MULTIPLE TRANSGENE ENGINEERING FOR MAIZE (*Zea mays* L.) DROUGHT AND SALT TOLERANCE

By

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ABSTRACT

MULTIPLE TRANSGENE ENGINEERING FOR MAIZE (*Zea mays* L.) DROUGHT AND SALT TOLERANCE

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Maize (*Zea mays* L.) is an important basic food and feed grain, and its stover is used for animal feed and biofuel. Increased soil-salinity and water-deficiency are the two major factors limiting the maize plant growth and development and subsequently affecting its grain and biomass yields, and the yield. The research in this dissertation is focused on multiple transgene engineering (transgene pyramiding) for drought and salt tolerance in maize.

In the research presented in this dissertation, genetic transformation of maize was performed via the gene gun bombardment of embryogenic immature embryos of maize using single and multiple constructs, pBY520 containing the barley (*Hordeum vulgare) HVA1* and the JS101 containing the bacterial mannitol 1 phosphate dehydrogenase (*mtlD*), both genes regulated by rice actin promoter and potato protease inhibitor II terminator. There were two gene linked cassettes in each of these two constructs, one cassette containing the abiotic stress tolerance gene of interest (*HVA1* or *mtlD*) and the other containing the *bar* herbicide resistance gene regulated by cauliflower mosaic virus 35S promoter and nos terminator.

The pBY520 and JS101 constructs were co-bombarded in 1:1 ratio into maize genome for transgene pyramiding. The confirmation of transgene integration and expression were made via molecular techniques, including polymerase chain reaction (PCR) for transgene integration, and reverse transcriptase (RT) PCR and Northern blotting for transgene transcription. Southern blotting was performed to find the number of copies of each transgene in transgenic plants.

Results showed stable integration and expression of the *HVA1, mtlD* and *HVA1-mtlD* in transgenic maize plants. Up to fourth generation transgenic (T3) plants were produced, with all progenies showing the co-integration of abiotic stress tolerance genes of interests and the *bar* gene with a frequency of 100%. The single *HVA1 or mtlD* transgenic plants showed higher leaf relative water content (RWC) and higher percent of plant survival as compared to their wild-type non-transgenic control plant counterparts under water withholding condition for 15 days followed by 7 days of re-watering. When exposed to different salt concentrations (0, 100, 200 and 300 mM NaCl) for 10 days, the *HVA1* and *mtlD* transgenic plants showed higher fresh and dry shoot and dry root biomass matter as compared to their wild-type non-transgenic control plants. The research also demonstrated that the *mtlD* transgenic plants that were salt tolerant, also accumulated mannitol in their cells. More research is needed on mannitol accumulation in transgenic plants to see whether the mannitol level measured by gas chromatography (GC) was not partially or totally representing sorbitol accumulation. Considering that mannitol and sorbitol are both osmoprotectants, the salt tolerance of the *mtlD* transgenic plants might be due to the accumulation of one or both osmoprotectants in *mtlD* transgenic plants.

Co-transformation of two transgenes (*HVA1+mtlD*) in maize plants also demonstrated the co-integration and co-expression of these two stacked genes in up to T3 plants, resulting in improved plant survival rate under 15 and 20 days of water withholding and enhanced shoot and root biomass weight at 100mM NaCl as compared to single transgene (*HVA1* or *mtlD*) transgenic plants and as compared to the wild-type control plants.

This dissertation also covers genetic transformation of maize plants with a construct containing the sorghum dehydration responsive element binding 2 (DREB2) transcription factor regulated by the *Arabidopsis rd29* drought inducible promoter and *nos* terminator. The preliminary results showed the integration of DREB2 into maize plants via PCR analysis. The DREB2 transgenic maize research will be continued in Sticklen’s laboratory by another researcher in the near future.

Although salt stress is predictable, drought is not predictable in most counties on earth. The dissertation research presented here is a step towards production of maize plants that can tolerate the harsh abiotic stress conditions of drought and salt.

DEDICATION

I dedicate this work to my wife, my son, and my daughter for their patience, love, encouragement and tireless support throughout my studies.

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KEY TO SYMBOLS AND ABBREVIATIONS

ABA: Abscisic Acid

Act1: Actin Rice Promoter

BAP: Benzylaminopurine

CaMV35S: Cauliflower Mosaic Virus (CaMV) 35S Promoter

DREB: Dehydration Responsive Binding Element

Hpt: Hygromycin phosphotransferase

Hsp: Heat Shock Protein

HVA1: *Hordeum vulgaris* abandant protein

IBA: Indole-3-butyric Acid

JS101: plasmid containing *bar* and *mtlD* gene

LB: Luria Bentani media

MS: Murashige and Skoog media

Nos: nopaline synthase terminator

pBY520: plasmid containing *bar* and *HVA1* gene

pC1301: plasmid containing *hpt* and *DREB2* gene

PCR: Polymerase Chain Reaction

PinII: potato proteinase inhibitor terminator

ROS: reactive Oxygen Species

RT-PCR: Reverse Transcription- PCR

INTRODUCTION

After wheat and rice, maize is one of the most important cereal crops in the world with a global production of 844.4 million tons. The United States is the largest maize producer in the world with average maize yield of 9.59 tons per ha and production of 316.16 million tons in 2010 (FAO, 2010). The yield and production of maize has been predicted to increase rapidly in the coming decades via the continuation of the use of plant breeding and biotechnology, including genetic transformation to meet the global increase demand for food, feed and industrial needs (Vega *et al.,* 2008).

Plant regeneration is the first important step which contributes to the success in plant genetic transformation. In the last years, maize scientists have tried to use different explants cultured and grown in different media, and then finally regenerated fertile plants (Sahrawat *et al.*, 2003; Shan *et al.,* 2009).

Maize tissue culture was initiated by Green and Phillips in 1975 when they used a compact type-I immature embryo callus from inbred line A188 in their studies. However, the problem with this callus type was that it grew very fast and therefore did not produce large number of somatic embryos. Then, Armstrong and Green cultured maize immature embryos on N6 media resulting in large number of somatic embryos. In their report, they explained the type I callus as-less embryogenic culture, but type II as highly embryogenic. In their research, regeneration and transformation of maize Hi II derived from a cross of A188xB73 (Armstrong et al. 1991) as it was the best choice for maize transformation due to it's highly type II embryogenic callus. In the author's dissertation, he used the type II HiII maize callus for his maize regeneration and genetic transformation.

Jones and Reiter (1992) regenerated maize plants from immature embryo-derived shoot meristems. However, this organogenic system has been less used due to the production of chimeric plants, plants that had transformed and non-transformed cells (Lowe *et al*., 1995).

A very efficient and genotype independent regeneration of maize was developed by the Sticklen team based on the plasticity and manipulation ability of mature seeds germinated shoot apical meristem primordium and their *in vitro* multiplication ability (Zhong *et al.*, 1992).

Maize transformation technology was initially developed in the 1990s for production of European corn border resistant plants. To date, the introduction of different genes into maize has been adapted to increase resistance to pests, herbicides and other biotic and abiotic stresses resulting in improved maize productivity for food, animal feed, biofuel and other expanding demand.

In the last decades, the development of several plant transformation methods was adopted to introduce genes into plant tissues. The concept of *Agrobacterium*-mediated transformation was developed in the 1970s and commonly used up to now in dicotyledonous plants because these plants could be infected by *Agrobacterium* (Zupan and Zambryski., 1995; Oneto *et al.*, 2010). However, in monocotyledonous plants including maize, this technique has shown the limitations of *Agrobacterium* infection (Komari *et al.*, 1998; Kriz and Larkins., 2009).

Biolistic or particle bombardment technology has been considered the best choice and used widely for maize transformation (Zhong *et al.*, 1996; Frame *et al.*, 2000; Aulinger *et al.*, 2003). Sticklen team used apical shoot meristem primordium bombardment for an efficient and relatively independent genetic transformation of maize. The success of this system is based on the plasticity and manipulation ability of this explant and its *in vitro* multiplication ability (Zhong *et al.*, 1996). In their technique, the team bombarded the inert particles such as tungsten or gold which were coated with genes of interest to penetrate into the plant meristem sub-epidermal cell layer (stem cell layer) because this layer has the potential to produce fertile transgenic plants (Sticklen and Oraby, 2005) using 22 caliber shells. Other scientists used the Biolistic device (gene gun) to bombard genes into immature embryos using helium gas instead of 22 caliber shells (Li *et al.,* 2003; Fadeev *et al.,* 2006).

Wu *et al.* (2002) used particle bombardment method and introduced up to nine independent genes at once, including selectable markers and expressed independently in transgenic rice. Then, the system was used for transformation of functioning artificial mini chromosome into maize (Yu *et al.,* 2007).

In addition to the method of DNA delivery system and explants used, other factors are important for high efficiency in plant genetic transformation. These include; the use of genotype, selectable marker and types of in vitro culture media (Decima *et al.,* 2010). Using suitable selection markers might be the most important factor in plant transformation (Sahrawat s*et al.,* 2003). The ideal selectable marker for dicotyledonous genetic transformation include; neomycin phosphotransferaseII (*nptII*) and hygromycin phosphotransferase (*hpt*) genes. In addition, the beta-glucuronidase (*gus*) gene has been utilized widely to select transgenics from non transgenic events via color indication. Nevertheless, the use of those markers in maize cell selection has not been efficient (Kriz and Larkins. 2009). However, the genes encoding a mutant form of acetohydroxy acid synthase (AHAS) protein or the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase have been used successfully in maize transformation (Peng *et al.,* 2006). However, the most successful selectable marker gene for maize transformation is the bialaphos resistance (*bar)* gene, a gene encoding phosphinothricin acetyltransferase (PAT) (Zhao *et al.,* 2001; Frame *et al.,* 2002; Li *et al.,* 2003; Valdez *et al.,* 2004; Huang *et al.,* 2005; Ishida *et al., 2007;* Stickle and Oraby, 2005; Decima *et al.,* 2010).

Among other factors, salinity and drought have been two major stress factors reducing maize seed yield, yield consistency and biomass yield. Salinity and drought play important stress factors affecting plant growth and development. Most of reports suggest that drought and salinity have similar effects on the plant cells as the input signal for drought is believed to be reduction of the turgor pressure due to water loss of the cells, and the input signal for salt stress is the high concentration of soluble salts inside cells.

The dissertation presented here is on the development of salinity and drought tolerance in maize genotypes via the transfer of the barley *HVA*1, the bacterial *mtlD,* and a combination of the *HVA1-mtlD* transgenes into maize genome. This dissertation also covers the transfer of the *Sorghum bicolor* Dehydration Responsive Element Binding (*sbDREB2*) transcription factor regulated by a drought inducible promoter into maize genome. The salinity and drought tolerance of transgenic maize plants showing the integration of *sbDREB2* will be completed and published elsewhere.

CHAPTER I

BARLEY GENE HVA1 CONFERS DROUGHT AND SALT TOLERANCES IN TRANSGENIC MAIZE (*Zea mays*. L)

1.1. LITERATURE REVIEW

1.1.1. Introduction

Maize is an important basic food and feed grain, and its stover (leaves and stems) is used for animal feed and biofuel. Increased soil-salinity and water-deficiency are the two major factors limiting the maize plant growth and development and subsequently affecting its yield and the yield. Maize is considered more sensitive to salt stresses at the early stages of its growth and development compared to all other cereal crops with the exception of rice (Cramer., 1994, US Salinity Laboratory, 2006). Also, maize requires adequate water in all stages of its physiological development to reach optimum productivity (Zhang *et al.*, 2008). In the past decades, many attempts to increase maize yield and yield stability have been taken through conventional breeding and selection under drought and salt conditions (Duvick and Cassman, 1999; Troyer, 1996). However, conventional maize breeding for drought and salt tolerances has not been very successful due to the complex interaction between genotype and environment (Johnson and Geadelmann, 1989). Therefore, maize genetic engineering for drought and salt tolerance is necessary not only to increase understanding of complex drought and salt tolerance, but also relatively quickly create commercial varieties tolerant to drought and salt stresses in order to meet the demands in planting maize in the salinity soil and drought stressed areas of the world. Plant drought and salt tolerance is reported to be related to a group of late embryogenesis abundant proteins.

1.1.2. LEA – Late Embryogenesis Abundant proteins

The plant hormone abscisic acid (ABA) regulates many key processes in plants including the response to abiotic stresses (Hubbard *et al*., 2010), and the processes induce the expression of genes encoding proteins involved in desiccation tolerance and dormancy in seeds (Hong *et al*. 1992; Gomez-Cadenas *et al*., 1999).

Under drought conditions, the ABA level increases in plants. Such increase in ABA results in the closure of stomata and therefore a decrease in transpiration from leaves. The role of ABA is regulating of multiple stress responsive genes related to drought or salt stresses in response to drought to protect the plant cells from osmosis (Finkelstein and Rock, 2002).

