss, shelf life decrease under saline condition. Interestingly, the taste, ripening time and fruit dry weight (at 6g/l NaCl stress) changed positively. The positive effect of moderate salt concentration has also been reported to be beneficial in terms of lycopene and carbohydrate content of tomato (Giannackoula and llias, 2013). At the same time malondialdehyde (MDA), H_2O_2 levels were higher, indicating stress response. In fact, there are reports of fruit weight loss even under moderate salinity. On top of that, yield and flowering percentages were shown to be significantly different for water sources with different salinity percentages (0 - 0.13) (Boamah *et al.*, 2011). Similar to previous studies, Ismail and Burrage (1994) had reported reduction in plant and leaf dry weights using nutrient film technique. They also noted stable photosynthesis rate with lowered leaf water potential and stomatal conductance. These results on stable photosynthesis rate under salinity stress is similar to Terry and Waldrone's (1984) report on sugar beet. They had concluded that total leaf area should be considered instead of photosynthesis rate per unit area. However, photosynthetic rates do not remain stable in all plants. Jamil and Rha (2013) showed such data with mustard.

1.7 Targets for salt tolerance improvement

To tackle salt stress plants use multiple approaches. As discussed earlier, salt stress harms the plant by inflicting ion toxicity and osmotic stress. Consequently, plants tackle both the issues with different independent approaches (Munns and Tester, 2008). These various mechanisms can be utilized to confer enhanced tolerance to plants. Here, the underlying mechanisms which are potential tools for developing salt stress tolerance by genetic manipulation are discussed.

1.7.1 Osmolytes

Osmolytes help to alleviate the imbalanced water potential experienced by plants under high salinity. The most discussed osmolyte is proline. Several studies has found that under saline conditions plants synthesizes more proline. In the recovery phase after stress, proline catabolism becomes noticeable (Sharma *et al.*, 2010; Szekeley *et al.*, 2008). From this evidence alone, the protective nature of proline becomes established. However, there has been differences in specifying the exact mechanism of proline's

activity (Ashraf *et al.*, 2007; Verbruggen *et al.*, 2008). Proline is considered to possess reactive oxygen species (ROS) scavenging, protein stabilizing, osmotic and chaperone activity. But for Glycine Betaine (GB, a quaternary ammounium compound) the role is purely osmoprotective (Guinn *et al.*, 2011; Raza *et al.*, 2007). Other osmoprotectants include sugars: trehalose, raffinose, fructans etc.; polyols: glycerol, mannitol, sorbitol; proteins: late embryogenic abundant proteins; amino acids and amides (Parvaiz and Satyawati, 2008).

1.7.2 Antioxidants

Levels of ROS increases under salt stress. Several antioxidants in different plants work to relieve plant cells from oxidative stress. Munns and Tester (2008) named superoxide dismutase, catalase, and peroxidases as the major antioxidants. They reduce ROS levels and keep a stable H_2O_2 level for signaling. Parvaiz and Satyawati (2008) additionally mentioned Monodehydro or dehydroascorbet reductase, ascorbet peroxidase, glutathione, Mn-SOD, Fe-SOD (mitochondrial) and Cu/Zn-SOD (chloroplastic).

1.7.3 Transporters

Numerous transporters are present in plant cells, often ubiquitously in various species to take up, transport and excrete Na^+. As they play pivotal roles in the maintenance of ion concentrations in root, shoot, xylem, phloem, cytosol and vacuole, they obviously hold potential to manage high salinity. Three transporters *SOS* (Salt Overly Sensitive), *HKT* (High Affinity Potassium Transporters) and *NHX* (Na^+/Proton Exchanger) are the most notable ones.

SOS1 is a plasma membrane Na^+/H^+ antiporter. This transporter expels Na^+ from cytosol and have been shown to confer tolerance against high salinity (Hoorie and Schroeder, 2004). High affinity potassium transporters (*HKT*s) are of two classes: K^+, Na^+ co-selective and Na^+ selective ones. The class I transporters function to reduce the shoot Na^+ influx. Though the exact mechanism of the process is under speculation (Maser *et al.*, 2002; Berthomieu *et al.*, 2003; Munns and Tester, 2008 and Sunarpi *et al.,* 2007) the potential role of *HKT1* to confront salinity stress has long been established. Among different *HKT1* sources, *OsHKT1* and *AtHKT1* are the most studied ones (Deinlein *et al.,* 2014 and Davenport *et al.,* 2007).

Apart from exclusion from cell itself, sequestration of Na^+ ions into vacuole is another strategy to cope with ion toxicity. A large number of studies were carried out using sodium proton antiporters of various sources into different plant species. Na^+/H^+ antiporters are vacuolar membrane antiporters. They sequester Na^+ into the vacuole and release H^+ into cytosol. Energy for such active transport of Na^+ is provided by vacuolar type ATPase (VATPase) and pyrophosphatase (VP). Six genes of this transporter family was found in *Arabidopsis*. The core transporting function is attributed to *AtNHX1*. *AtNHX2* has regulatory functions (pH maintenance). Though literatures mostly focus on Na^+ transporting activity of *AtNHX1*, it also mediates other strategies: accumulation of proline, higher K^+/Na^+ ratio, increased chlorophyll activity (Deinlein *et al.*, 2014). However, Xingyu and his colleagues (2010) has put forth a conclusion that transportation of K^+ is the primary function of vacuolar *NHX*. Above that, they also reported cytosol K^+ ion studies showing that K^+ remains sequestered in the vacuole.

Cheeseman in 1988 put light on the thought that there needs to be a mechanism that controls the ion transportation from root to shoot (Cheeseman, 1988). Shi and colleagues in 2002 presented such a control mechanism. In their discourse it is presented that *SOS1* shows Na^+ up taking activity in low salt concentration to allow mesophyll vacuoles to sequester excess Na^+ and efflux activity when salt concentration is far greater for sequestration to be effective. On the other hand, wheat *HKT1* in the roots becomes blocked when salt stress is detected. For K^+ uptake, the root cells switch to other low affinity K^+ transporters. This shows the intricacy and fidelity of the natural tolerance mechanisms present in plants.

1.7.4 Transcription factors

Munns and Tester (2008) in their review stated three types of tolerance mechanisms: tolerance to osmotic stress, Na^+ exclusion from shoots and tissue tolerance to Na^+. Apart from these, other reviews broadly discussed about transcription factors as well (Deinlein *et al.* 2014). Upon salt stress several pathways are initiated to avoid and reduce toxic effects. Growth promoting signals (Munns and Tester, 2008) are down regulated and inorganic ion uptake, osmolyte synthesis are upregulated (Deinlein *et al.*, 2014). Notable transcription factor families are: basic leucine zipper (bZIP) (Yang *et al.*, 2009), WRKY transcription factor (Jiang *et al.*, 2009a), APETALA2/Ethylene Response Factor (AP2/ERF) (Kasuga *et al.*, 1999), MYB or myeloblastosis

transcription factor (Cui *et al.*, 2013), basic helix–loop–helix (bHLH) (Jiang *et al.*, 2009b) and NAC (NAM, ATAF, and CUC) transcription factors (Tran *et al.*, 2004). In the discussion below, the utilization of all these mechanisms to fight salt stress is presented.

1.8 Transformation studies on salinity stress tolerance using various genetic resources

To tackle the Global problem of salinity a lot of focus of the scientific community has been aimed at developing high salinity stress tolerant plant development using numerous genetic resources. The current researches have moved beyond exploiting all the basic mechanism of salinity tolerance found in plants (section 1.7). So far experiments using transcription factors, transporters, osmolytes and antioxidants have provided promising results.

As salinity induces osmotic and oxidative stresses, several transformation studies incorporated transcription factors, osmoprotectants and antioxidants. Oh and his team (2005) transformed rice cv. Nakdong with Arabidopsis CBF3/DREB1A and ABF3 separately under constitutive promoter (Oh *et al.,* 2005). The transformed plants up-regulated numerous genes. As a result, all the health indicators of transformed plants were better than non-transformed ones. Above that no growth deficiencies were observed. The transcription factor DREB1B has also shown similar results conferring salinity tolerance under an inducible Promoter rd29 (Responsive to Dehydration 29A) (Datta *et al., 2012*).

Osmoprotectants are also an attractive choice among other genes to fight high salinity. Interestingly, Jang and his colleagues used a bi-functional fusion of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase genes (Jang *et al.,* 2003). Under constitutive expression, the fusion protein showed 200 times more production and without any notable phenotypic problems. Choi and his colleagues, on the other hand, used xyloglucan endo-trans-gluco-sylase/hydrolase CaXTH3 in *Arabidopsis* (Choi *et al.,* 2011). It has been hypothesized that CaXTH3 confers high salinity and drought tolerance by cell wall remodeling.

The above two studies reported salinity tolerance upto 100 mM NaCl. Antioxidants, however, have been reported to provide tolerance above 150 mM NaCl concentration. Cu/Zn Superoxide dismutase from grey mangrove and Monodehydroascorbate reductase from holly mangrove genes (*Sod1* and *AeMDHAR*) conferred tolerance against 150 mM NaCl in rice (Sultana *et al.,* 2012). And *katE* from *E. coli* provided 200mM and 250mM NaCl tolerance in BR5 (Moriwaki *et al.*, 2008) and kasalath (Prodhan *et al.*, 2008) rice varieties. Moriwaki *et al.* (2008) also reported successful flowering and fruiting in 100mM NaCl. In addition, Prodhan *et al.* (2008) reported two fold increase in plant growth compared to controls under 200mM NaCl.

1.9 Studies applying *AtNHX1* for achieving tolerance against high salinity stress

Apart from the genetic resources mentioned in the previous section (section 1.8) sodium proton antiporters have also been widely used for similar tolerance studies. Apse and colleagues in 2003 successfully established the importance of *AtNHX1* in K^+ and Na^+ sequestration and thereby proving its role in salinity tolerance (Apse *et al.,* 2003). Since then *AtNHX1* has been successfully proven to provide tolerance to high salinity in different plants. However, vacuolar antiporters of various origin have been given notable importance in researches on developing tolerance to high salinity. *SbNHX1* (Pandey *et al.*, 2016), *TaNHX1* (Khoudi *et al.*, 2009), *PgNHX1* (Bhaskaran and Savitramma, 2011), *OsNHX1* (Yasmin *et al.*, 2015) related studies were noticeable in the recent years. All these studies have invariably showed their efficiency as antiporters in tackling salinity stress.

The level of tolerance along with positive physiological features that *AtNHX1* provides to tackle salinity can be realized from recent studies. In canola, *AtNHX1* provided tolerance up to 200 mM NaCl for 30 days (Dorani-Uliaie *et al.*, 2012). The study also reported higher K^+ concentrations in leaves and three times greater proline accumulation. Same level of tolerance and proline production was achieved with kiwi fruit (Tian *et al.*, 2011). On top of that, they also reported higher flavonoid in all parts of transgenic plants and higher ROS elimination. In peanut, photosynthetic parameters: stomatal conductance, photosynthesis rate and transpiration rate almost doubled in transgenic plants (Banjara *et al.*, 2012) under 150mM NaCl. This shows the capability

of sodium sequestration by *AtNHX1* in maintaining a favorable condition for plant under salt stress. This also may be the cause of retained growth (measured by fresh weight) in transgenic plants which has been reported in these studies. Similar profiles were presented in mungbean (Sahoo *et al.*, 2016) and groundnut (Asif *et al.*, 2011). In addition to MDA, proline and chlorophyll results showed less root water content loss in the mung bean study.

Stable integration of transgene and their passage towards progenies of later generations has always been a major concern of transformation related projects. That is why quite a few studies were also conducted to find out about the stability of *AtNHX1*. Moghaieb *et al.* (2014) presented that wheat carrying *AtNHX1* showed 3:1 Mendelian segregation at second generation. On an impressively longer scale, transgene stability for six generations in soybean with no loss of yield and 100mM NaCl tolerance was reported by Li and colleagues (2010).

Moreover, the importance of V ATPase and H^+PPase for *AtNHX1* cannot be exaggerated as antiporters can never function without energy provided to them (Zhou *et al.,* 2010). In correlation to this dependence of *AtNHX1* on energy source, Shen and his team studied the result of co-overexpression of AVP1 and *AtNHX1*in cotton. As hypothesized all physical and production related results from the transgenic plants were better than control plants (Shen *et al.*, 2014). So, this sort of co-overexpression is a fine way to provide salinity tolerance two plants of economic significance. Lastly, Zhang and his colleagues compared the results between transgenic plants overexpressing *AtNHX1* and *HvBADH1*. Though *HvBADH1* expressing plants had a better profile of proline, soluble sugars and chlorophyll, *AtNHX1* expressing calli showed better survival under severe salt stress (Zhang *et al.,* 2015). From this experiment the effectiveness of *AtNHX1* as the gene of interest compared to other genetic resources for providing salinity tolerance becomes strongly established. On top of that, form all these studies and findings it can be well understood that *AtNHX1* is a remarkable gene with proven effectiveness in conferring salinity tolerance, stability in various plants and without any adverse effect on economically important crops.

1.10 Studies on salinity tolerant plant development in Bangladesh

A notable number of transgenic studies has taken place within the borders of Bangladesh to develop salinity tolerant crop. From rudimentary studies, such as, factor optimization for *Agrobacterium* - mediated transformation of different species, tomato (Islam *et al.,* 2010 and Sarker *et al.,* 2009), maize (Sarker and Biswas, 2002), rice (Hoque *et al.,* 2005), jute (Sarker *et al.*, 2008; Sajib *et al.*, 2008), potato (Sarker *et al.,* 2009), lettuce (Ahmed *et al.*, 2007) and salinity tolerance level of calli (Liza *et al.*, 2013) to sophisticated transformation and cloning studies have been undertaken. A brief and comprehensive review is presented below. *In silico* studies to find out the synchronized role of various transcription factors, genes and enzymes in abiotic (salinity included) stresses were performed in Bangladesh (Razzaque *et al.,* 2014). Even the structural analysis of *AtNHX1* and *AtNHX2* was performed using bioinformatics tools (Feisal and Islam, 2017). These studies show the priority given to salinity tolerance related research in Bangladesh since the rise of salinity has become a major impediment to crop production.

Development of salinity tolerant lines of rice and jute applying a set of genetic resources has been reported from Bangladesh. *SNAC1* gene from pokkali rice cultivar (*OsSNAC1*) has been cloned for genetic transformation (Al-emran *et al.*, 2010). Upon transformation with *OsSNAC1* BRRI Dhan – 55 showed drought tolerance for 20 days and salinity tolerance at seedling stage. For another similar transformation study, *in planta* method was proven four times more efficient for rice variety binnatoa (Parvin *et al.*, 2015). Similarly, *SOS1* was cloned from pokkali (Razzaque *et al.*, 2013) and transformed BRRI Dhan-28 with overexpressing the *OsSOS1* gene (Yasmin *et al.*, 2015). The transgenic lines showed better phenotypic results compared to controls. Yet, shoot length reduction, chlorophyll reduction and root length data were not impressive at all. The authors probably have suggested co-overexpression of *NHX1* and *SOS1* based on such results.

Three antiporter genes: *OsNHX1*, *AtNHX1* and *AtNHX2* were cloned (Razzaque *et al.*, 2014) to develop salinity tolerant plants along with an inducible promoter *Adh* from *Arabidopsis* (Ashraf *et al.*, 2014). Among these extensive study was performed on *OsNHX1 OsNHX1* was over expressed in Binnatoa under either Actin 1D or CaMV35S promoters. They proved to provide same level of seedling stage growth and yield under

saline condition (Islam and Seraj, 2009). However, reasoning with the notion that 35S promoter is not best suitable for monocots, Islam *et al.* (2007) cloned upstream regions of Na^+/H^+ antiporter gene from pokkali and IR64 rice variety. Both showed high expression levels compared to 35S in calli whereas only IR64 promoter resulted in uniform expression of GUS gene in leaves. Another study, this time with addition of 3' UTR to *OsNHX1*, showed significant increase in yield profiles and salinity tolerance (Amin *et al.*, 2016). Despite all these positive indicators, Biswas and his team reported a decrease in salinity tolerance at both seedling and reproductive stage when transgenic binnatoa was successfully crossed with BRRIDhan-28 (Biswas *et al.*, 2014). This shows the level of difficulty in breeding, even after successful transformation.

The rice variety binnatoa was also tested with overexpressing *PgNHX1* (Islam *et al.*, 2010) and *PDH45* (Amin *et al.,* 2012) genes. Both yielded similar result: salinity tolerance with improved production and growth rates. The latter yielded almost double production than the control in one of the transformed lines. Lastly, *KatE* gene from *E. coli* conferred salinity tolerance (150mM NaCl for 9 weeks) in jute (Islam *et al.*, 2013).

1.11 Transformation studies on tomato

Tomato has been a mentionable target for genetic transformation related studies for developing salinity tolerant lines. However, in Bangladesh, tomato has become the focus of such studies due to its increasing economic value (Section 1.3). Though the most notable studies of this country were performed on rice, tomato related studies have passed the rudimentary phase and will be focusing on post-transformation analyses. Sarker and his colleagues (2009) established the regeneration protocol for tomato varieties: Pusa Ruby and BARI Tomato 3. They used cotyledonary leaves as explant. They also carried out transformation of the explant with plasmid *pBI121* and regenerated tomato plants using the protocol. Following the proper growth of the regenerated plants antibiotic resistance and GUS expression was seen. Their regeneration and transformation protocol was thereby, a successfully established one. Using the same plasmid but different regeneration protocols Das *et al.,* (2015); Chowdhury and Islam (2012); Islam *et al.,* (2010) performed *Agrobacterium*-mediated transformation for tomato (section 1.12). The 2015 study reported a least GUS

expression percentage of 60 whereas the 2010 study showed a highest GUS expression percentage of 90.

Unlike research on tomato in Bangladesh, several studies from abroad have reported success in developing salinity tolerant lines. Their choice of transgene has been diverse and results highly appreciable for commercial release. El Awady and his colleagues established optimization of the transformation protocol for tomato variety edkawy from kingdom of Saudi Arabia. They successfully transformed the tomato with *AtNHX1* gene for tolerance and *BAR* gene for selection (El Awady *et al.,* 2014). However, no tolerance levels of the transformed plants were reported. Yarra and his colleagues transformed ped variety with antiporter from wheat *TaNHX2*. Such genetic manipulation conferred tolerance to salinity (150mM salt concentration) with detectably higher relative water content, higher rate of photosynthesis and higher chlorophyll content (Yarra *et al.,* 2012). To aid antiporter (*TNHX1*) vacuolar pyrophosphate (*TVP1*) is present in wheat. Both of these genes were overexpressed separately in rio-grande variety by Khoudi and his team (2009). Though transformation studies did not cross 10%, the transformed plants showed tolerance at 200mM salt solution.

Indian tomato variety Pusa Ruby were transformed with osmotin and bacterial *codA* gene (Goel *et al*., 2010 and Goel *et al*., 2011). The over-expression of osmotin gene resulted in higher relative water content, chlorophyll, proline, leaf expansion and survival of transformed plants in 150mM salt concentration for 10 days. The bacterial *codA* gene on the other hand showed similar results by over production of glycinebetaine. Another osmoprotectant *Mannitol-1-phosphate* was overexpressed in tomato variety Pusa Uphar (Khare et al., 2010). This study reported a seed germination in 200mM salt supplemented MS basal media along with other positive indicators for salinity tolerance. These results show just how rewarding these transformation studies are and will motivate any research team to take up an initiative to replicate such attempts to develop a transgenic line in Bangladesh.

The studies on tomato do not stop there. A set of studies were carried out to find possible genetic options against high salinity within the tomato genome. Fortunately, manipulating tomato's own genetic resource has been proven to be rewarding just as much. Isopentyl transferases from tomato *SlIPT3* and *SlIPT4* have been found to help in maintaining high cytokinin levels in plant. When overexpressed, higher cytokinin

provided tolerance to higher salinity (Zizkova *et al.,* 2015). Pan and his colleagues assessed the tolerance levels provided by overexpression of *SlERF5*, a new AP2/ERF transcription family member. The results indicated the involvement of this transcription factor in salinity and drought stress (Pan *et al.,* 2012). Similar results of abiotic stress tolerance was found with overexpression of *SlAREB1,* another transcription factor. Additionally, *SOS1 and NHX2* from tomato genome (*SlSOS1* and *LeNHX2*) was shown to have significant direct roles in salinity tolerance (Olias *et al.,* 2009; Rodriguez-rozales *et al.,* 2008). *SlSOS2,* however, up-regulated *SlSOS1*, *LeNHX2*, *LeNHX4*, vacuolar ATPase etc. to respond to high salinity when overexpressed (Huertas *et al.,* 2012).

1.12 *In planta* method as a choice for transformation of tomato

Surprisingly, all the studies and experiments on tomato discussed above were done through tissue culture dependent *Agrobacterium*-mediated transformation. However, the number of *in planta* transformations targeting embryos was not insignificant (Keshamma *et al.*, 2008; Subramanyam *et al.*, 2013; Sunderesha *et al.*, 2009 and Manickavasagam *et al.*, 2015). This is because *in planta* technique can provide numerous advantages in certain scenarios. Through literature review, it was seen that some tissue culture dependent studies reported regeneration percentage above 75 (Goel *et al.,* 2010; Yarra *et al.,* 2012; Sarker *et al.,* 2009; El Awady *et al.,* 2014) whereas, germination percentages in tissue culture independent studies usually ranged from 60 to 75% (Hasan *et al.,* 2008; Jaganath *et al.,* 2014; Subramanyam *et al.,* 2015). Nonetheless, the overall transformation efficiency in both the strategies contain a considerably similar in range (Sunderesha *et al.,* 2010; Hasan *et al.,* 2008; Supartana *et al.,* 2006; Das *et al.,* 2015; Rai *et al.,* 2012). However, considering the time consumed in tissue culture dependent protocols, tissue culture independent transformation strategies, such as, *Agrobacterium*-mediated transformation of embryos is certainly advantageous.

However, in Plant Tissue Culture Lab of BRAC University, the deciding factor for switching to *in planta* transformation technique was the regeneration percentage in selective media for tissue culture dependent transformation. The regeneration percentage under the established protocol was remarkably higher (around 90%) (Mukta,

2014). In contrast, when the same protocol was used to regenerate from *Agrobacterium* infected explants, the percentage dropped to 50% (approx.) for pBI121 and to 20% (approx.) for pH7WG2_OsNHX1_1.6 for different varieties of tomato from BARI and BINA (Mohsina, 2014; Mukta, 2014). The low percentage of regeneration had to be overcome for successful transformation of tomato in the lab. In 2015, Datta reported the advantages of *in planta* transformation in rendering a high percentage of the putative transformed plants. In her study, three constructs (pBI121, pK7WG2_AtNHX1_1.6 and pK7WG2_OsNHX1_1.6) were used for the transformation of five tomato varieties. This comparative study of tissue culture based transformation and *in planta* transformation revealed that *in planta* transformation can produce almost twice as much transformed plants as the tissue culture based transformation.