Late embryogenesis abundant (LEA) proteins are encoded by ABA-induced genes, first discovered as accumulating during late embryogenesis of wheat and cotton and subsequently they were found in different other organs of various plant species (Chen *et al*., 2002).

LEA proteins are generally divided into several groups based on the structural domains or chemical characteristics, and found to be expressed under cellular stress conditions such as drought, desiccation, and cold, osmotic stress and heat (Wise, 2003). Many genes encoding LEA proteins have been successfully isolated under such conditions. For example, according to Hundertmark and Hincha (2008), 55 protein encoding genes have been isolated and classified into nine different groups from the *Arabidopsis* thaliana genome. The expression of all these genes was detected in various organs in which half of them displayed high expression levels.

The accumulation of LEA proteins in different transgenic plants has been demonstrated to confer resistance to various abiotic stresses including water deficiency. For instance, Liu *et al*. (2009) reported that in transgenic tobacco, a group of two *LEA* genes called *BhLEA1*and *BhLEA2*, encoding group 4 LEA proteins were induced by dehydration and signaling molecules including ABA, resulting in drought and salt tolerance. Transgenic tobacco plants expressing

BhLEA1 and *BhLEA2* had higher relative water content of leaves, higher photosystem II activity, and lower membrane permeability than their wild-type control plants. Moreover, high accumulation of *BhLEA1* and *BhLEA2* proteins was found under drought tolerance. The research by this group confirmed the important role of LEA protein accumulation in transgenic tobacco in the protection and maintenance of plant cell membrane during dehydration.

LEA gene from Tamarix transferred to blueberry (*Vaccinium corymbosum*) via *Agrobacterium tumefaciens* method resulted in an increase cold tolerance of transgenic plants (Zhao *et al*., 2011). Transgenic plants of this experiment exposed to 0^{o}C for 24 hrs showed improved activities of peroxidase and superoxide dismutase, and also significantly reduced level of lipid peroxidation as compared to non-transgenic plants.

Different LEA protein genes have been isolated and conferred cold, drought or salinity tolerance in rice (*Oryza sativa* L.) (Xiao *et al*., 2007); lettuce (*Lactuca sativa*) (Park *et al*., 2005a); cabbage (*Brassica oleracea*) (Park *et al*., 2005b); *Arabidopsis* (Figueras *et al*., 2004); and tobacco (*Nicotiana tabacum*) (Ziat *et al*., 2011). The LEA protein gene from barley (*Hordeum vulgare* L.) has been studied the most.

1.1.3. Expression of the Barley *HVA1* gene conferring abiotic stress tolerance in plants.

HVA1 is a group 3 LEA proteins that specifically expresses and accumulates in barley seed aleurone layer and embryo during late seed development. Several scientists initially suggested that the expression of *HVA1* gene in transgenic plants could confer various abiotic stress tolerance including drought and salinity tolerance. Hong *et al*. (1992) reported that the expression of *HVA1* was rapidly induced in young seedlings under salt or ABA treatments. This phenomenon was also associated with the *HVA1* an increase in the levels of *HVA1* mRNA in both shoot and root of the young seedlings under cold and heat stresses, but such expression dramatically decreased in old seedlings (7 day old seedlings).

Accumulation of *HVA1* protein has been known for its important role in the understanding of the mechanism of dehydration tolerance in plants. Babu *et al*. (2004) transferred *HVA1* to rice, and reported on the role of this gene as related to the dehydration tolerance in transgenic rice plants. This group reported that relative water content of transgenic plants maintained at high percentages (92%) after 28 days of water stress, whereas relative water content decreased to 51% in non-transgenic plants under the same conditions. The dry shoot and root biomass matter were more and the percentage of cell membrane leakage was less in *HVA1* transgenic plants as compared to their wild-type control plants. This suggested that the expression of the *HVA1* gene encoding group 3 LEA proteins confers dehydration tolerance in transgenic rice by protecting plant cell membranes.

Moreover, the expression of *HVA1* gene in transgenic mulberry (*Morus indica*) plants resulted in higher cellular membrane stability (70%) as compared to non-transgenic plants (50%) under 48hrs of 2% polyethylene glycol (PEG) osmotic treatment (Lal *et al.*, 2008), and transgenic plants showed 1.5-fold higher relative water content after 10 days of water stress and accumulated higher 5-19-fold higher proline under 400mM NaCl stress condition. Checker *et al*. (2012) also reported that the expression of *HVA1* gene in transgenic mulberry plants enhanced proline accumulation, membrane stability and photosynthetic yield after 30 days of water deficit, or when exposed to 200mM NaCl for 30 days. The authors also reported similar results under cold stress (10^{o}C) condition.

The expression of *HVA1* in T3 transgenic rice seedlings driven either by a rice actin constitutive (*Act1*) or inducible (4ABRC) promoters resulted in enhanced drought and salt tolerance. In this experiment, transgenic plants showed greater seedling growth performance, more shoot height and shoot weight, and lesser ion leakage than in wild-type non-transgenic plants under drought and salinity stress conditions (Rohila *et al.*, 2002).

Xu *et al*. (1996) studied salt stress tolerance in transgenic rice expressing the Barley *HVA1* gene regulated by the rice *Act1* promoter, and reported that shoot height and root fresh weight of transgenic plants increased significantly after totally 30 days of salt treatment at the rotation of 10 days for 200mM-water-50mM NaCl, as compared to non-transgenic control plants that were maintained under the same conditions. *HVA1* transgenic rice plants also showed high survival rate (60-80%) whereas all of the non-transgenic control died after extending more number of days of salt treatment. Results also revealed accumulation of *HVA1* protein in transgenic rice leaves and roots.

Studying fourth generation (T3) *HVA1* transgenic wheat, Sivamani *et al*. (2000) reported an increase in crop biomass and a higher water use efficiency under moderate water deficit condition as compared with their wild-type non-transgenic control plants. In this experiment, both homozygous and heterozygous lines of T3 generation showed up to 55% higher root dry biomass and up to 17% higher shoot dry weight as compared to their wild-type non-transgenic control plants.

Similarly, Bahieldin *et al*. (2005) confirmed that the expression of *HVA1* gene in transgenic spring wheat lines conferred drought tolerance, and most of the transgenic plants showed higher plant height, total biomass as well as higher grain yield, as compared to those of *HVA1* non expressing plants (Plants that had integrated, but had not expressed the *HVA1* gene) and those of the wild-type non-transgenic control plants.

Oraby *et al*. (2005) found that under different salinity levels, T3 oat plants expressing *HVA1* transgene showed better performance than non-transgenic plants on flower induction, seedling growth, and the number of kernels per plant. Under 200mM NaCl treatment, the panicle length and number of spikelets per panicle of control plants were less by 50% as compared to transgenic plants. The kernel yield was also reduced by 40% and 90% for transgenic and wild-type control plants respectively. Furthermore, when *HVA1* gene in transgenic creeping bentgrass (*Agrostis stolonifera var. palustris*) was up-regulated under water deficit condition, plants showed higher leaf water content, and significantly less leaf wilting (Fu *et al*., 2007).

The role of LEA protein accumulation in plants as well as the role of *HVA1* gene encoding group 3 LEA proteins have been well known as evidence for abiotic stress responses in a vary of crop species. Table 1 represents the expression of the barley *HVA1* gene conferring drought and/or salt tolerance in different transgenic crops.

Table 1: The expression of *HVA1* gene in different transgenic crops

Species	**Phenotype**	**Reference**
Rice	Drought and salinity tolerance	Xu *et al*., 1996
	Drought and salinity tolerance	Rohila *et al*., 2002
	Drought tolerance and stability of cell membrane	Babu *et al*., 2004
Wheat	Increased biomass and water use efficiency under stress	Sivamani *et al*., 2000
	Drought tolerance	Detvisitsakun *et al*., 2001
	Improved plant water status and yield under field drought conditions	Bahieldin *et al*., 2005
Oat	Drought tolerance	Maqbool *et al*., 2002
	Salinity tolerance in increasing yield per plant	Oraby *et al*., 2005
Creepingbentgrass	Drought tolerance	Fu *et al*., 2007
Mulberry	Salinity and drought tolerance	Lal *et al*., 2008
	Drought, salinity and cold tolerance	Checker *et al*., 2012

1.2. RESEARCH GOAL AND OBJECTIVES

The goal of this research was to express the barley *HVA1* gene in maize plants and breed transgenic plants for development of drought and salt tolerance. The following specific objectives were to be met in order to reach the above goal.

The specific objectives of the research were:

(i) Transfer the Barley *HVA1* gene into the maize genome.

(ii) Determine the segregation of the linked *bar* and *HVA1* genes and verify their proper expression and stability of transmission to up to four generations.

(iii) Evaluate the effects of the *HVA1* transgene on maize seedling development, plant height, and fresh and dry biomass matter as compared to those of the wild-type non-transgenic control plants under drought and salt stress conditions.

1.3. MATERIALS AND METHODS

1.3.1. Explants

Maize Hi II immature zygotic embryos were ordered from Plant transformation facility, Department of Agronomy, Iowa State University. Calli were established and maintained in an induction media containing 4g/L of Chu N6 base media with vitamins supplemented with 30g/L sucrose, 2.76g/L proline, and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Type II calli were transferred to fresh media every 2 weeks for proliferation and for gene gun bombardment.

1.3.2. Gene construct

Plasmid pBY520 (Figure 1) derived from pBluescript II KS (+) (Stratagene, 11011 North Torrey Pines Rd, La Jolla, CA 92037) was generously provided to our laboratory from the late Professor Ray Wu of Cornell University, and used for the transformation of maize. This construct contains *HVA1* coding sequences driven by 1.3 kb upstream region of the rice *Act1* translation codon (McElory *et al*., 1990; Zhang *et al*., 1991) and terminated with the polyA 3\` terminal of potatoes proteinase inhibitor II (*pin II*) gene (Keil *et al*., 1986). The pBY520 also contains the bacterial phosphinothricin acetyl transferase (*bar*) structural gene, as a selectable marker in maize transformation. In this construct, the bar coding sequences are driven by the cauliflower mosaic virus (CaMV 35S) promoter and terminated by the nopaline synthase nos 3\` region (Bevan *et al*., 1983).

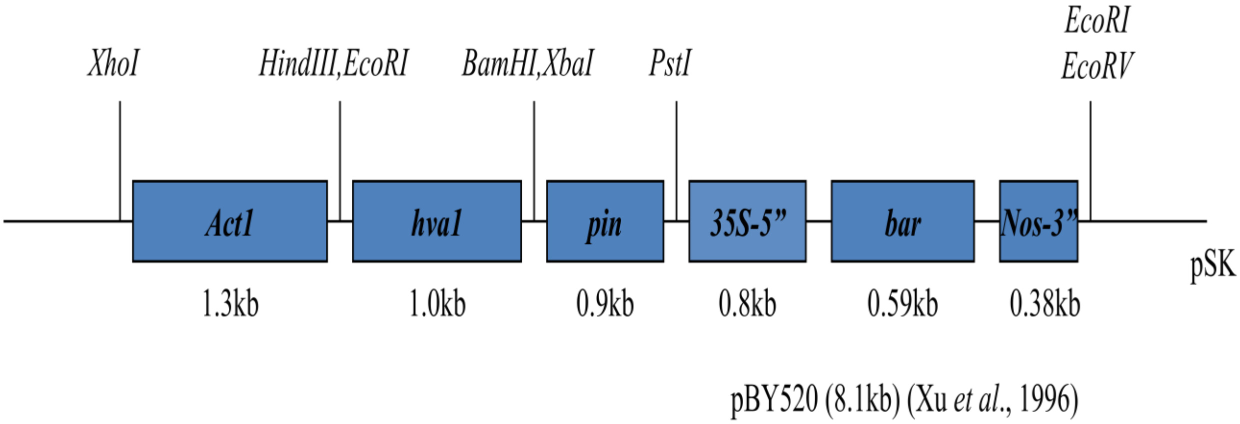


Figure 1: Linear map of pBY520 construct. Rice actin promoter (*Act1*), Barley (*Hordeum vulgare* or *HVA1* LEA3 gene, Cauliflower Mosaic Virus 35S (*35S*) promoter, *Bar* Liberty herbicide resistance gene (*bar*) and nopaline synthase terminator (*Nos*). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

1.3.3. Transformation of plasmid vector into *E. coli* competent cells

The pBY520 plasmid was transferred into *E. coli* for multiplication by heat shocking for 45 seconds in a water bath at a temperature of 42^0C containing 2 µl of plasmid DNA mixed with 50 µl of DH5α *E. coli* competent cells (Sigma). The mixture was then immediately placed on ice for 2 min and added 700 µl of Luria Bertani (LB) medium into the tubes. This mixture was then incubated for 1 hour at 37^0C with 250 rpm shaking. The cultures were placed on solid LB selection media contained 100mg/L ampicilin, and incubated overnight at 37^0C. Single colonies were taken and placed into glass flask containing 50 ml of LB media. This was then incubated at 37^0C with 250 rpm shaking overnight. The plasmid DNAs were isolated and purified using Qiagen plasmid purification kit (Cat. No.12123) as per manufacturer's instruction. Purified plasmid was then used for biolistic bombardment.