1.13 *In planta* transformation parameters

In the recent days *in planta* transformation optimization and its application has been widely seen. *In planta* transformation for local tomato varieties of Bangladesh has been optimized through several studies. In these studies, factors influencing transformation efficiency has been identified.

The major influential parameters are: *Agrobacterium* suspension OD_{600}, infection period, co-cultivation period and selection pressure. Paramesh *et al.* (2010) cited the studies indicating to the importance of several factors in making an efficient transformation technique. Likewise, Hasan *et al.* (2008) showed the importance of bacterial density in fruit infiltration based *in planta* transformation in tomato. Complementary to their results, Yasmeen *et al.* (2009) showed 48 hour incubation to be the most effective. The helpful effect of pre-culture is reported both for tissue culture dependent and independent transformation strategies (Paramesh *et al.,* 2012). In addition, Khoudi *et al.* (2009) showed the influence of explant type for tissue culture dependent *Agrobacterium*-mediated transformation. According to the study, primary leaves of tomato was the best explant for *Agrobacterium* infection.

The transformation studies in Bangladesh did not include any use of acetosyringone but, such use of acetosyringone is shown to have positive impact onto the overall transformation efficiency (Raj *et al.*, 2005; Yarra *et al.,* 2012).

Das *et al.* (2015) established that best transformation rates were got by using *Agrobacterium* suspension of OD_{600} 0.8 for 15 minutes infection and 3 day co cultivation. The tomato varieties used was BARI Tomato 8. A similar study with 3 Bangladehi varieties (BINA Tomato 3, BINA Tomato 5 and BAHAR) and one Indian variety (Pusa Ruby) established the same results 5 years earlier (Islam *et al.,* 2010). Both these studies mentioned the benefits of preculture. But for transformation of Bangladeshi varieties by using embryos as explants the parameters were different. With the three varieties used in this dissertation, Datta showed that, Optical Density of 1.0-1.2 at 600nm with 30 minute infection and 24 hour co cultivation period gave the best results (Datta, 2015).

1.14 Research objectives

Previous discourse have firmly established the ever increasing importance of tomato and the threat of salinity in crop production. By relating the potential of vacuolar antiporter: *AtNHX1* from *Arabidopsis* and the methodology at hand, an obvious option of genetic engineering can be opened up. In this age of technology, such attempts to address agricultural issues should become a regular practice. From this mindset, the present study was aimed at getting the following objectives secured:

- Detecting the effects of transformation in seedling (*in vitro*) stage
- Finding out the morphological changes in mature putatively transformed plants
- Assessment of the reproductive nature of positively selected plants and comparison to control plant's nature
- Testing the leaf tissue for salinity tolerance levels

Chapter 2

Materials and Methods

Materials and Methods

2.1 Plant material

In this study, three varieties of tomato seeds were used. Namely, BINA Tomato 2, BINA Tomato 3 and BAHAR. All of them are developed by (BINA) Bangladesh Institute of Nuclear Agriculture. BAHAR was developed the earliest, in 1992. A local variety was turned into a mutant variety by gamma radiation. The mutant plant was named "Anobik". Fruits from the mutant was found to be sour and small in size. For further improvement, this mutant was crossed with "Oxheart" variety. The resulting variety was BAHAR. Its fruit turned out to be large and delicious and hence registered.

Similarly, another local variety under gamma radiation at BINA produced "S1" mutant. To improve the quality of the mutant, it was crossed with BAHAR. The two varieties selected from the cross were BINA Tomato 2 and BINA Tomato 3. They were officially registered by National Seed Board in 1997. The comparative view of the three varieties is given in Table 2.1.

Table 2.1 Comparison among BINA Tomato 2, BINA Tomato 3 and BAHAR

Features	BINA Tomato 2	BINA Tomato 3	BAHAR
Season	Summer	Summer	Winter
Height (in cm.)	75-80	80-85	80-85
Fruit (shape and weight in gm.)	Round, 50	Slightly tubular, 82	Round, large, 110
Fruit per plant	18-22	12-14	16-20
Time required for fruit setting from plantation	55-60	60-65	85-95
Yield per hectare (in ton)	36-40	38-42	60-75
Vitamin C content (mg/100g)	18	19.5	21

Figure 2.1 Seeds of BINA Tomato 2 (A), BINA Tomato 3 (B) and BAHAR (C)

2.2 *Agrobacterium* and plasmid vector

Agrobacterium tumefaciens strain LBA4404 carrying the plasmid *pK7WG2_AtNHX1_1.6* was used for in planta transformation. The plasmid vector (binary) incorporated the gene of interest *AtNHX1_1.6* and nptII for selection. *AtNHX1* encodes the protein for vacuolar sodium proton antiporter of *Arabidopsis thaliana*. *NptII* (neomycin phosphotransferase II) gene encodes an aminoglycoside phosphotransferase that can inactivate a range of aminoglycoside antibiotics including kanamycin. Additionally the construct also confers tolerance against streptomycin and spectinomycin for selection among bacteria. Both the genes are under the strong constitutive promoter 35S and T35S terminator.

2.3 Germination medium

For *in vitro* germination and growth of control plants (not infected) solidified Murashige and Skoog (1962) medium without any hormones was used.

2.3.1 Preparation of MS medium

MS medium consists of several stock solutions of defined strength. The composition of MS medium is mentioned in table 2.2. Preparation procedures of the stock solutions are delineated later. Firstly, all the glassware to be used are washed with detergent, tap water and distilled water sequentially. All the stock solutions (Macro, micro, organic, iron EDTA) were added to a minimal volume (1/3 of the total medium volume) of distilled water and dissolved properly before adding the next solution. Then, sugar and myo-inositol was added and dissolved properly. After, adding the remaining amount of

water, pH of the solution was adjusted to 5.8±0.2 with the addition of NaOH or HCl as required. The solidifying agar powder was added at last and dissolved evenly by melting in oven.

Table 2.2 The composition of MS solidified medium

Component	Amount required per liter
Macro 10X	100 ml
Micro 100X	10 ml
Organic 100X	10 ml
Fe-EDTA 100X	10 ml
Sucrose	30 grams
Myo-inositol	0.1 grams
Agar	7-8 grams

The prepared MS medium was poured into flasks and autoclaved within the shortest period of time. Finally the flasks containing the sterilized medium were kept at 25ºC away from light.

2.3.2 Preparation of the stock solutions

For all the stock solutions, the components were added in distilled water according to their concentrations and dissolved properly one after another. In case of Fe-EDTA the stock was kept in amber bottle or foil paper wrapped reagent bottle. All the stocks were autoclaved and kept at 4ºC for preservation.

Table 2.3 Composition of Macro 10X

Component	Amount in grams/liter
KNO3	19
NH_4NO_3	16.5
$MgSO_4.2H_2O$	3.7
$CaCl_2.2H_2O$	4.4
KH_2PO_4	1.7

Table 2.4 Composition of Micro 100X

Component	Amount in milligrams/liter
KI	83
H_3BO_3	620
$MnSO_4.4H_2O$	2230
$ZnSO_4.7H_2O$	860
$Na_2MoO_4.2H_2O$	25
$CuSO_4.5H_2O$	2.5
$CoCl_2.6H_2O$	2.5

Table 2.5 Composition of Organic 100X

Component	Amount in milligrams/liter
Nicotinic acid	0.5
Pyridoxin HCl	0.5
Thaimin HCl	0.1
Glycine	2.0

Table 2.6 Composition of Iron EDTA

Component	Amount in milligrams/liter
$FeSO_4.2H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3

2.4 Co cultivation medium

For co-cultivation of infected seeds with *Agrobacterium*, solidified Murashige and Skoog (1962) medium in petri plates without any hormones was used.

2.5 Selection medium

For selection through kanamycin resistance and to inhibit *Agrobacterium* growth following co-cultivation, MS medium was supplemented with 20 mg/l kanamycin and 100 mg/l Cefotaxim (bacteriostatic). From the stock antibiotics, specific amount of these antibiotics were added to melted MS solidified medium at temperatures lower than 50ºC (table 2.7).

2.5.1 Antibiotic stock preparations

Cefotaxim, kanamycin, streptomycin and spectinomycin (Duchefa bioc) antibiotics were used in this study. The antibiotic powder were added to specific amount of double distilled water according to desired concentrations (Table 2.7). Then mixed properly and filtered with microfilter. The whole preparation took place in laminar air flow. The stocks were kept in sterilized eppendorf tubes at -20ºC.

Table 2.7 Concentrations of antibiotics in stock and in media

Antibiotic	Concentration of stock (mg/ml)	Concentration in media (mg/l)	Amount to be added in media
Cefotaxim	100	100 in MS	100µl in 100ml MS
Kanamycin	25	20 in MS 50 in YEP	80µl in 100ml MS 100µl in 50ml YEP
Streptomycin	20	25 in YEP	100µl in 50ml YEP
Spectinomycin	25	50 in YEP	63µl (approx..) in 50ml YEP

2.6 *Agrobacterium* liquid culture medium

YEP (Yeast extract peptone) medium, supplemented with streptomycin, spectinomycin and kanamycin was used as liquid culture medium. Components of YEP were dissolved in distilled water and the pH was set to 7±0.2 and sterilized. Just before using the medium, antibiotics were used according to Table 2.7.

Table 2.8 Composition of YEP medium.

Components	Concentration (g/l)
Yeast extract	10
Peptone	10
NaCl	5

2.7 *Agrobacterium* solid culture medium

YEP with antibiotics, similarly as the liquid culture was used with 0.7-0.8% agar to prepare solid culture medium in petri dishes.

2.7.1 Preparation of *Agrobacterium* culture

As a routine *Agrobacterium* culture in solid medium were subcultured to new plates every 4-5 weeks.

YEP medium supplemented with antibiotics was inoculated with bacterial colonies from solid cultures. Inoculated medium was incubated at 28ºC for 13-16 hours in orbital shaker. The desired OD_{600}, 0.6 to 1.4 was achieved within this time.

2.8 Optical Density measurement

OD of liquid cultures were measured at 600 nm with spectrophotometer. While measuring YEP medium with antibiotics was used as control.

2.9 *In planta* transformation

For transformation all the following procedures were completed under aseptic condition. Afterwards the seeds were grown till their first true leaves were large and strong.

2.9.1 Sterilization of seeds

The seeds were first visually selected to exclude all the blackened and abnormally shaped ones. Firstly, the sterilization protocol required 5 minutes of 70% alcohol treatment. Then another 5 minute treatment with 30% Clorox (table 2.10) mixed with a drop of Tween-20 detergent. Between the treatments autoclaved distilled water washes were performed twice and afterwards the seeds were washed till all traces of detergent was removed.

The sterilized seeds were put into flasks covered by foil paper and shaken in autoclaved distilled water over night by orbital shaker at 120 rpm. Incubation temperature of 28°C was applied throughout the overnight shaking period.

2.9.2 Infection with *Agrobacterium*

After overnight shaking the seeds were placed onto petri dishes (in laminar air flow). Then, *Agrobacterium* culture of OD_{600} between .6-1.4 was poured into the dishes. *Agrobacterium* infection was applied for 15 minutes and 30 minutes with occasional shaking from the sides.

2.9.3 Co-cultivation

After infection, the infected seeds were placed onto co-cultivation medium. Co cultivation period was set to 24 hours. The non-infected set or control seeds were kept in water.

2.9.4 Placement in selection medium

After completion of co-cultivation for 24 hours, the infected seeds were placed in selection medium. The controls were placed in Germination medium alongside.