1.3.4. Biolistic^{TM} bombardment

For gene gun bombardment, the embryogenic type II friable calli from one week callus cultures were selected and placed together in a 1x1 cm area on top of the osmotic induction media at least 4 hours prior to bombardment. About 6µl of the plasmid mixture containing DNA coated tungsten particle (M10) were used for bombardment as described by Frame *et al*. (2000). The bombardments were carried out twice per plate by using a Helium PDS 1000HE device (Biotechnology Systems Division, Wilmington, DE) with 1100 psi acceleration pressure. Bombarded calli were then cultured on the induction medium for 1 week while maintained in darkness at 27^0C before transferring them to selection medium contained 2.5 mg/L of glufosinate ammonium. Cultures were sub-cultured at two-week intervals.

All DNA precipitation and bombardment steps were performed under sterile conditions under a laminar flow hood at room temperature. For plant regeneration, the selected embryonic calli were transferred to Murashige and Skoog (1962) or MS medium containing 1 mg/L 6-benzylaminopurine (BAP). Regenerated shoots were rooted on MS medium containing 1 mg/L Indole-3-butyric acid (IBA). Plantlets were transferred into small square container pots containing BACCO Professional Planting Soil Mix, and covered using transparent plastic bags for keeping the humidity for acclimation into their new environment. The plastic covers were removed gradually over a period of 4 days and the plantlets were transferred into 30cm diameter pots and grown to maturity in a greenhouse.

1.3.5 Confirming the integration and expression of transgenes in putatively transgenic plants

1.3.5.1. Polymerase Chain Reaction (PCR) analysis

Genomic DNA was isolated from young leaf tissues using CTAB method. *HVA1* F, 5`-ACC AGA ACC AGG GGA GCT AC-3` (forward primer) and *HVA1* R, 5`-TGG TGT TGT CCC CTC CCA TG-3` (reverse primer) were used to detect *HVA1* gene for T0-T3 plant regenerations. DNA amplifications were performed in a thermo cycler (PerkinElmer/ Applied Biosystem, Forster City, CA). Optimized PCR conditions were 94^{0}C for 3 min for initial denaturation, 35 cycles of 30 s at 94^{0}C, 30 s at 56^{0}C, 45s at 72^{0}C and a final 10 min extension at 72^{0}C. The PCR product was loaded directly onto a 1% (weight/volume) agarose gel stained with 2ul ethidium bromide and visualized under UV light.

1.3.5.2. Southern Blot Hybridization Analysis

Southern blot hybridization analysis was carried out to determine the stability of transgenic event and gene copy number of *HVA1* and bar transgenic plants. 15 µg of maize genomic DNA from each putatively transgenic line was digested with *Hind*III or *Bam*HI enzymes in the proper buffer [500 mM Tris-HCl (pH 6.5), 1 mM EDTA] in a total volume of 250 µl overnight at 37^{0}C. The digested DNA was precipitated with 70% ethanol at $–20^{0}$C overnight, pelleted by centrifugation at 13,000 rpm for 10 min, allowed to dry and then re-dissolved in sterile distilled water before electrophoresis. The digested DNA was run on electrophoresis at 70 v on 0.8% agarose gel and transferred to a Hybond-N+ membrane

(Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA) at an energy level of 2,000 J. Gene-specific probes were synthesized using *HVA1* forward and reverse primers and purified using the DNA clean and concentrator -5 Kit (ZYMO, RESEARCH, D4004), and labeled with α-[^{32}P]-dCTP using Random Prime labeling kit (GE Healthcare) according to the manufacturer's instructions. Membranes were hybridized at 68^{0}C overnight with the radiolabled probe and autoradiographed on premium autoradiography films (Hyblot CL, Denville, Scientific INC, E3018) at –80^{0}C for overnight.

1.3.5.3. Reverse Transcription-PCR (RT-PCR)

PCR positive plants of *HVA1* gene were used in RT-PCR analysis. A total of 200 mg young leaf tissues were grinded into liquid nitrogen for each 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA). 0.2 ml chloroform was added and vortexed for a few seconds. The tubes were placed into a centrifuge and spun at maximum speed for 15 min at 4^{0}C. An aqueous phase was then transferred into fresh tubes and added 0.5 ml of cold isopropanol. Samples were incubated at -20^{0}C for 1 hour and centrifuged at maximum speed for 10 min at 4^{0}C. The supernatant was thrown away leaving the RNA pellet. This was washed with 700ul of 70% ethanol then spun in a centrifuge at 12,000prm for 5 min at temperature of 4^{0}C. The RNA pellets were dried at room temperature and dissolved in RNase-free water and quantified using a spectrophotometer. 2µg RNA obtained was used for cDNA synthesis using the Superscript$^{\mathbf{TM}}$ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The same primers and PCR conditions for *HVA1* as described above were used.

1.3.5.4. Northern Blot

Northern blot analysis was performed using Random Prime labeling kit (GE Healthcare) as per described in southern blot to assay the *HVA1* gene expression of the transgenic plants. A total of 15 µg of RNA per sample was loaded onto a 1.2% (w/v) agarose-formaldehyde denaturing gel as described by Sambrook *et al*. (1989) and transferred to an Hybond-N+ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA) at an energy level of 1200 J. Same DNA α-[^{32}P]-dCTP labeled probe as in southern was used for detection of transcripts.

1.3.6. Biological activity tests

1.3.6.1. Biological assay for ***bar***

Five different concentrations (200, 250, 300, 350, and 400mg/L) of glufosinate ammonium (Aventis, Strasboug, France) corresponding to 200, 300, 350, and 400 µl/L of Liberty herbicide dilutions (1.67 pounds of active ingredient glufosinate ammonium per gallon of Liberty herbicide) were applied for leaf painting using a cotton swab to determine the kill curve (lowest concentration that could kill maize leaf). The level of 350mg/L of glufosinate ammonium solution was used and applied to leaves using brush to find the segregation ratios of the transgenic progenies. The tips of young leaves of 3-leaf stage seedlings were selected and painted (Lee *et al.*, 2007). The leaf injury caused by herbicide application was observed one week after painting treatment.

1.3.6.2. Drought tolerance test

A total of 30 seeds from the fourth generation (T3) transgenic line and a non-transgenic line were planted in the greenhouse in 15 cm diameter pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX) under greenhouse conditions of 29 and 25^{0}C corresponding to day and night temperature; and 16-hour day length with light intensity of 600-700 mu mole m^{-2} s^{-1}. A dilution of commercial 20-20-20 fertilizer solution was applied to seedlings twice per week. Four week old seedlings were selected and used for drought tolerance test. Seedlings were exposed to 15 days of no water treatment (drought) followed by 7 days of re-watering. Data were collected for leaf relative water content (RWC) at 10 days and 15 days after water stress (before re-watering for recovery) and percentage of plant survival was determined 7 days after re-watering.

1.3.6.3. Salt tolerance stress test

A total of 40 seeds of T3 of a transgenic line and a non-transgenic line were sown in the round clay pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX). This experiment was replicated in two locations of the same greenhouse. To test the herbicide resistance of transgenic plants to assure the presence of *HVA1* gene (linked to the *bar* gene cassette), the transgenic seeds were allowed to germinate under 15mg/L of glufosinate ammonium. The surviving seedlings were then daily treated with equal volume of four different concentrations (0, 100, 200 and 300 mM) of NaCl by gradually increasing 50mM per day to reach the final concentrations in 10 days. The commercial 20-20-20 fertilizer was supplemented into salinity solution for nutritional needs. Seedling samples were taken after 10 days of salt treatment for shoot and root length, and shoot and root fresh and dry weights data collections. After weighting for fresh shoot and root biomass, these tissues were dried out in an oven at 80^{0}C for two days and weighed to determine for shoot and root dry weights.

1.3.6.4. Measurement of leaf relative water content (RWC)

The leaf samples from transgenic and non-transgenic plants were taken for the determination of leaf RWC after 10 days and 15 days of water withholding. In each of the two repeated experiments, about 10 cm^2 leaf discs of three randomly selected plants were collected at 8AM, their mid-ribs were cut and discarded, and the rest of the leaves were immediately placed in plastic bags to limit water evaporation. Then, each leaf section was removed from the plastic bag and weight for its fresh weight (FW), and then leaf samples were soaked into distilled water for four hrs to full turgidity at normal room condition. After hydration, the samples were placed between two tissue papers to remove any water on the leaf surface and then immediately weighed to obtain fully turgid weight (TW). Samples were then dried in an oven at 80^0C overnight, placed in a desicator for 15 min to cool down, and then weighed to determine their dry weight (DW). Leaf relative water content (RWC) was calculated by the following formula (Gaxiola *et al*., 2001)

$$\text{RWC (\%)} = [(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})] \times 100$$

1.3.6.5. Statistical analysis

All data were collected and statistically analyzed using a completely randomized design (CRD) using PROC GLM (SAS version 9.2 software package). Analysis of variance (ANOVA) was conducted to test the statistical significance at an alpha level of 0.05.

1.4. RESULTS AND DISCUSSIONS

1.4.1. Maize tissue culture and regeneration

Results of maize *in vitro* culture and plant regeneration, followed by self pollinator are shown on Figure 2 below.

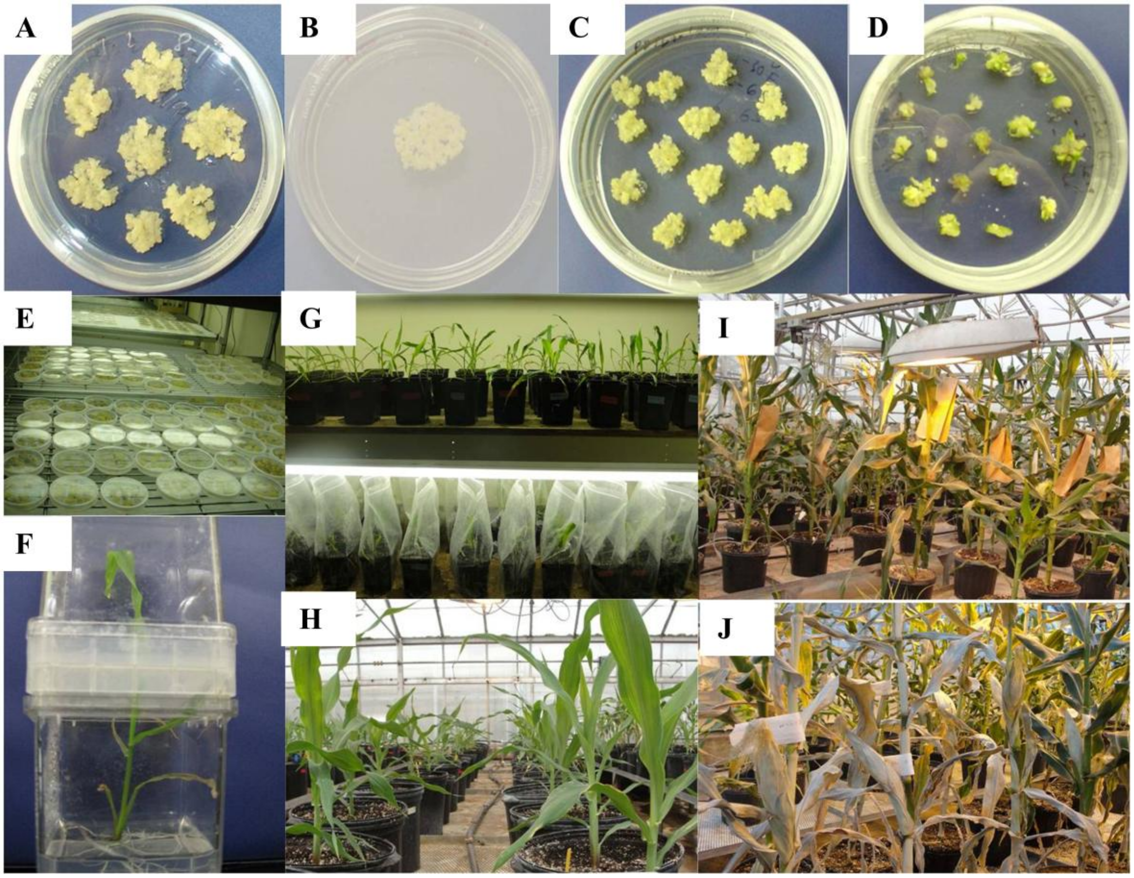


Figure 2: Maize in vitro culture, plant regeneration and breeding of transgenic plants: (A) Highly embryogenic Hi II immature embryos (B) Highly embryogenic immature embryos after transgene bombardment; (C) Embryogenic tissue growth in selection medium; (D) and (E) embryogenic tissues in regeneration media; (F) plantlet growth in rooting media; (G) Acclimatization of plantlets in growth chamber; (H) plants growing in greenhouse; (I) Plant breeding, and (J) breeding of mature plants.

Transformation experiments were performed by using Hi II immature embryos-derived calli via Biolistic^{TM} bombardment. The bombarded calli grew well in the N6 osmotic medium at 25^{0}C in complete darkness for the first two weeks after bombardment (Figure 2B). Selection of herbicide resistant cultures took place in 8-10 months, when cultures were selected on a culture medium containing 2.5mg/L of glufosinate ammonium, where the transgenic embryogenic calli proliferated rapidly and displayed somatic embryos in form of white and fast growing granular sectors. There were also some non-proliferating and partially necrotic mother calli (brown sectors) that did not survive the selection. Under fluorescent light condition of 16hr light/ 8 hr dark and 25^{0}C, somatic embryos quickly proliferated in regeneration medium containing 1mg/L of Benzyl amino purine (BAP) and regenerated into normal shootlets which produced primary and secondary roots 4 weeks after they were placed in rooting medium containing 1mg/L of Indol butyric acid (IBA). The rooted plantlets were transferred to soil, and pots were covered with plastic bags for one week to allow plantlets to be acclimated to normal culture room conditions. Plantlets were then transferred to 30 cm diameter soil pots and placed in our maize greenhouses where they were grown to maturity (Figure 2 F-J above).

The optimum conditions for maize transformation via particle bombardment using immature zygotic embryos vary in literature. Scientists (Petrillo *et al*., 2008, Oneto *et al*., 2010) reveal that using 2ug of DNA plasmid mixed with particle of 1um in size, 6 cm distance between the loaded gene construct and target, and twice shot with pressure of 1100Psi are optimum conditions for maize genetic transformation via the Biolistic ™ device. In this candidate's research, the bombarded calli were transferred from the N6 medium to MS medium where they regenerated within four weeks (Amstrong *et al*., 1994, Oneto *et al*., 2010). The purpose of N6 osmotic treatment was to minimize the damages of the cell walls caused by bombardment (Wang *et al*., 2003a). Such osmoticum media assists in an increase in transformation efficiency by decreasing the damage made to the cells (Vain *et al*., 1993; El-Itriby *et al*., 2003).

1.4.2. Integration of *HVA1* and *bar* transgenes via PCR and Southern blot analysis

The PCR results showed positive integration of *HVA1* and *bar* transgenes in T0, T1, and T2 generations (Figure 3, 4, 5).

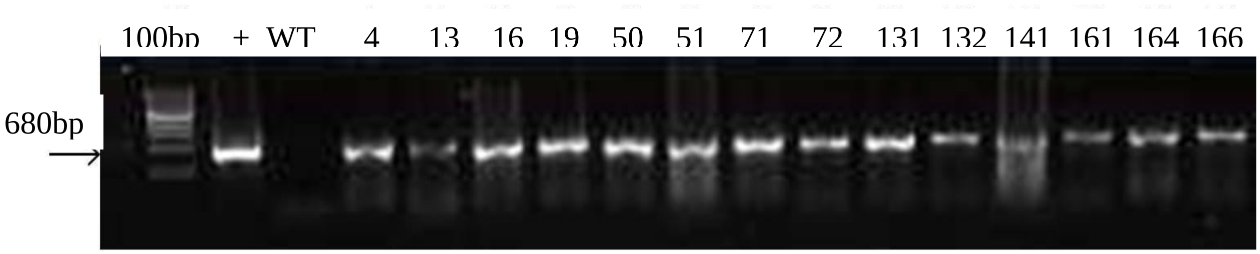


Figure 3: PCR analysis of T0 *HVA1* transgenic plants confirming the correct expected band size of 680bp.

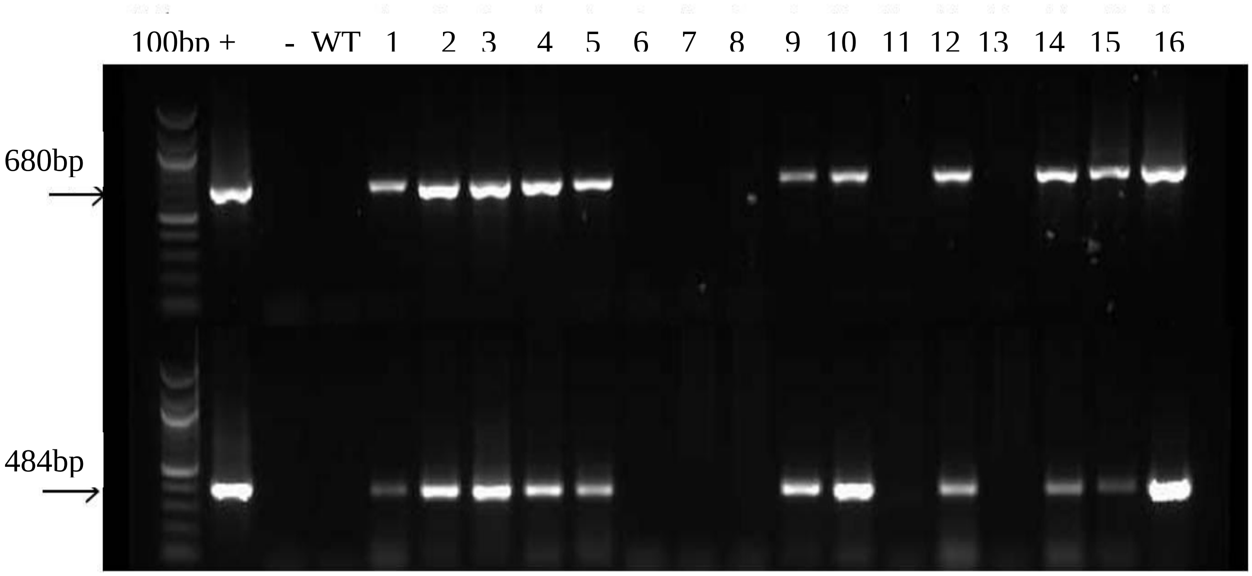


Figure 4: PCR analysis of T1 *HVA1* and *bar* transformants (line#132) showing the expected band size of 680 bp for *HVA1* and 484bp for *bar* gene.

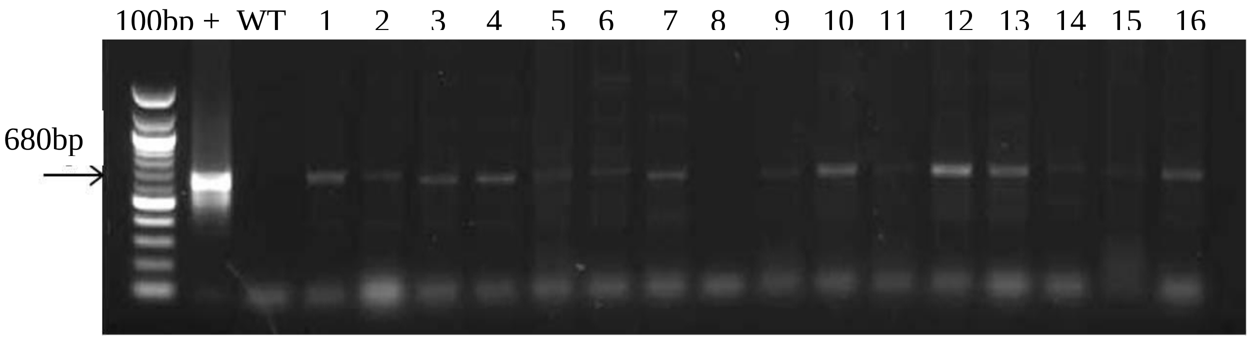


Figure 5: PCR analysis of T2 of *HVA1* transformants (line#132) with correct expected band size (680bp).

The results of the PCR analysis confirm the integration of *HVA1* gene across T0-T2 generations (Figure 3, 4 and 5). The co-existence of *HVA1* and *bar* genes in the transgenic plants is due to the transcriptional linked *HVA1* and *bar* gene cassettes, therefore transgenic plants showed the integration of both *HVA1* and *bar* genes together at 100% frequency (Figure 4). Similar observations were reported by Maqbool *et al*. (2002), Oraby *et al*. (2005) and Kwapata *et al*. (2012) who reported stable co-integration of two linked genes in the subsequent transgenic progenies with a frequency of 100%.

Southern blot hybridization confirmed the integration of *HVA1* transgene in T0 transgenic maize plants (Figure 6 below). Southern blot also revealed the copy number of *HVA1* transgene in transgenic plants (described below).

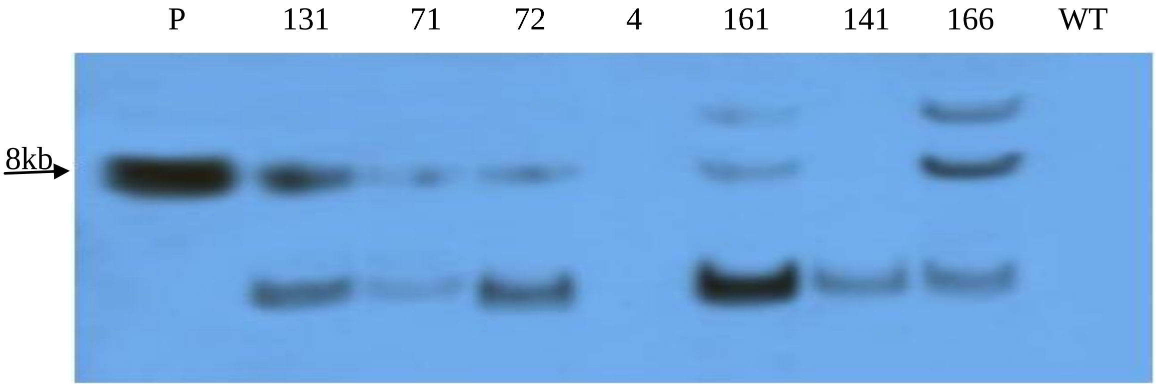


Figure 6: Southern blot showing integration of *HVA1* gene in T0 transgenic plants. P: pBY520 plasmid as positive control. WT: wild-type. Digestion was performed using the *Hind*III restriction enzyme.

Southern blot analyses results showed the integration of *HVA1* gene with two to three inserted gene copies (Figure 6), except for the transgenic line 141 that shows one inserted transgene copy. Although the T0 line #4 plant showed the integration of *HVA1* transgene using the PCR technology, Southern blot did not confirm the same because the amount of transgene might have not been sufficient to show in Southern blotting, or plant may have been chimerically transformed (the piece taken for PCR analysis, but not the whole plant contained the transgene).

Several studies have shown that the use of particle bombardment for genetic transformation can result in multiple integrated transgene copies in transgenic plants, and also can cause the rearrangement of the transgenes (Pawlowski *et al*., 1996; Kohli *et al*., 1998; Dai *et al*., 2001).

1.4.3. *HVA1* transcription confirmed by RT-PCR and Northern blot analysis

Figure 7 represents the *HVA1* transgene transcription in T1 plants confirmed via RT-PCR analysis.

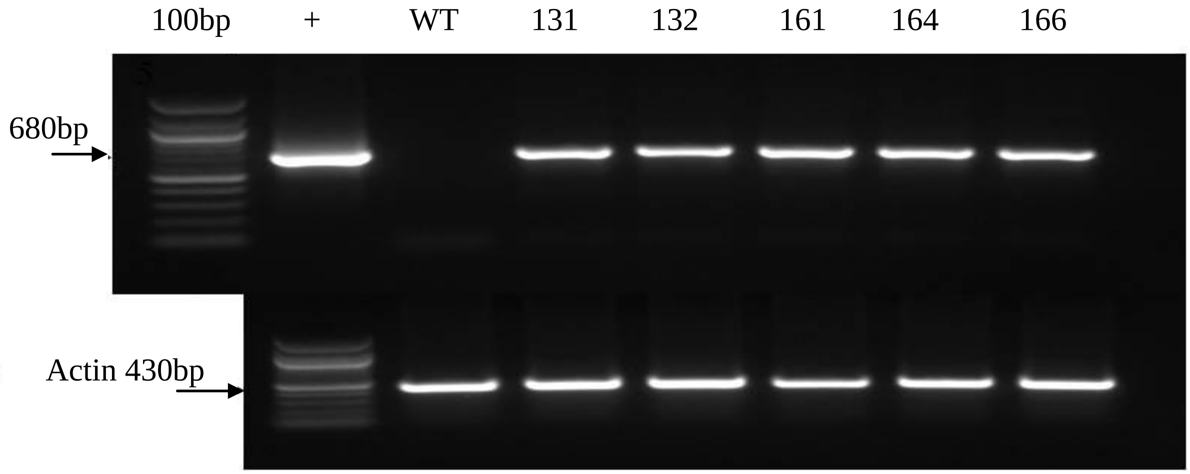


Figure 7: RT-PCR showing expression of *HVA1* transgene in T1 transgenic plants; + pBY520 plasmid positive control, wt: wild-type. The expected band size for *HVA1* gene is 680bp. The lower portion of this figure shows the expression of maize endogenous actin1 gene with an expected band size of 430bp is loaded as control to show that the RT-PCR has been working well.