2.10 Acclimatization

Following sufficient growth, the seedlings in germination medium or selection medium were taken out and washed with tap water thoroughly to wash off any medium sticking to them. Then they were planted into pit mixed soil in plastic cups. The soil was cautiously hydrated and plastic bags were put on to secure moisture for plants. Holes in the plastic bags were made for minimal air exchange. The planted seedlings were placed in tissue culture room under lights and 25°C.

2.10.1 Nutrient shock

The first phase of acclimatization is basically depriving the plantlet of instant carbon. In this condition the plant is forced to photosynthesize. If necessary, diluted MS medium (one tenth) was added in the soil. At this stage, the insides of polybags were continuously moistened by spraying water. The plants overcome this stage in 10-15 days.

2.10.2 Moisture shock

The plastic bags were partially opened by removing the rubber bands holding them. It had to be carefully checked if any plant was unable to take the stress. If so, the bags were closed again for 1-2 days. When the plantlets could withstand the partially open environment successfully, the plastic bag was completely removed. Plantlets under too much stress were put under open plastic bags again. After successful transpiration had been achieved by the plants a few days were spared before the next step. Usually, moisture shock takes 15-20 days.

2.10.3 Temperature shock

The plants are subjected to outside environment (not under direct sunlight) for 2 hours each day for 7 days. Depending on their response to outside environment gradually the time outside the culture room was increased in the next 7 days. After strong adaptation to outside temperature was observed, the plantlets were put under direct sunlight on an hourly basis for 7 days. The hours under sunlight were gradually increased over the next 14 days.

2.10.4 Transferring to the net-house

Successfully acclimatized plants were taken to net house, planted in large clay pots with soil mixed with sufficient amount of pit. Overhead polythene sheet was used to avoid water logging from rain and bamboo sticks were used when plant heights increased.

2.11 Maintenance in net house

All the plants were labeled properly denoting their variety and dates. The plants were watered routinely, checked for weeds, insects and infestations. If infestations were growing, appropriate counter measures would be taken. Special care was taken when flowers and fruits started to set. All the data regarding morphological and reproductive

nature was recorded. After tomatoes turned red and ripened, they were picked and submitted to laboratory authority for seed collection.

2.12 Sterilization optimization of leaves for leaf disc senescence assay

Different treatment sets (30 seconds to 3 minutes) with 70% alcohol, 30% Clorox and 70% alcohol, 0.1% mercuric chloride was tried to sterilize leaves collected from mature tomato plants. Autoclaved distilled water was carried out between and after treatments with alcohol and Clorox or mercuric chloride.

2.12.1 Reagents for sterilization

30% Clorox and 0.1% mercuric chloride and 70% ethanol was used in sterilization optimization and sterilization of leaves for leaf disc senescence assay. Ethanol 70% was commercially available. Clorox was added to defined volume of distilled water to get the concentration of 30%.

While preparing 0.1% of mercuric chloride, extra caution was taken by wearing gloves. After measuring the defined amount was added to distilled water. The salt was dissolved by stirring with magnetic stirrer.

Table 2.10 Preparation of reagents for sterilization

Reagent	Concentration	Amount needed for 1 litre reagent
Clorox	30% (v/v)	300 ml
Mercuric chloride	0.1% (w/v)	1 g

2.13 Leaf disc senescence assay

All the mature putatively transformed plants were assayed in leaf disc senescence assay. Controls were assayed to determine the usual tolerance levels against salt.

After 8 weeks of plantation, fully grown fresh leaves were selected for leaf disc senescence assay. Leaves were picked from different sides of the plant which were same in age and morphology. The leaves taken from plants were carried in moistened zip lock bags washed vigorously in tap water followed by distilled water.

The leaves were sterilized according to optimized protocol. They were firstly, treated with 70% ethanol for 30 seconds and washed twice with autoclaved distilled water. Then, 1 minute treatment with mercuric chloride was carried out. The leaved were thoroughly washed again with autoclaved distilled water. 1cm (in diameter) sized discs were cut with cork-borer from thick mid portion of leaves with no major veins in the middle. The discs were placed in salt solutions of concentrations 0mM, 25mM, 50mM, 75mM and 100mM in petri dishes.

The placed leaf discs were checked every day for stress signs, yellowing, discoloration, browning, necrosis and bleaching for 15 days.

2.13.1 Preparation of salt solutions

NaCl crystals were measured in electronic balance and added to a defined amount of water. The solutions were sterilized and cooled before using.

Table 2.11 Amount of salt required for salt solutions

NaCl concentration in MS media		Amount of NaCl added in 100ml of water in mg
0mM	0 dS/m	0
25 mM	2.5 dS/m	146.3
50 mM	5 dS/m	292.6
75 mM	7.5 dS/m	438.9
100 mM	10 dS/m	585.2

Chapter 3

Results

Results

The research work presented here was focused on transforming three tomato varieties (BINA Tomato 2, BINA Tomato 3 and BAHAR) with *Arabidopsis* vacuolar antiporter and assessing different aspects of the putatively transformed plants. The aspects under consideration included several indicators of morphology and reproductive nature. Finally, leaf tissue response under salinity stress was documented for characterizing the tolerance level of putative transformed plants.

This study incorporated the optimized parameters established by previous studies and provides the evidence for protein level activity of the putatively transformed plant. In this section the results will be presented step by step.

3.1 Germination response following *Agrobacterium* infection

In this study, the sterilized seeds were divided into two groups: control and test group. The control group was not infected with *Agrobacterium* and therefor placed in MS medium for germination. Test groups were infected with *Agrobacterium* (OD_{600} between 0.5-1.4) for fifteen and thirty minutes. After infection, one day co-cultivation was maintained in antibiotic free germination medium. Then, the seeds were placed into MS medium supplemented with 100 mg/l cefotaxim and 20 mg/l kanamycin. Different number of trials (at least five) were carried out for three different varieties.

During germination the control groups and test groups showed significant differences in several ways. The control seeds germinated quickly compared to the test groups of all varieties. The data is given in table 3.1. All the control seeds of all three varieties germinated within the first five days and their average is just around three days. The infected seeds took more time to germinate; around seven days on average. Seeds of BINA Tomato 2 took the least time to germinate among all three varieties.

The percentage of seed germination or germination efficiency, however, was less varying. Control and test groups of BINA Tomato 2 and BINA Tomato 3 varieties showed similar germination efficiencies. The control and test groups of BAHAR however showed a minor difference (9%). Even this difference can be considered insignificant as the standard variation was high. BINA Tomato 2 showed the highest germination rate (90%) while no germination efficiency was less than 70% (table 3.2).

The growth of the seedlings after germination also showed significant difference just as the time taken for germination (table 3.1). The control seedlings quickly grew revealing true leaves and reaching a proper height. However, the test group seedlings had to overcome the stress from infection and grow in selection medium. So, test group seedlings took longer time for similar growth regardless of the variety (genotype). On an average, BINA Tomato 2 control seeds took the least time, followed by BINA Tomato 3 and BAHAR seedlings. Among the test groups BINA Tomato 3 took the least time while BAHAR test groups took the longest time to achieve first true leaves.

Table 3.1 Time required for germination and development of first true leaves

Varieties	Treatments	Time period required for germination (in days)	Average time requirement for germination (days)	Time required for proper growth of first true leaves (days)	Average time required for proper growth of first true leaves (days)
BINA Tomato 2	Control	2-5	3.2 (±0.92)	7-19	13.9 (±3.69)
	Test	2-10	6.83 (±1.37)	26-31	28.32 (±1.68)
BINA Tomato 3	Control	2-5	3.06 (±0.93)	8-23	14.64 (±3.81)
	Test	2-12	7.65 (±2.17)	21-29	26.03 (±2.28)
BAHAR	Control	2-5	3.13 (±0.92)	12-20	16.96 (±2.16)
	Test	3-13	7.61 (±2.31)	25-38	32.67 (±3.49)

Standard deviation is given within parentheses. The data is collected from four trials of each variety.

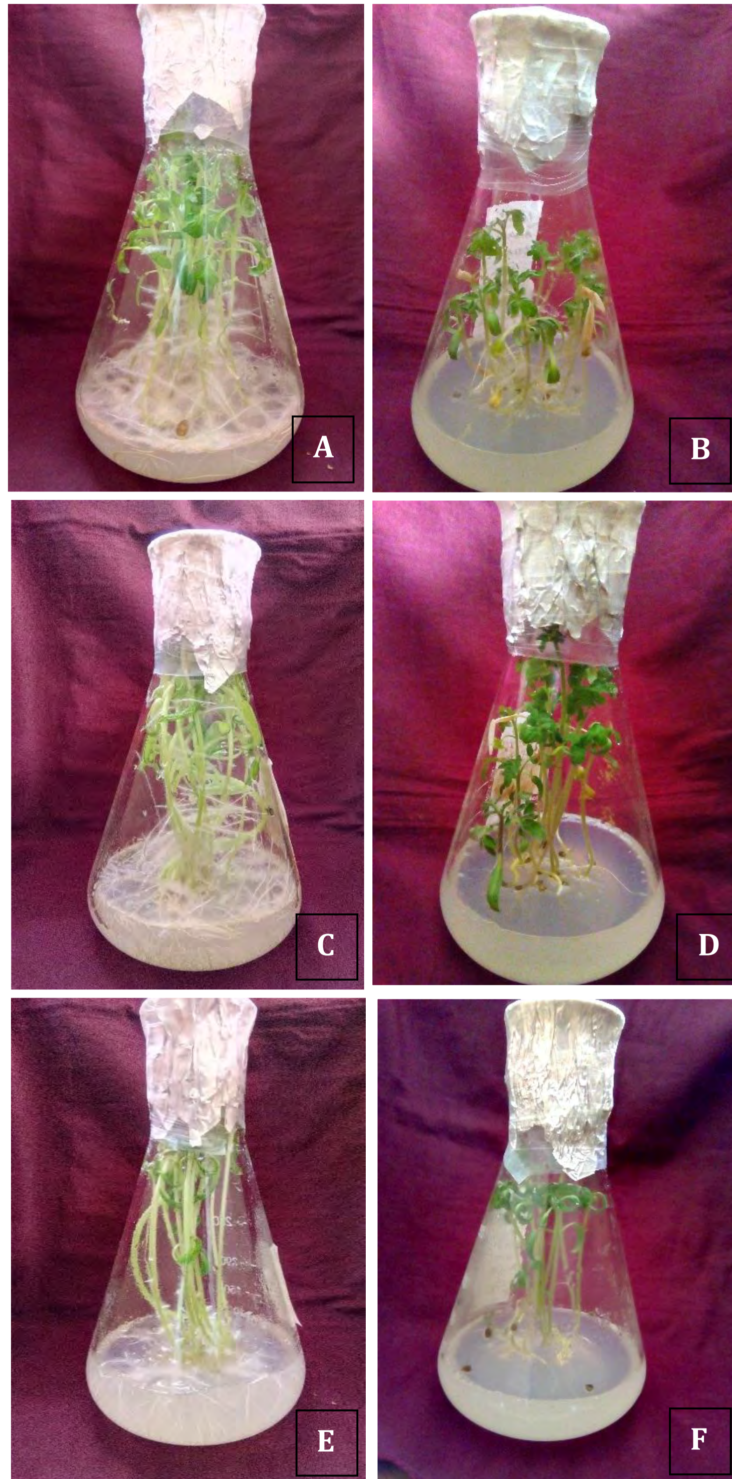


Figure 3.1 *In vitro* growth of control seedlings; (A) BINA Tomato 2, (C) BINA Tomato and (E) BAHAR; and test groups following transformation; (B) putative transformed plants BINA Tomato, (D) BINA Tomato 3 and (F) BAHAR. Photos were taken 4 weeks after seed placement on media.

Table 3.2 Germination efficiencies of control and test groups of the three tomato varieties

Varieties	Treatments	Number of trials	Total number of seeds taken	Total number of seeds germinated	Germination efficiency
BINA Tomato 2	Control	5	88	81	92.5 (±7.06)
	Test	5	164	154	93.9 (±6.18)
BINA Tomato 3	Control	6	98	77	78.6 (±18.74)
	Test	7	210	158	75.2 (±11.0)
BAHAR	Control	6	69	50	72.5 (±11.78)
	Test	5	171	139	81.3 (±17.88)

Standard deviation is given within parentheses.

3.2 Morphological findings after *in vitro* growth

After the completion of *in vitro* growth, seedling height and root length were noted. These two parameters show the growth pattern *in vitro*. Both parameters showed significant difference between control and test groups. For all the varieties, seedling height and root length were greater in the control groups indicating better growth than test group. Visual observation also clearly showed the sturdy growth of controls and weak structure of test group plants. BINA Tomato 2 control showed the longest (on average) seedling height whereas BINA Tomato 3 controls showed the longest (on average) root. In all the varieties, average root length of the control groups were close to 4 cm and for the test groups it was 2 cm BAHAR showed the lowest average height for both control and test groups (table 3.3). BINA Tomato 2 and BINA Tomato 3 control group average heights were just above 9 cm and test group average heights were

above 5 cm Nonetheless, this comparatively weak growth pattern of the test groups was overcome in the next hardening stage.

It should also be mentioned here that all the test groups showed unbranched tap root whereas the controls always had long branched tap roots. Moreover, adventitious roots were present only in all the control groups only. Another interesting feature of the test group plants (regardless of variety) was that all the roots had a bent structure. Such pattern was not seen in any control group plant. Pictures of the different root morphologies are given in figure 3.2. Such pattern is the mark of the stress endured by the infected seeds.

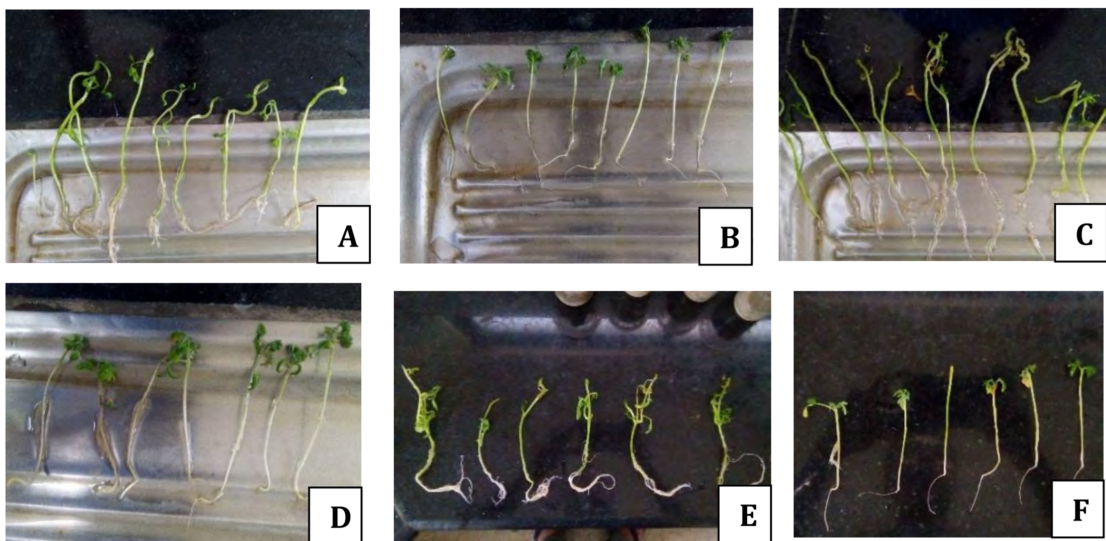


Figure 3.2 Root morphology: Control seedlings showing highly branched tap roots; (A) BINA Tomato 2, (C) BINA Tomato 3 and (E) BAHAR; and putative transformed plants showing unbranched shorter tap roots (B) BINA Tomato 2, (D) BINA Tomato 3 and (F) BAHAR.

3.3 Acclimatization success percentage after *in vitro* growth

Upon sufficient growth, the seedlings were taken out of the flask (*in vitro* set up) and acclimatized to be transferred to the natural environment. During this stage a large number of seedlings died. In all the control groups half of the seedlings died. The reason behind this is the inability of plantlets to cope up with the changing environment in the acclimatization stage. The test groups had almost similar acclimatization success rate.

Only BINA Tomato 2 test group showed a success rate of just above 30%. Such results were got even after providing sufficient care. The standard deviation values were high in all the groups. Table 3.4 shows the data. The acclimatization stage took around 3-5 weeks to complete.

Table 3.3 Seedling height and root length of *in vitro* grown seedlings

Varieties	Treatments	Number of seedlings	Seedling height (after 30 days; in cm)	Average seedling height (after 30 days; in cm)	Root length (after 30 days; in cm)	Average root length (after 30 days; in cm)
BINA Tomato 2	Control	52	4.5-12	9.3 (±1.8)	3-4.5	3.92 (±0.51)
	Test	94	3-7	5.39 (±1.12)	1.5-3.5	2.28 (±0.59)
BINA Tomato 3	Control	43	4.5-12	9.03 (±1.68)	3-5	4.11 (±0.58)
	Test	72	3.5-8	5.56 (±1.19)	1.5-3.5	2.21 (±0.56)
BAHAR	Control	46	4-11	7.52 (±2.12)	2-4	3.62 (±0.52)
	Test	77	3-6.5	4.43 (±0.96)	1.5-3	2.01 (±0.49)

Standard deviation is given within parentheses.

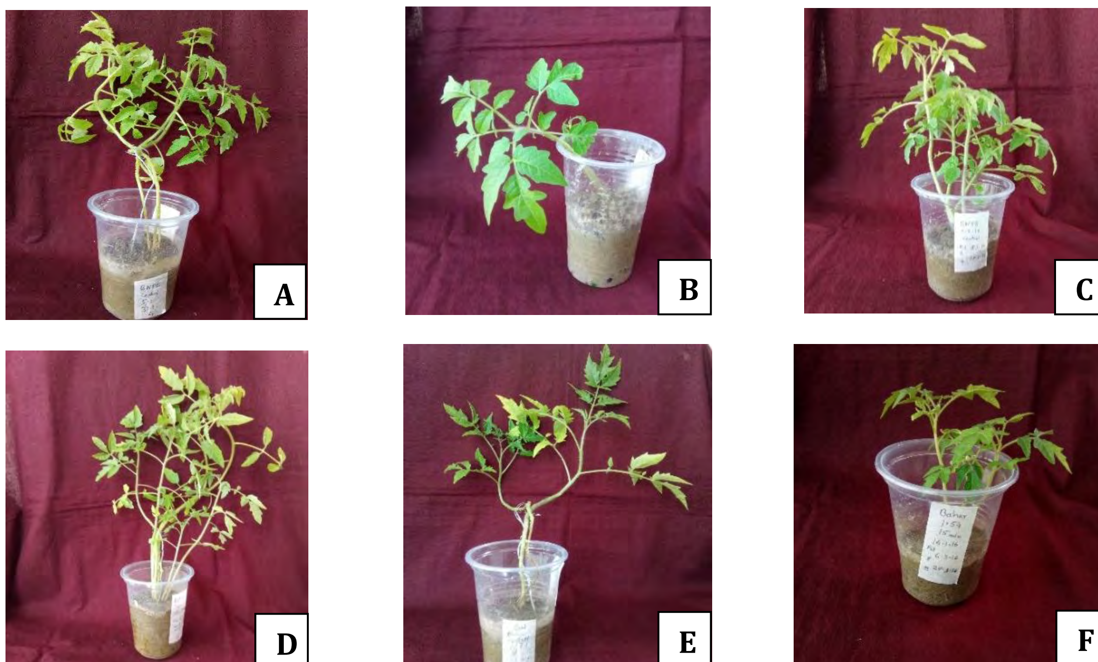


Figure 3.3 Plants after acclimatization: Control plants (A) BINA Tomato 2, (C) BINA Tomato 3 and (E) BAHAR; putative transformed plants (B) BINA Tomato 2, (D) BINA Tomato 3 and (F) BAHAR.

3.4 Survival percentage

The acclimatization stage took around 3-5 weeks. After acclimatization the plants were transferred to natural environment. Some of the plants could not survive this stage but the rest grew, set flowers and fruits. The survival rate was documented. For BINA Tomato 2 and BAHAR, about 90% of the controls survived and 80% of the test group plants survived. For BINA Tomato 3 the percentages were lower (around 75%). Table 3.5 presented ahead shows the data.

From germination to plantation in natural environment, the number of surviving plants decreased in stages. For BINA Tomato 3 and BAHAR varieties the patterns were almost same.

Figure 3.4 Acclimatization and transfer to natural environment of control and test group plants; (A) showing the nutrient shock phase of control; (B) putative transformed plant growing in nutrient shock phase; (C) plants under moisture shock phase of acclimatization (D) plants after successful moisture shock phase of hardening; (E) plants after successful temperature shock stage of hardening; (F) plants planted in natural environment.

The numbers of plants came down to thirty percent at three successive stages: germination *in vitro*, acclimatization, and plantation in natural environment. The acclimatization stage is responsible for most of the mortality. Only for BINA Tomato 2, the pattern shows a difference between the percentages of plants surviving at the end. Graphs 1 (A – C) shows the patterns observed for BINA Tomato 2, BINA Tomato 3 and BAHAR varieties respectively. The graph indicated that overall acclimatization protocol needs to be further adjusted.

Table 3.4 Acclimatization success percentages of control and test groups of the three tomato varieties

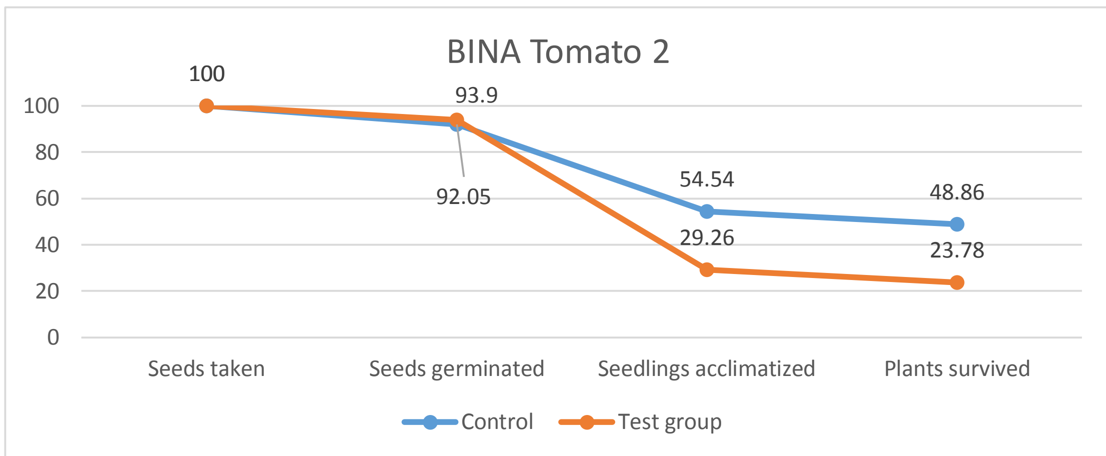
Varieties	Treatments	Total number of seedlings taken for acclimatization	Total number of plantlet surviving acclimatization	Acclimatization success percentage
BINA Tomato 2	Control	81	48	59.2 (±14.23)
	Test	154	48	31.2 (±34.34)
BINA Tomato 3	Control	77	42	54.5 (±28.83)
	Test	158	76	48.1 (±22.15)
BAHAR	Control	50	26	52 (±20.1)
	Test	139	72	51.8 (±20.71)

Standard deviation is given within parentheses.