Northern blotting of plants confirmed the transcription of *HVA1* gene in T0 and T1 transgenic plants (Figure 8 and 9).

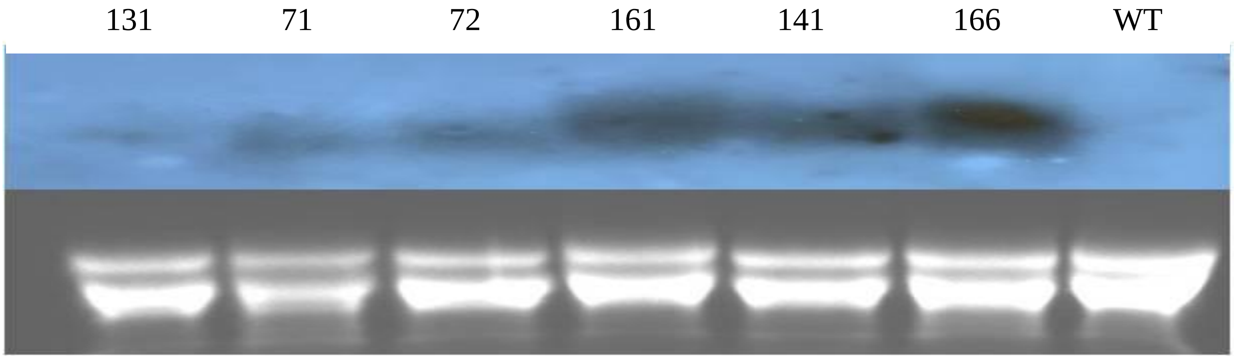


Figure 8: Northern blot expression of *HVA1* transgene in T0 transgenic lines; WT: wild-type (non-transgenic plant).

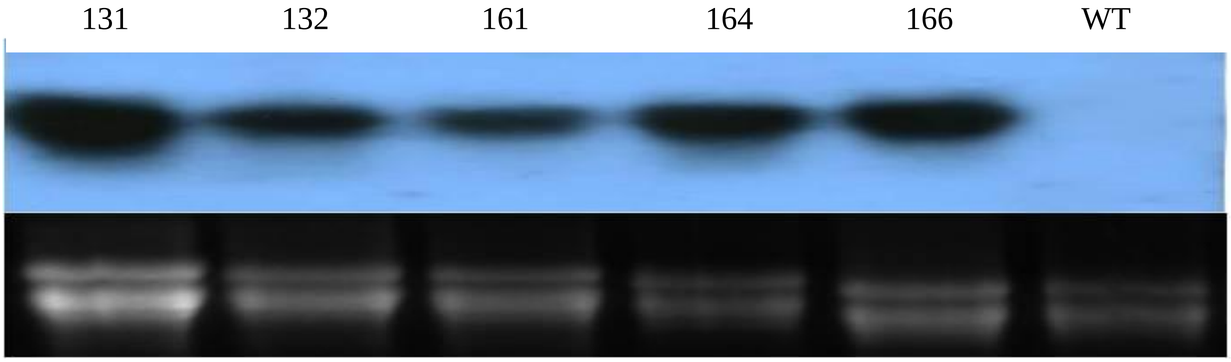


Figure 9: Northern blot expression of *HVA1* transgene in T1 transgenic lines; WT: wild-type (non-transgenic plant).

The above RT-PCR analysis shows the transcription of *HVA1* transgene in T1 plants (Figure 7) and confirmed by northern blot analysis (Figure 9). Figure 8 and 9 shows that *HVA1* transgene show different levels of transcriptions, while T1 show higher transcriptions. The reason for various levels of transcription might be due to different site of insertion of transgene in maize genome, and the reason that T1 plants show higher level of transcription is because the T0 plants were self bred towards homozygosity.

1.4.4. Confirmation of herbicide resistance in *bar* transgenic plants

We developed a kill curve test painting the wild-type non-transgenic maize leaves with different concentrations of commercial Liberty herbicide (containing 18.2% glufosinate anominum). This experiment showed that the minimum concentration of glufosinate anominum that killed the leaf tissues was 350 mg/L (Figure 10).

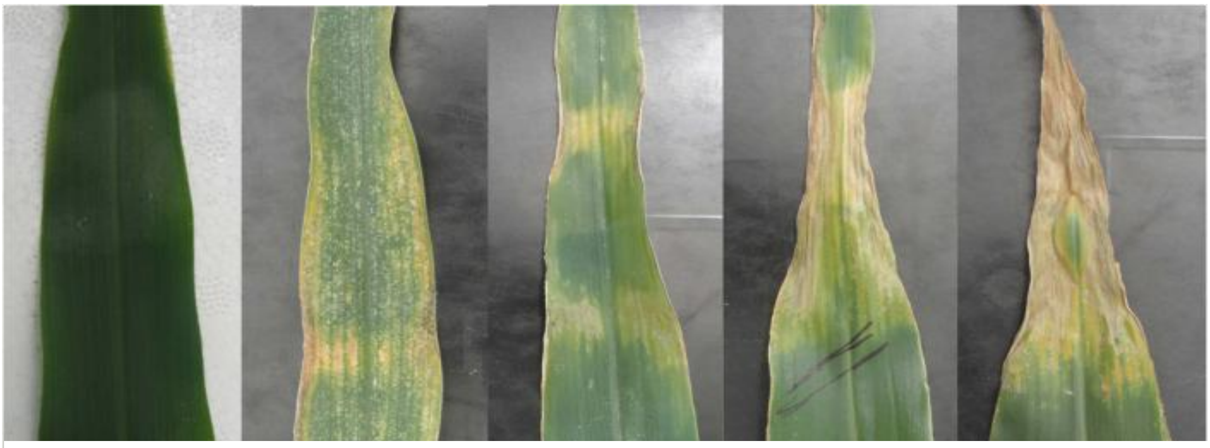

WT200mg WT250mg WT300mg WT350mg WT400mg

Figure 10: Kill curve for herbicide selection: Young leaves of wild-type non-transgenic plants painted with 5 different concentrations of commercial Liberty herbicide containing 18.2% glufosinate anominum. The concentration of 350mg/L glufosinate anominum was used to apply for transgenic plants.

By using the herbicide kill curve test (Figure 10), this candidate found the optimum concentration of herbicide for leaf painting to be 350mg/L glufosinate ammonium**.** This kill curve was used in transgenic plants to determine whether the bar gene segregation follows the Mendelian inheritance. For this purpose, the Chi-square was used to determine the transgenes (linked) segregation ratio (3:1) in T1 progeny. However, Chi-square test result showed that the bar gene segregation did not follow Mendelian segregation. This might be because most transgenic plants had integrated more than one transgene inserts, and therefore the multiple copies of transgene insertion have caused the transgene not to follow the Mendelian segregation.

Figure 11 (below) represents the effect of 350 mg/L glufosinate anominum on leaf painting of a T1 *bar* transgenic line versus that of a wild-type non-transgenic control plant.

WT
Non-transgeneic Transgeneic

Figure 11: Leaf painting (at 350mg/L glufosinate ammonium after 7 days) showing herbicide injury symptom on non-transgenic plant versus T1 transgenic plant confirmed resistant.

1.4.5. Confirmation of drought stress tolerance in T3 plants

Among all transgenic T1 plants that showed *HVA1* transcription, line #132 was selected for drought tolerance test because other lines did not produce sufficient seeds or did not produce kernels for self breeding. The T1 line transcribed the *HVA1* gene (Figure 9), and the Northern blot positive of its line 132 produced over 150 seeds (which became T2 plants), each producing sufficient kernels for self breeding.

The percentage of leaf RWC of transgenic line #132 and wild-type non-transgenic control plant was calculated after 10 and 15 days of withholding of the water irrigation are shown on Figure 12.

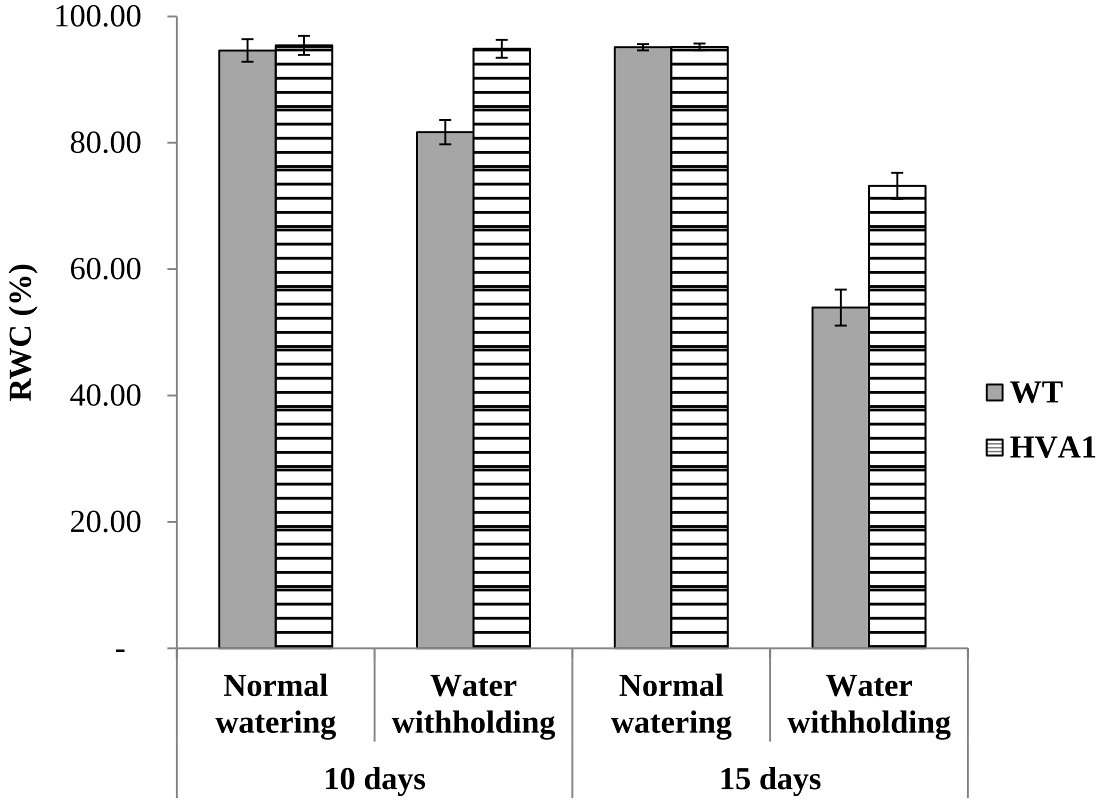


Figure 12: Percentage of leaf relative water content (% RWC) of *HVA1* transgenic line #132 after 10 and 15 days of water deficit (withholding).

After withholding the water for 10 days, non-transgenic plants started wilting whereas most of the transgenic plants showed normal growth performance. However both transgenic and non-transgenic control plants showed symptoms of drought stress i.e. leaves wilted, burned at the leaf edge and tip, and the upper leaves turned yellow after 15 days of water withholding. The wild-types showed more severe symptoms of drought stress with most of the leaves being wilted and dehisced compared to transgenic plants after 15 days of water withholding treatment.

Figure 12 also shows that the leaf RWC measured in wild-type leaves was decreased to 81.7% whereas leaf RWC in transgenic plants still maintained as high as 94.9%. After 15 day of water withholding, leaf RWC in the wild-type control plants was as low as 53.9% as compared to the leaf RWC in transgenic plants that was 73.2%. There was no significant difference in leaf

RWC between the control and transgenic plants that were watered regularly, and the transgenic plants after 10 days of water withholding. However, the leaf RWC of *HVA1* transgenic line was less after 15 days of water withholding as compared to that of 10 days water withholding, but far less in the leaves of the wild-type control plants.