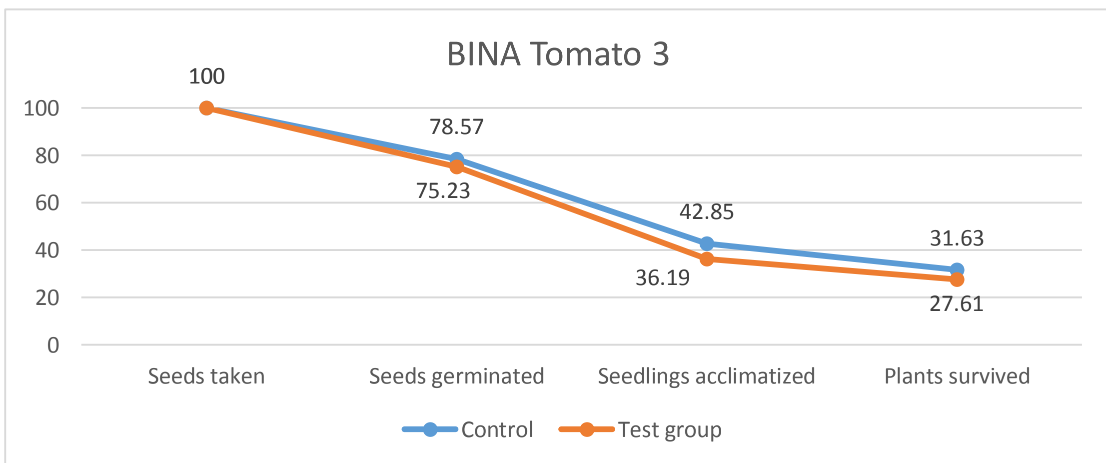
Table 3.5 Survival rate in natural environment of control and test groups of the three tomato varieties

Varieties	Treatments	Total number of plantlets transferred to natural environment	Total number of plants in natural environment (after three weeks)	Survival percentage
BINA Tomato 2	Control	48	43	89.6 (±7.39)
	Test	48	39	81.3 (±3.34)
BINA Tomato 3	Control	42	31	73.9 (±30.21)
	Test	76	58	76.3 (±39.02)
BAHAR	Control	26	23	88.5 (±22.08)
	Test	72	58	80.6 (22.24)

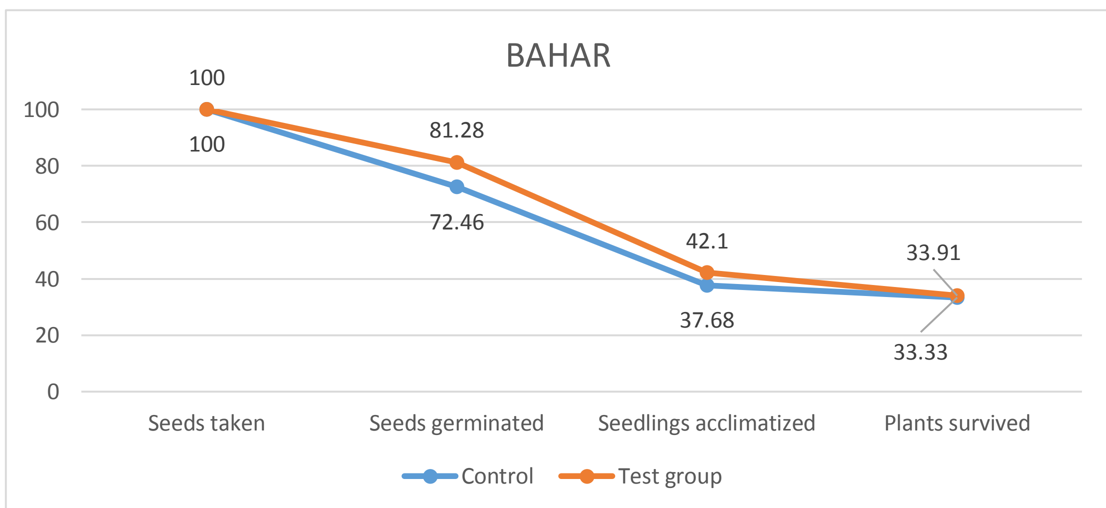
Standard deviation is given within parentheses.



Graph 1-A Seed germination, acclimatized seedlings and survival percentages showing a pattern of decline from the original number of seeds.



Graph 1-B Data showing the pattern for BINA Tomato 3 variety similar to graph 1-A.



Graph 1-C Data showing the pattern for BAHAR variety.

3.5 Morphological findings from fully grown plants

Significant differences were not seen between test groups and control groups of all the varieties with respect to plant height, flowering and fruiting response as it did before. BINA Tomato 2 control and test group plants had an average height of 20.8 cm. BINA Tomato 3 plants had an average height close to 24 cm. BAHAR controls had a slightly higher average height than control (table 3.6). Then again, standard deviation values were higher than the differences. In a nutshell, the height of putatively transformed and control plants did not vary significantly. This is an indicator of the reversion of the putative transformed plants from weaker and stunted growth pattern seen in the previous stages.

Table 3.6 Height of mature plants after 2 months of growth in natural environment

Varieties	Treatments	Average plant height (in cm)
BINA Tomato 2	Control	20.81 (±3.86)
	Test	20.81 (±4.32)
BINA Tomato 3	Control	24.03 (±5.26)
	Test	23.87 (±6.18)
BAHAR	Control	21.56 (±4.81)
	Test	19.96 (±3.72)

Standard deviation is given within parentheses.

Unlike plant height, the total leaf surface data revealed significant difference between control and test groups in BINA Tomato 3 and BAHAR. The putatively transformed plants of both these varieties had significantly higher total average leaf surface area. The control and test group of BINA Tomato 2 variety on the other hand had pretty close total surface area. The test groups of BINA Tomato 3 and BAHAR also had average total leaf surface area close to the total surface area of BINA Tomato 2 (table 3.7). However, this difference did not have any dramatic effect in the reproductive nature of the different groups (section 3.5).

Table 3.7 Total average leaf surface area of fully grown plants

Varieties	**Treatments**	**Number of plants documented**	**Average total leaf surface area in centimeter squared**
BINA Tomato 2	Control	30	487.68 (±25.94)
	Test	30	490.10 (±27.01)
BINA Tomato 3	Control	30	403.58 (±26.54)
	Test	30	503.06 (±32.71)
BAHAR	Control	25	402.88 (±31.02)
	Test	25	489.42 (±28.03)

Standard deviation is given within parentheses.

3.6 Reproductive response of regenerated plants in natural environment

Upon sufficient growth the plants started to flowers and then set fruits. The percentage of plants that flowered (table 3.8), fruited (table 3.9) and the time required to do so were documented. All the plants of test group of BINA Tomato 2 and BINA Tomato 3 set flowers. BINA Tomato 2 putative transformed plants retained the percentage in setting fruit also. The control groups had lower flowering and fruiting percentages. However, the percentages did not go below 80%.

Thus, the reproductive responses of the controls and putative transformed groups were roughly of the same level. If analyzed to the details, it is evident that the reproductive performance of putatively transformed plants was better.

There was very little difference between the groups in terms of time needed to set flowers and fruits. However, the time required for both flowering and fruiting varied only to a small extent among varieties. BINA Tomato 2 was the earliest and BAHAR was the slowest in this scenario. The data are given in the tables below (tables 3.8 and 3.9). Among all the temporal measurements only the groups of Bahar variety showed significant difference in fruiting response.

Figure 3.5 Control plants (on the left) and putative transformed plants side-by-side after two months in natural environment. (A) BINA Tomato 2, (B) BINA Tomato 3 and (C) BAHAR.

Figure 3.6 Floral inflorescence in mature plants: Control plants (A) BINA Tomato 2, (B) BINA Tomato 3 and (C) BAHAR and putative transformed plants (D) BINA Tomato 2, (E) BINA Tomato 3 and (F) BAHAR.

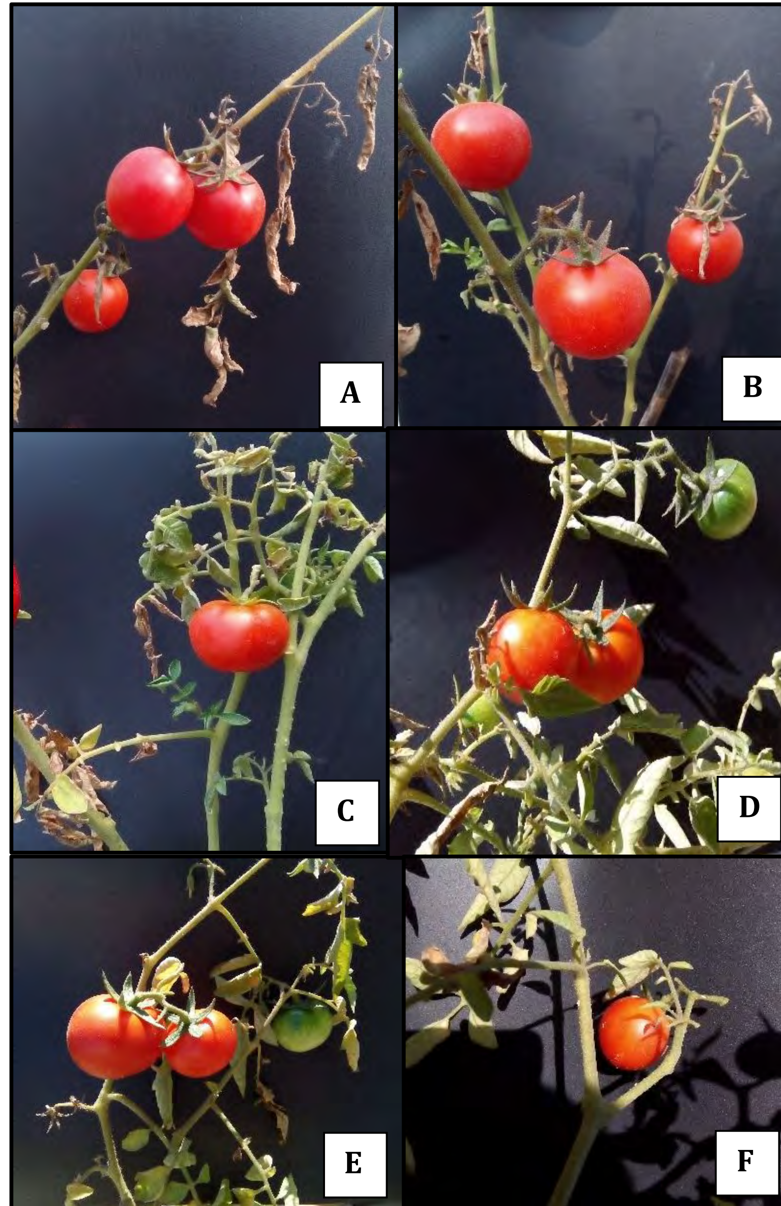


Figure 3.7 Ripened fruit on mature plants: For control plants (A) BINA Tomato 2, (B) BINA Tomato 3 and (C) BAHAR and putative transformed plants (D) BINA Tomato 2, (E) BINA Tomato 3 and (F) BAHAR

Table 3.8 Flowering response in control and putative transformed plants of the three varieties

Varieties	Treatments	Number of plants	Number of plants setting flowers	Percentage of flowering response	Time needed for flowering (weeks)	Average time required (weeks)
BINA Tomato 2	Control	20	19	95	7-9	8.1 (±0.64)
	Test	30	30	100	7-10	8.23 (±1.02)
BINA Tomato 3	Control	20	18	90	8-9	8.74 (±0.45)
	Test	30	30	100	8-9	8.61 (±0.49)
BAHAR	Control	20	16	80	8-10	9.24 (±0.83)
	Test	25	23	92	9-11	9.56 (±0.79)

Standard deviation is given within parentheses.

Table 3.9 Fruiting response in control and putative transformed plants

Varieties	Treatments	Number of plants	Number of plants setting fruits	Percentage of fruiting response	Time required for fruit setting and ripening (weeks)	Average time needed (weeks)
BINA Tomato 2	Control	20	17	85	10-13	11.43 (±1.16)
	Test	30	30	100	11-14	12.58 (±1.06)
BINA Tomato 3	Control	20	18	90	11-14	12.14 (±1.01)
	Test	30	28	93	11-14	13.23 (±0.84)
BAHAR	Control	20	16	80	11-13	11.95 (±0.92)
	Test	25	21	84	12-14	13.12 (±0.82)

Standard deviation is given within parentheses.

3.7 Optimization of leaf tissue sterilization for leaf bioassay

To assess the tissue tolerance against salinity stress, leaf disc senescence assay was carried out. But to perform leaf disc assay, leaf sterilization was a very important preparatory task. Here, the sterilization of leaf tissue was optimized. A combination of alcohol (70%) and Clorox (30%) was first used. Then, alcohol (70%) and mercuric chloride (0.1%) was used.

Two factors of the sterilization was: contamination and tissue health. If the sterilization is not strong enough contaminations cannot be removed. Again, with very strong sterilization the leaf tissue will lose its composure and won't show the proper response in leaf disc senescence assay.

Table 3.10 Sterilization of leaf tissue

70% ethanol treatment (minutes)	30% Clorox treatment (minutes)	0.1% Mercuric chloride treatment (minutes)	Remarks on contamination	Remarks on leaf tissue health/status after treatment
1	3	0	Yes	Not bleached
1	5	0	Yes	Not bleached
2	3	0	Yes	Bleached
2	5	0	Yes	Bleached
1	0	2	No	Bleached within 24 hours
1	0	3	No	Bleached within 24 hours
2	0	2	No	Bleached within 24 hours
2	0	3	No	Bleached within 24 hours
0.5	0	3	No	Bleached within 24 hours
0.5	0	2	No	Bleached after 2 days
0.5	**0**	**1**	**No**	**Not bleached**
0.5	0	0.5	Yes	Not bleached

Data was obtained from triplicate trials.

The optimization showed that 70% ethanol treatment for 30 seconds, followed by a 1 minute treatment with 0.1% mercuric chloride removed contamination completely without damaging the tissue. Table 3.10 shows the optimization.

3.8 Leaf disc senescence assay

Finally, leaf disc senescence assay was performed to assess the salinity tolerance levels of control and putative transformed plants. Understandably, the control plant leaf tissue will bleach earlier at lower salt concentration. On the contrary, retention of green color and healthy tissues at high salt levels will indicate tolerance. This will technically confirm the expression of the gene of interest. However, widespread sensitivity to salinity of the putative transformed plant tissues will imply the absence of expression.

Table 3.11 Leaf disc senescence assay of control and putative transgenic plants

Varieties	Treatments	Number of plants documented	Total number of plants tolerant at 25mM NaCl	Total number of plants tolerant at 50mM NaCl	Total number of plants tolerant at 75mM NaCl	Total number of plants tolerant at 100mM NaCl
BINA Tomato 2	Control	5	5	1	0	0
	Test	20	20	3	0	0
BINA Tomato 3	Control	5	5	2	0	0
	Test	15	15	5	0	**1**
BAHAR	Control	5	5	2	0	0
	Test	20	20	6	0	0

All results were obtained after 12 days of salt treatment.

All the control plant tissue showed high levels of damage at 70mM and 100mM salt solutions within 7 days. And in 50mM salt solution only a handful of plants showed tolerance. This effectively sets the control plant tolerance levels at 50mM NaCl. Among all the plants from the three varieties, only one plant from the test group of BINA Tomato 3 survived at 100mM salt solution for 12 days. This indicates to the transgene

expression in that particular plant. Furthermore the successful tolerance was in much higher extent in putative samples compared to controls.

This tolerant plant was infected with *Agrobacterium* of OD_{600} 0.5 for 30 minutes with the plasmid *pK7WG2_AtNHX1_1.6*. The plant bore flower and fruit without any phenotypic alteration. So, it can be inferred that constitutive expression of the transgene did not adversely affect the reproduction of the putatively transformed plant. Table 3.11 shows the results of leaf disc senescence assay.

3.9 Percentage of putative transformed plants and transgene expressing putative transformed plants

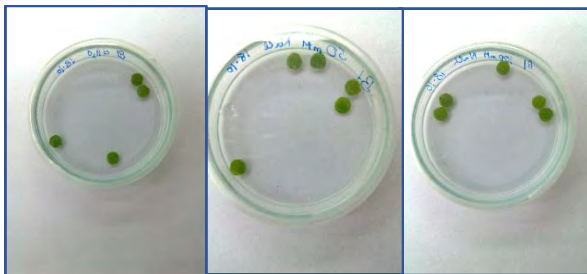
This study was aimed at developing transgenic plant. *In planta* method was applied with the assumption of getting sufficiently enough putative transformed plants. Putative transformed plants were identified by their survival in the selection media and in natural environment. Based on the best three trials, results from all three varieties showed that around half of the seeds infected grew to be putatively transformed. On top of that, the percentage was more or less at the same level regardless of the variety. Among them, BAHAR had the highest percentage of 53 and BINA Tomato 3 had the lowest percentage, 49.2.

Next, percentage of plants expressing the transgene becomes a focus. 20 putative transformed plants of BINA Tomato 2 and BAHAR varieties and 15 BINA Tomato 3 putative transformed plants were assayed on the basis of leaf disc senescence. As table 3.12 depicts, none of the putative transformed plants showed salinity tolerance from BINA Tomato 2 and BAHAR varieties. BINA Tomato 3 had a percentage of 6.66 that showed enhanced salinity tolerance.

Non-transformed control plants

Putatively transformed plants

Day 1:



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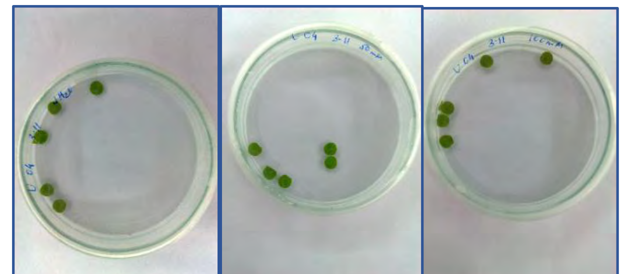
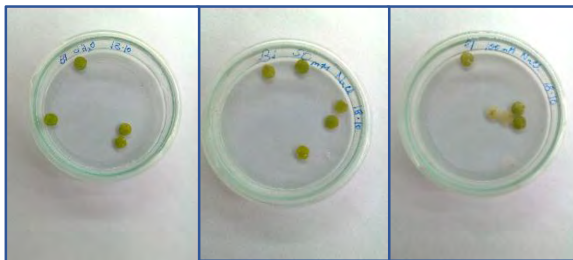
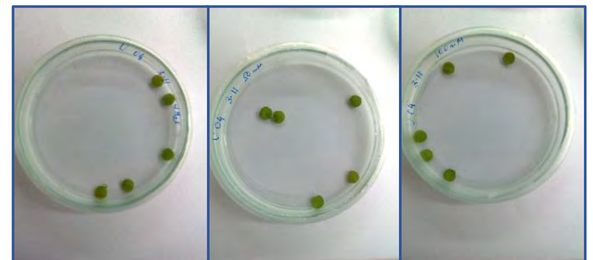


0mM NaCl 50mM NaCl 100mM NaCl

Day 7:

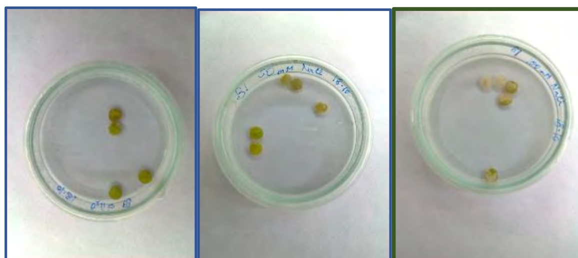


0mM NaCl 50mM NaCl 100mM NaCl

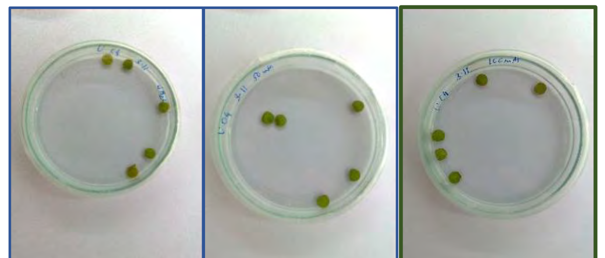


0mM NaCl 50mM NaCl 100mM NaCl

Day 12:



0mM NaCl 50mM NaCl 100mM NaCl



0mM NaCl 50mM NaCl 100mM NaCl

Figure 3.8 Leaf disc senescence assay showing putative transformed BINA Tomato 3 plant tissue healthy at 100 mM NaCl for 12 days. But the control plant tissue showed senescence at day 7 under 50 mM salt concentration.

Table 3.12 Percentage of putative transformed plants and transgene expressing putative transformed plants

Varieties	Number of seeds infected	Number of seedlings surviving antibiotic selection and growing into mature stage	Percentage of putatively transformed plants	Number of plants showing salinity tolerance in leaf tissue and the number of plants assayed	Percentage of putative transformed plants showing significantly greater salinity tolerance
BINA Tomato 2	62	32	51.61	0 (20)	0
BINA Tomato 3	59	29	49.15	1 (15)	6.66
BAHAR	83	44	53.01	0 (20)	0

Chapter 4

Discussion

Discussion

In the current study, seeds from three tomato varieties BINA tomato 2, BINA tomato 3 and Bahar were transformed using *in planta* method. After infecting the embryos with *Agrobacterium*, the seeds went through *in vitro* development and acclimatization stages. Only around 1/3 of the total seeds infected survived to the last stage (natural environment). Their reproductive response was a major area for interest in the study. Finally, leaf disc senescence assay revealed the salinity tolerance levels of the plant leaves.

4.1 Selection process

Detection of transformed from non-transformed plants is absolutely crucial in any transformation study. For *in planta* transformation, selection should be done at t_1 generation (Keshamma *et al.,* 2012). However, in this study, due to the challenges of achieving a t_1 generation and time limitation, selection was done at t_0 generation. The aim of the experiment was to select the infected embryos that expressed the inserted genes for the first 3-4 weeks.

At this stage, several attempts have been reported; such as, Subramanyam with his team used a two stage selection process. Once after germination and again after fifty three days have passed (Subramanyam *et al.,* 2013). This successfully eliminates all the escapes. More than two stages of selection can be done with ease by supplementation of antibiotics to media. Bhuiyan and his colleagues used a three stage selection process throughout which the selection pressure (antibiotic concentration) was gradually increased in successive stages. Another factor prioritized in that study was the delayed exposure of the infected tissue to selection agent (antibiotic) (Bhuiyan *et al.,* 2011). A gap between co-cultivation and selection gives the infected tissue enough time to recover from *Agrobacterium* infection and grow. The growth of the infected tissue is important as single transformed cells divide and produce a group of transformed cells. This leads to reduced elimination of transformed cells which were weak and had lower expression. Das and his team used 200mg/l kanamycin for selecting transformed BARI tomato 8 following the gradual increment of selection pressure without any intermission (Das *et al.,* 2015). But Sarker and his colleagues used the interval for the above

mentioned reasons and gradually increased antibiotic concentrations to 200mg/l for BARI tomato 3 (Sarker *et al.,* 2009a). However, all these reports are on tissue culture based regeneration following transformation.