Figure 13 represents the survival rate of transgenic versus wild-type control plant after 7 days of water recovery treatment.

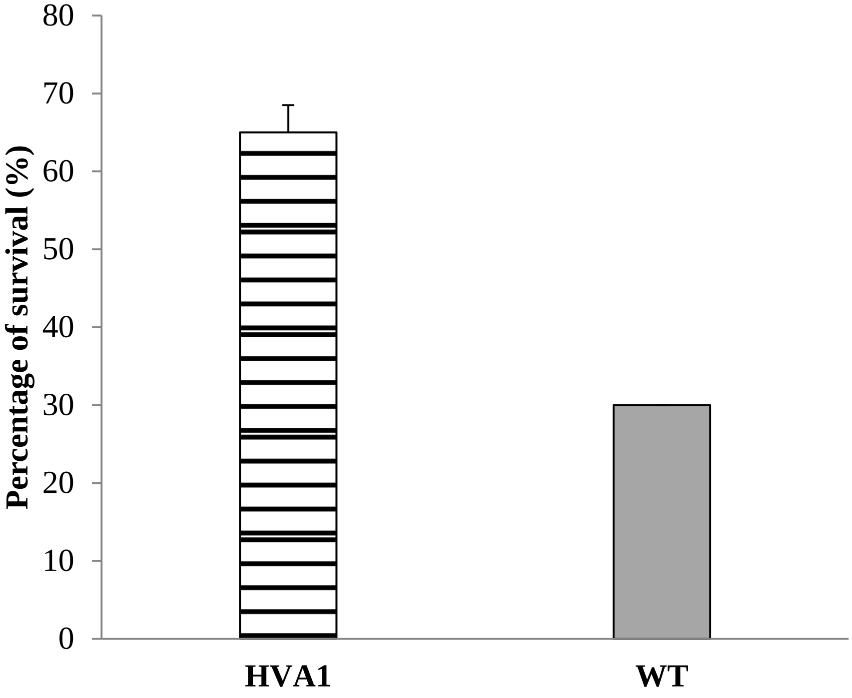
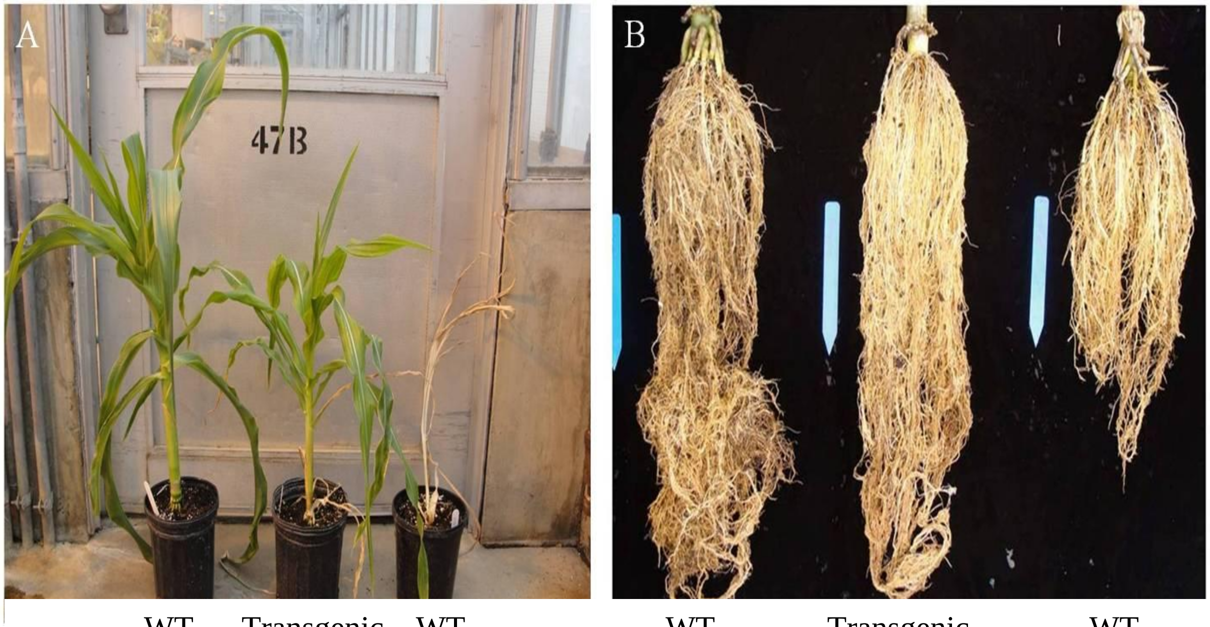


Figure 13: Percentage of survival rate of transgenic versus wild-type control plant after 7 days of water recovery treatment

Results show that when plants were re-watered for 7 days after 15 days of water withholding for drought stress recovery, transgenic plants showed a quick (2-3 days) recovery and performed high percentage of survival (65% recovery) compared to wild-type (30% recovery).



WT Normal watering | Transgenic Under drought | WT Under drought

WT Normal watering | Transgenic Under drought | WT Under drought

Figure 14: Effect of drought stress on plant growth and mature root system of *HVA1* transgenic maize versus its wild-type control plants. The pictures were taken for drought stress tolerance of T3 *HVA1* transgenic plants after one week of water recovering (A), and mature roots (B)

Maize is very susceptible to drought and requires water for cell elongation across all stages of its growth and development. However, the amount of water requirement will vary depending on the growth stages in which increases during vegetative stages and reduces during reproductive stage. It has been reported that drought stress during early vegetative stage of maize growth cause a constant leaf wilting resulting in 5 to 10% yield losses (Thelen, 2012).

There are several mechanisms explained for drought tolerance ability of plants (including maize) under water deficit conditions. One of these mechanisms is the high accumulation of abscisic acid (ABA) found in drought tolerant lines as compared with drought sensitive lines (Wang *et al*., 2003b). ABA is known to regulate stomata opening and other responses resulting in tolerance to drought in plants. In the results [presented here, it is possible that accumulation of ABA may have played a role.

The expression of *HVA1* gene encoding the Barley LEA3 protein in this study is in agreement with several other reports on other *HVA1* transgenic crop species showing higher leaf RWC, significantly increased plant growth, and drought tolerance of transgenic plants as compared to their non-transgenic control plant counterparts (Xu *et al*., 1996; Babu *et al*., 2004; Fu *et al*., 2007).

1.4.6. Salt stress tolerance in T3 plants

The salt treatment was conducted after seed germination (i.e. seven days after sowing the seeds). Four different salt concentrations (0, 100, 200, and 300mM NaCl) were applied daily as 250 ml solution per pot while increasing the salt concentration at 50mM per day until it reached its final concentration. Transgenic and wild-type plants showed normal development up to one week after being exposed to 100mM NaCl, but wild-type leaves started showing injurious symptom after the NaCl treatment. After increasing the NaCl concentration to 200 mM, the wild-type plants were severely affected as compared to those treated with 100mM. At 300mM NaCl concentration, both transgenic and wild-type plants showed severe leaf injury after one week of salt treatment.

Figures 15-20 (below) display the results of the effect of different salt concentrations on T3 *HVA1* transgenic versus non-transgenic control plants on shoot and root length, and shoot and root fresh and dry weight biomasses.

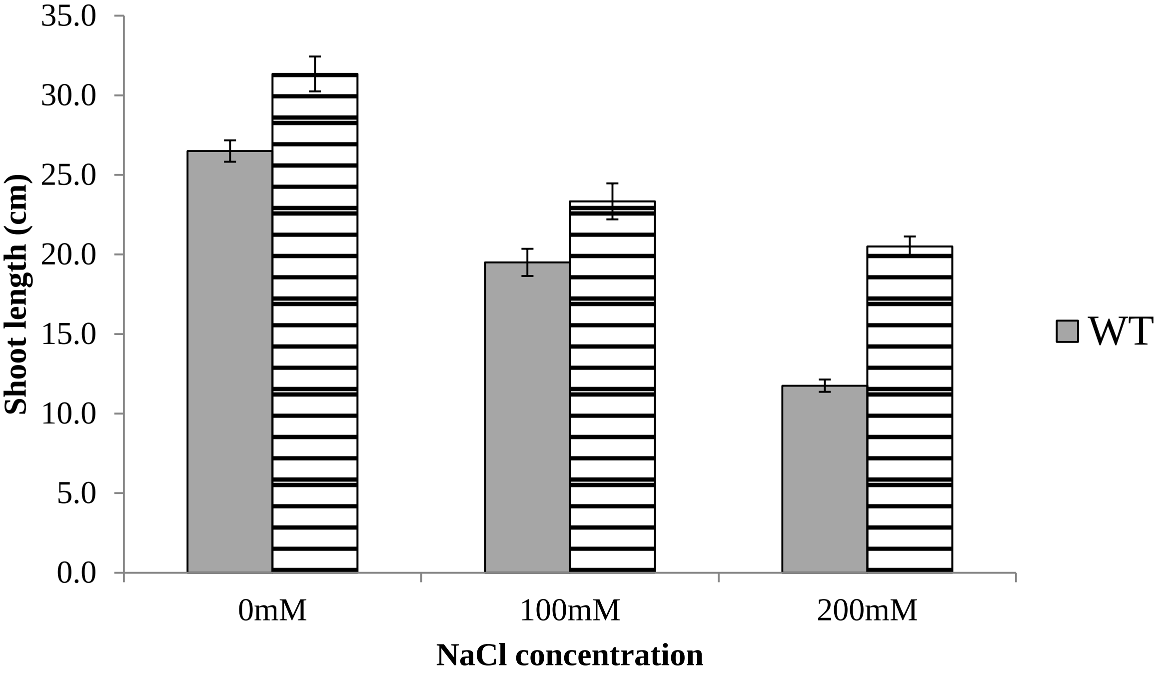


Figure 15: Effect of salt treatment on shoot length of T3 plants.

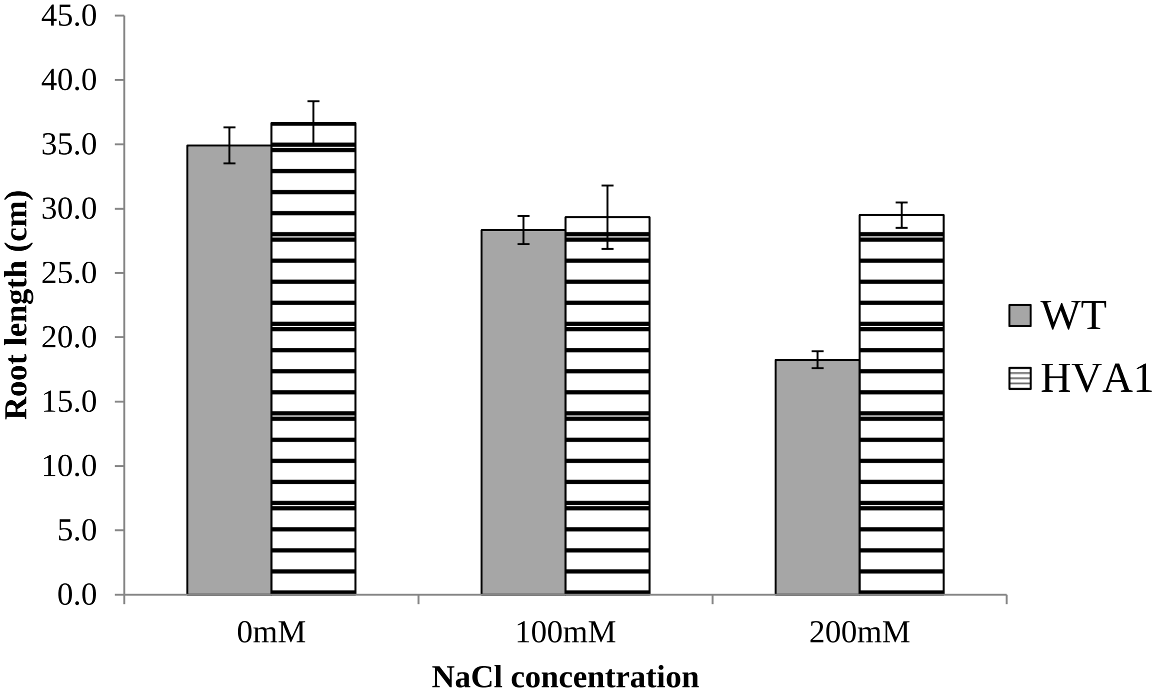


Figure 16: Effect of salt treatment on root length of T3 plants.

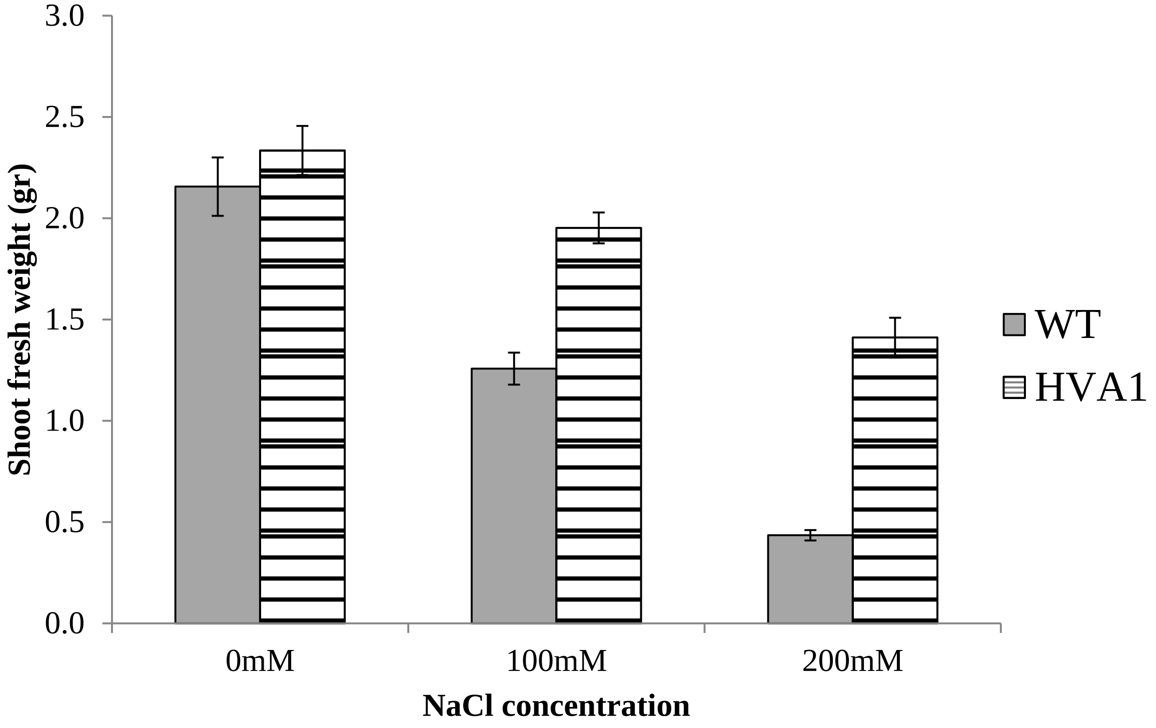


Figure 17: Effect of salt treatment on shoot fresh weight of T3 plants.

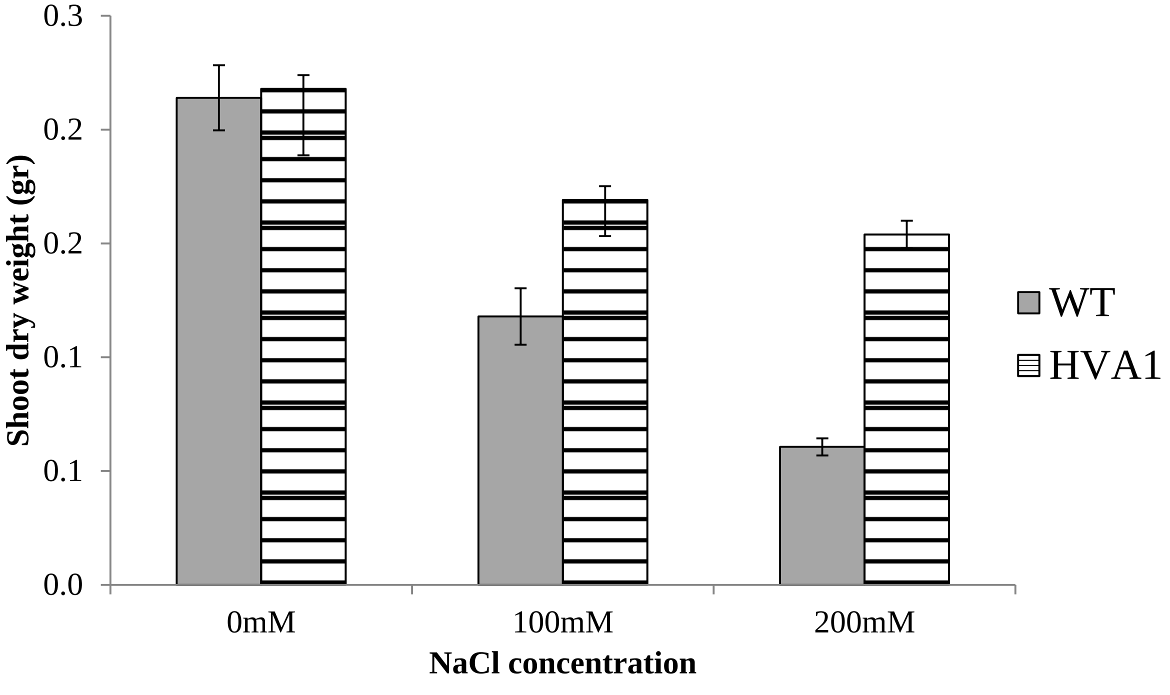


Figure 18: Effect of salt treatment on shoot dry weight of T3 plants.

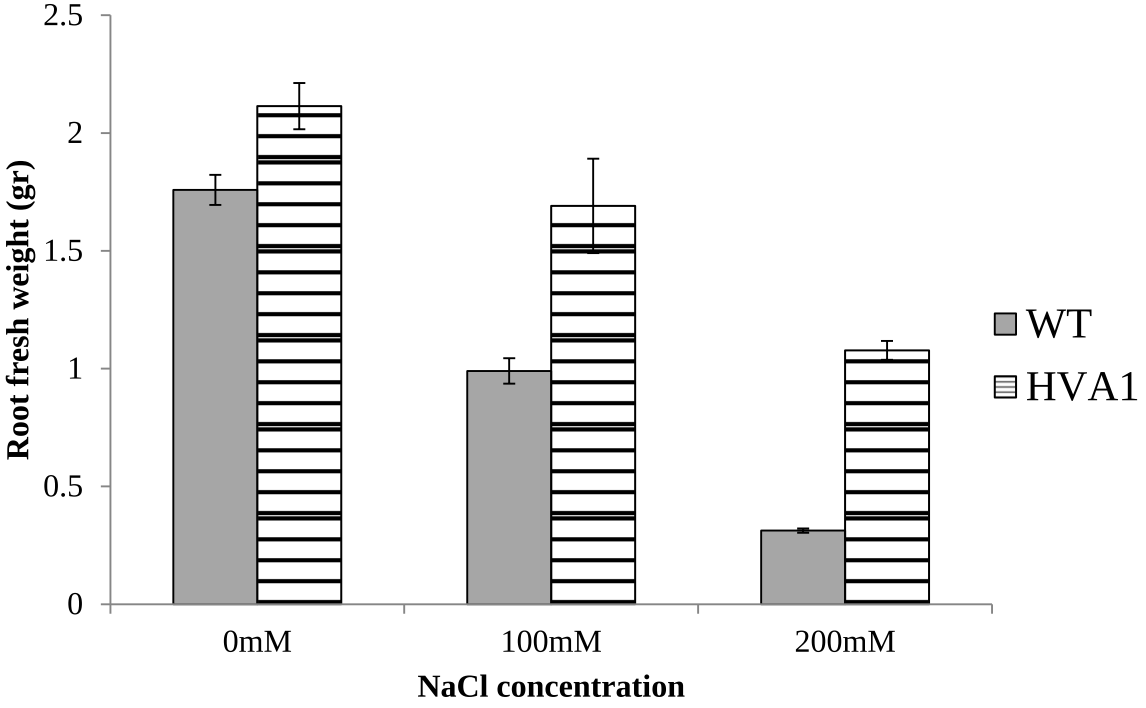


Figure 19: Effect of salt treatment on root fresh weight of T3 plants.

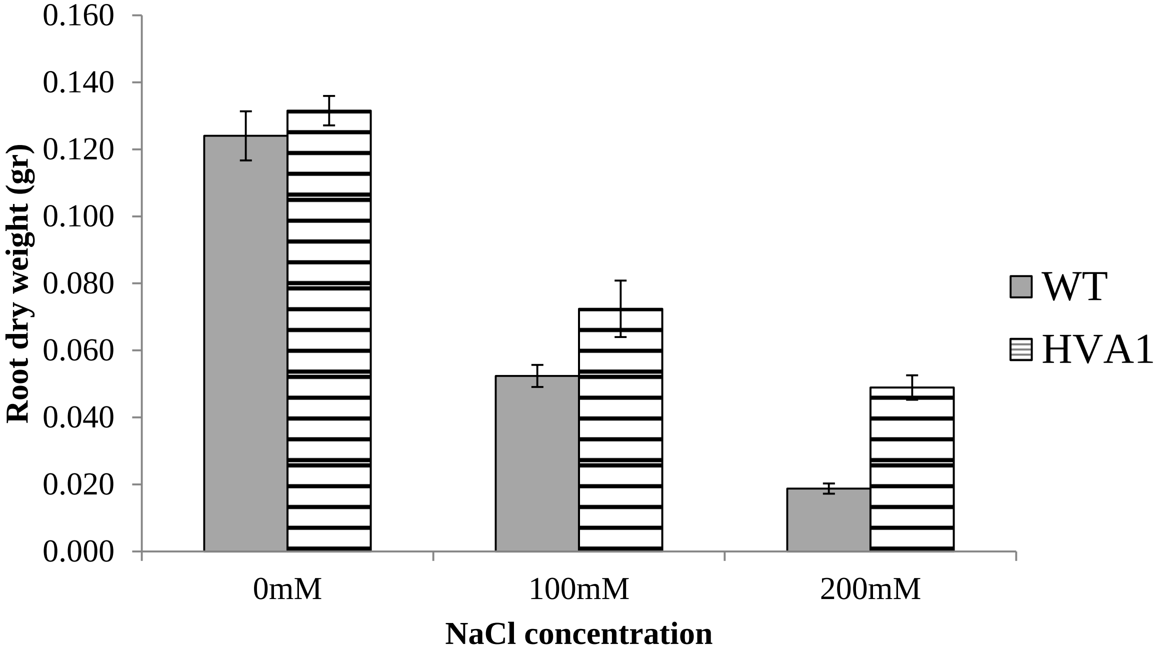


Figure 20: Effect of salt treatment on root dry weight of T3 plants.

Table 2 shows that the reduction in shoot length of both transgenic and wild-type plants were respectively at 25% and 26% when exposed to 100 mM NaCl. However at 200mM, percentage of shoot and root length reduction in wild-type plants increased significantly to 55.7% and 47.7%, respectively (Appendix 8 and 9) whereas the reduction of shoot and root length were respectively 34.6% and 19.5% in transgenic plants. Similarly, fresh and dry shoot of wild-type control plants were reduced by 42% and 45% at 100mM and up to 80% and 72% at 200mM NaCl concentration. Salt stress also reduced by 85% of root dry weight in wild-type compared to 63% reduction in transgenic plants at 200mM NaCl. Over all, the results show that the percentage of reduction of shoot and root length and their fresh and dry biomass were affected by different salinity concentrations, with a maximum reduction at 200 mM NaCl treatment.

In general, the growth of seedlings and fresh and dry biomass of transgenic plants were less affected by different NaCl levels as compared to their wild-type control plants.

Table 2: Reduction in shoot and root length and their biomass affected by salt treatments

Salt concentration	**100mM**		**200mM**	
Genotype	**T3 *HVA1***	**WT**	**T3 *HVA1***	**WT**
% shoot length reduction	25.5b	26.4b	34.6b	55.7a
% root length reduction	20.0b	18.9b	19.6b	47.7a
% shoot fresh weight reduction	15.3b	41.5b	39.2b	79.8a
% shoot dry weight reduction	22.4c	44.9b	29.4c	71.7a
% root fresh weight reduction	20.0d	43.7c	49.0b	82.2a
% root dry weight reduction	44.9c	57.8b	62.8b	84.9a

Shoot and root length, and shoot and root fresh and dry biomass weights of both transgenic and wild-type seedlings (10 days after salt treatment) were decreased while increasing salt concentrations. However, wild-type seedlings showed more severe injury on leaves and roots resulting in reduced shoot and root length and fresh and dry matters significantly compared with transgenic plants.