Unfortunately, in the present study, single and simple selection methodology was followed which led to rapid seed germination and plantlet development. Here, a lower antibiotic concentration was used which helped the survival percentage at that stage to rise. Unfortunately, this one stage selection also made it possible for transiently transformed seedlings to be selected.

Above that, using strict selection after infection could have resulted in very low germination efficiency. Though stringent selection is often considered; the use of low selection pressure can be backed up by the insights from the experiments of Pandey (2010) and Ombori (2013) with their colleagues. Ombori showed, how GUS markers can show results that can be unrelated to the final transformation efficiency. And Pandey stated the complexity of kanamycin selection process to find out untransformed cells among transformed ones and vice versa.

4.2 Parameters of different factors in this study

The parameters used in this study was based on previous studies carried out to establish an optimized procedure of *in planta* transformation. Here, 15 and 30 minutes of infection time, 0.8 to 1.2 OD_{600} and 24-28 hours of co cultivation was used. Each parameter has been shown to provide the best transformation success rates (Datta, 2015) for all the three tomato varieties. All of these parameters act together to produce healthy transformed tissue or seedling (Paramesh *et al.,* 2010). Liu and colleagues (2009) showed with optimization studies that too much deviation from the optimized level of one parameter can bring the transformation efficiency to a mere zero. In transforming soybean through ovary dip method, when they removed the stigma instead of cutting style, none of the trials were successful.

In this study, pricking was avoided. Previous results of Dattta (2015) and Margaret along with her research team suggested that wounding did not help with the transformation studies (Margaret *et al.,* 2004). On the other hand, several other studies reported strategies to enhance transformation.

Pandey and his colleagues (2016) used vacuum infiltration chamber for transformation of cumin seeds while performing *in planta* transformation. Manickvasagam with his team on the other hand, used both sonication and vacuum chamber (Manickvasagam *et al.,* 2015). Infecting seeds in vacuum chamber reduces the atmospheric pressure and lets the air within the seed come out. This free space can then be occupied by *Agrobacterium* solution enhancing transformation efficiency. On the other hand, sonication creates micro-pores or channels in seeds making paths for bacteria to reach the meristematic cells of the zygotic embryo. In the present study, natural imbibition led transformation was used.

4.3 Germination, *In vitro* growth, physiological and reproductive response related findings

In the *in vitro* stage, the time required for germination and proper growth of the test group took almost double time compared to the control plants. Above that, overall height and root length also proved to be significantly different. Such differences can be explained by the presence of antibiotic kanamycin (selection agent) and cefotaxim (bacteriostatic) in the medium. The stress from the infection can also be attributed to have caused such pattern. The roots of all test group plant varieties were short and thin. But most startlingly, they were bent at the initiation point of shoot (figures 3.2). This can also be explained by antibiotic stress.

Datta (2014) reported 42% - 48% regeneration percentage after *Agrobacterium* infection for 3 varieties. For *in planta* transformation, the germination percentages in selection medium were 96%, 92% and 93% for BINA tomato 2, BINA tomato 3 and Bahar respectively. Compared to her result, the percentages in this study were 92%, 75% and 72% for BINA tomato 2, BINA tomato 3 and Bahar respectively. However, germination efficiency of two groups (control and test) of BINA tomato 3 and Bahar varieties were close. So, it is evident that the factors which caused slow and retarded growth in the *in vitro* stage did not significantly impede the germination process. In fact mature plant height of the putative transformed plants after the same time in natural environment was the same as control plant height. This suggests that even though there was significant differences in *in vitro* stage, they did not persist in mature plants. The

initial stress of the transformation procedure used in this study was successfully overcome by the plants regardless of varieties.

Before we move on to discussing the physiological results, the acclimatization stage should be mentioned. The mortality rate was the highest in that phase. Understandably, all the plants could not cope up with the changing nutrient, moisture, temperature and light intensity (Chandra *et al.,* 2010). As a result a lot of seedlings died. After acclimatization, only a small number of plants died in the natural environment. For this reason, in future more emphasis is to be given to establishing a more efficient system for acclimatization.

Average total leaf surface area data revealed no difference in control and putatively transformed plants for BINA Tomato 2. The case was different with the other two varieties. Significant difference was seen between the groups. Interestingly, in both varieties the putative transformed plants had a larger average total surface area and the test groups of all the three varieties had similar figures. However, this pattern did not result in any major health concerns for the plants. So it can be said that this difference has been pretty much inconsequential.

Flowering and fruiting percentages in both groups of all varieties were very high. But the percentages were always higher in putatively transformed ones. This can be explained by the selection of the fittest plants. As the putative transformed ones had gone through a stressed phase, the surviving ones are supposed to be better responsive when in natural environment. The time needed for flowering varied among the varieties but stayed almost same in control and putative transformed groups. Here, this might be the varietal difference at work.

Several studies previously reported anomalies in the reproductive nature of transformed plants in t_0. Ombori and his team (2013) reported sterility in maize and Yasmeen and her colleagues (2008) reported failure in setting fruit for tomato.

In the present study, several important morphological and reproductive data showed similarity between control and putatively transformed plants. Such similarities are very common in transformation studies. Under non-stress conditions, control group plants and transformed plants tend to yield similar results. One report (Asif *et al.,* 2011) had no phenotypic abnormality in test group plants under normal condition. Another (Parvin *et al.,* 2015) report showed that chlorophyll, Na^+, K^+ concentrations of transformed

plants remained same under non stress conditions. On top of that, Tian (Xing) *et al.* (2010) reported no morphological dissimilarities between wild type and transgenic plants constitutively expressing atnhx1 in groundnut.

Lastly, the time required for the whole procedure is a major concern for any plant transformation related studies. For the transformation of tomato, both tissue culture based and tissue culture independent methods are published widely. Here, tissue culture independent *in planta* method was used to get two major advantages: a greater number of putative transformed plants (section 1.12) and less time requirement. Subramanyam and his colleagues (2013) reported that only 27 days were needed for getting transformed brinjal. In this study the test group plants took 7-8 weeks to be ready for plantation. The reason lies in the protocol used. Though this study followed tissue culture independent method, it incorporated an *in vitro* stage. That is why a significantly longer time was needed. Nonetheless, compared to the time required in a tissue culture based protocol, *in planta* method with an *in vitro* stage was found to be less time consuming.

4.4 Leaf tissue senescence assay

This part of the study reveals the expression of the transgene. Though flowering and fruiting results have been highly satisfactory, the leaf disc senescence assay did not yield results as expected. 0mM, 25mM, 50mM, 75mM and 100mM NaCl concentrations were used. Most of the controls could only tolerate upto 25 mM. However, a significant number of control plants could tolerate up to 50mM NaCl stress for over 10 days. So the highest level of tolerance in control plants were set at 50mM NaCl. If any putative transformed plant showed tolerance more than this level, it would be considered the tolerance level provided by the overexpressed vacuolar antiporter. Unfortunately, only one plant showed leaf tissue tolerance at levels greater than 50mM. That putative transformed plant leaf tolerated 100mM NaCl for 12 days whereas most putative transformed plant leaves could only tolerate 50mM NaCl for 7 days. It is noteworthy that overall tolerance of the putative Transformed leaves were more than the control.

The plant showing higher tolerance was a BINA tomato 3 plant. It was infected with *Agrobacterium* (OD_{600} 0.5) for 30 minutes. Though OD_{600} was below the optimized

bacterial density, this plant was the only one showing higher salinity tolerance in its leaf tissue. Such deviation can only be reasoned by the often uncertain response of different biological system. Even using the best parameters cannot guarantee success. Here the response of *Agrobacterium* and the plant tissue altogether might have caused highly inefficient transformation or protein expression. That will be discussed in the next part. Now, other leaf disc senescence assay results are presented (table 4.1) in the next page.

From the results it is obvious that the tolerance level achieved in this study endured a lower concentration of NaCl, but for a longer time. The possibility of a higher tolerance cannot be ignored. However, this assumption needs further experimentation.

As mentioned before, no putative plants from BINA tomato 2 and Bahar showed better tolerance than control plants. The reasons can be divided into two types. First is the lack of transgene expression and second, no transgene integration at all.

Jan in his review (2016), mentioned chimerism and instability of transgene as the major problems of any *in planta* transformation. If the leaves used in assay were from untransformed regions of the plant, the assay will surely provide negative result. Usually in *in planta* transformation studies such as this one, protein level assays are done at t_1 generation. If the transgene is integrated into undifferentiated meristematic cells which are destined to develop sympodial branches, seeds obtained from these reproductive structures would produce stable transformants in t_1. Chimeric results have been reported by Keshamma *et al.* (2008) and Moghaieb *et al.* (2014).

Another factor that is very crucial for proper gene expression is the copy number of the integrated genes. Single copy integrations result in proper gene expression. But multicopy gene insertions will alter gene expression for certain in most cases. Sultana and her colleagues reported multicopy insertion with *AeMDHAR* gene conferring salt tolerance resulted in sterile plants (Sultana *et al.,* 2012). Ombori (2013) in his transformation study got 2-6 copy numbers. He also mentioned the benefits of single copy integration in harboring less gene silencing and increased stability. Islam and Khalequzzaman (2015) reported all their transgenic plants carrying single copies of genes conferring tolerance to cadmium. A similar report was made by Bhuiyan and his team (Bhuiyan *et al.,* 2011). Keshamma and his colleagues on the other hand reported in their transformation study that all integration were no single copy integrations

(Keshamma *et al.,* 2012). Kohli and his colleagues (2010) in their review expressed how unstable multiple copy integration is and how it might silence the gene.

Table 4.1: Comparison of different levels of tolerance assessed from leaf tissue senescence assay.

Target plant and variety	Gene of interest and its source	Gene expressed protein function	Leaf disc senescence assay result	Reference
Rice; BRRI dhan 55	SNAC1 from rice cv. pokkali	Transcription factor for abiotic stress	200mM salt tolerance for 3 days	Parvin *et al.*2015
Rice; Taipei 309	MDHAR from *Acanthus ebracteatus*	Monodehydro reductase; ROS scavenger	150mM salt tolerance for 4 days	Sultana *et al.*2011
Rice; BRRI dhan 28	SOS1 from pokkali rice cv.	Plasma membrane antiporter	150mM salt tolerance for 7 days	Yasmin *et al.*2015
Tomato; Pusa Ruby	CodA from *E coli*	Component of Glycinbetaine biosynthesis pathway	200mM salt tolerance for 7 days	Goel *et al.*2010a
Tomato; Pusa Ruby	Osmotin gene from tobacco	Osmoprotectant	200mM salt tolerance for 7 days	Goel *et al.*2010b

Finally, it can be summarized that an attempt was made to achieve putatively transformed plants carrying *AtNHX1* gene for tolerance against high salinity in this study. Backed by the current situation in agriculture of Bangladesh, this was a very timely attempt. However, the protein level assay indicated to the achieved tolerance. Tolerance to 100mM salt solution for 12 days is not an insignificant result. Further studies are needed to characterize the genetic makeup of the transformed plants. From this study it can be taken as suggestion to prioritize protocol improvement for better transformation efficiency.

Chapter 4

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