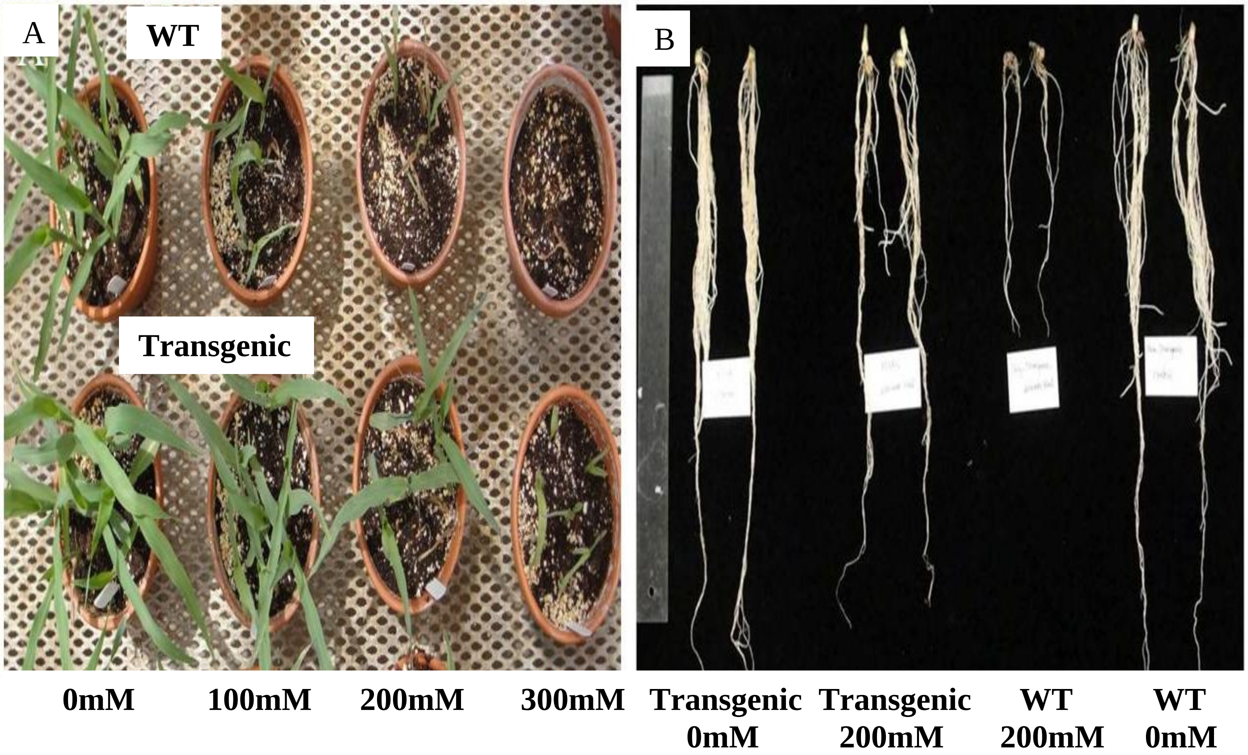


Figure 21: Effect of salt stress on seedling growth (A) and root system (B) of *HVA1* transgene and wild-type plants after 10 days of NaCl treatment.

To determine the further effect of the different salinity concentrations on the growth of plants and on the number of leaves per plants, additional study was performed with 3 -leaf stage plants exposed to four different NaCl concentrations (0, 100, 200, 300mM) for 6 days (after all treatments reached to the final NaCl concentration). The results are shown on the Table 3 (below)

Table 3: Effect of salinity stress on number of leaves per plant after 6 days of NaCl treatment

	Number of leaf (leaf/plant)			
NaCl levels	0mM	100mM	200mM	300mM
HVA1	4.8 ± 0.01a	3.9 ± 0.01b	3.7 ± 0.04c	3.4 ± 0.07d
WT	4.8 ± 0.04a	3.3 ± 0.07d	3.1 ± 0.01e	3.0 ± 0.00e

Means within columns followed by the same letter are not significantly different (P<0.05)

Table 3 indicated that number of leaves per plants in the wild-type was significantly reduced more than that in *HVA1* transgenic plants under NaCl stress. Under 300mM NaCl level, the youngest leaves (the fourth leaf) of wild-type plants were not produced or rolled and were dead, whereas the fourth leaf in transgenic plants were produced and extended normally to become a new normal leaf (Figure 22). The effect of NaCl stress resulted in decreased number of leaves per plant in wild-type plants from 3.3 to 3.0 at 100mM and 300mM corresponding to 30% to 37% of reduction, respectively.

The average number of leaves in transgenic plants was reduced from 3.9 to 3.4 leaves per plant at 100mM and 300mM (19% and 29% reduction), respectively. Whereas there was no difference of number of leaves per plant between transgenic plants and wild-type non-transgenic control plants.

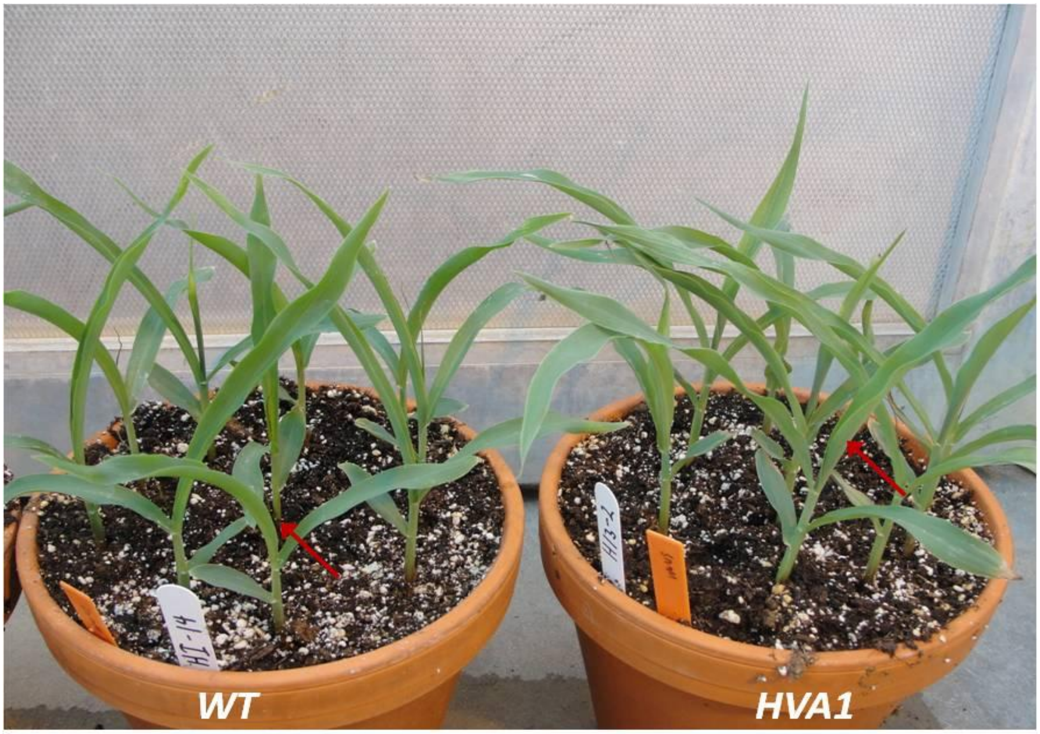


Figure 22: Effect of salinity stress on the development of leaves in transgenic and wild-type plants. Picture was taken after 6 days of NaCl treatment

In the research presented here on the effect of salinity treatment of the maize seedlings agrees with the report on *HVA1* transgenic versus control non-transgenic oats, indicating that the wild-type control plants tended to be shorter and the shortest height for most genotypes was obtained at 200mM NaCl (Oraby *et al*., 2005). Similar findings were reported by Xu *et al*. (1996) in transgenic rice and wheat (Checker *et al*., 2011) expressing the *HVA1* gene. Furthermore, the relationship between accumulation of LEA 3 proteins and abiotic stress tolerance has been confirmed in studies for transgenic wheat (Sivamani *et al*., 2000; Bahieldin *et al*., 2005), rice (Rohila *et al*. 2002; Babu *et al*., 2004), and Oat (Maqbool *et al*., 2002; Oraby *et al*., 2005).

CHAPTER II

BACTERIAL *MTLD* GENE CONFERS SALT TOLERANCE AND ACCUMULATION OF MANNITAL IN TRANSGENIC MAIZE (*Zea Mays*. L)

2.1. LITERATURE REVIEWS

2.1.1. Introduction

Plants often face adverse effects of extreme environmental conditions such as drought, high salinity or low temperature. One of the mechanisms against abiotic stresses is that plants can withstand the impacts of these conditions by synthesizing and accumulating sufficient amounts of osmoprotectants inside their cells. However, many crop species cannot naturally synthesize and accumulate enough of these compounds in their cells. Therefore, it is possible to transfer certain heterologous genes coding for enzymes associated with the production of these osmoprotectants into the susceptible crop species for development of abiotic stress tolerance (Rontein *et al.*, 2002). The mechanisms of drought and salt tolerance are explained below.

2.1.2. Biosynthesis of compatible solutes in plants for drought and salt tolerance

Plant responses to unfavorable environments include escape, avoidance or tolerance to the conditions, meaning that plants usually avoid or escape the harsh abiotic stresses, tolerating and surviving the conditions. Many previous studies have focused on the mechanisms that plants can tolerate abiotic stresses, especially the water deficit and high salt concentrations. For instance, halophytes plants are able to live under very high salinity concentrations, via the function of exclusion of both Na^+ and Cl^- ions during their water uptake or these plants may have the ability of storing salt inside their cells in a way that such salt concentration does not affect their growth and development (Garthwaite *et al.*, 2005).

Abiotic stress tolerant plants have the capability to go through biosynthesis and accumulation of compatible solutes in their cells to balance the concentration of those solutes between their cells and the solutes in their surrounding environment (Mimura *et al.,* 2003). The compatible solutes produced inside the water stressed cells are called osmoprotectants. The osmoprotectants prevent the cellular dehydration and maintain plant cell turgor pressure in favor of plant survival during osmotic stresses (Valliyodan and Nguyen, 2006).

Plant osmoprotectants consist of sugars (raffinose, galactinol, sucrose; trehalose; sorbitol and mannitol); amino acids (proline, arginine, citrulline, orthinine); glycine betaine (GB); polyamine (putrescine); and amides (glutamine, asparagines). Although the basic roles of these compatible solutes are to stabilize proteins and cell membranes of plants during osmotic stresses, each of them also has other functions (Valliyodan and Nguyen, 2006).

It has been reported that increases in concentration of sugar alcohols or polyols (glucose, mannitol, or trehalose) in chickpea tissues caused accumulation of proline, an amino acid needed to balance carbon nitrogen under stress conditions (Sheveleva *et al.*, 1997) and improved yield in chickpea (*Cicer arietinum* L.) (Bhatnagar *et al.,* 2009). It has also been reported that certain osmoprotectants improve tolerance to water deficit in wheat and soybean (Vendruscolo *et al.*, 2007; De Ronde *et al.*, 2004), and rice (Xu *et al.*, 2008). In addition, the synthesis of complex sugars such as trehalose can stabilize the plant lipid membranes, proteins and other biological structures during the dehydration stress conditions. For example, Zhang *et al*. (2005) transferred a trehalose synthase gene in tobacco and reported that the increased trehalose accumulation in transgenic tobacco plants resulted in increased drought and salinity tolerance of transgenic tobacco plants.

Mannitol biosynthesis pathway is begun with the photosynthetic carbon reduction cycle, where glucose-6- phosphate is converted into fructose-6-phosphate, and the fructorse-6-Phophate into Mannitol-1-Phophate via the mannitol phosphate dehydrogenase (*mtlD*) enzymatic activity.

Then the Mannitol-1-Phosphate is converted into mannitol via the Pase (phosphatase) enzymatic activity (Figure 20).

Figure 23: A summary of the pathway for the synthesis of mannitol.

Mannitol is a sugar alcohol that is naturally synthesized as a natural osmotic protectant, contributing to salt tolerance in many crops. For example, celery (*Apium gravelens* L.) is a salt tolerant plant that is known to accumulate mannitol under salt stress conditions (Everard *et al.*, 1994).

The *mtlD* enzyme (encoded by *mtlD* gene) has been found in various bacteria, known to help in generating NADPH. The bacterial *mtlD* gene was first transferred into tobacco (*Nicotiana tabacum* L.) by Tarczynski *et al*. (1992) who reported an enhanced mannitol accumulation in transgenic plants, along with significantly higher growth performance compared to non-transgenic plants under high salt concentration condition.

Another enzyme associated with mannitol biosynthesis in manotol-6-phosphate reductase (*M6PR*). When the celery *M6PR* gene was transferred to *Arabidopsis*, transgenic plants showed accumulation of mannitol, enhancing the transgenic plants salt tolerance at a concentration of 100mM NaCl. However, when transgenic plants were exposed to 200mM NaCl, transgenic plants showed higher survival rate and higher dry biomass matters as compared to control non-transgenic plants, however they did not produce seeds (Abebe *et al.*, 2003).

2.1.3. Expression of bacterial *mtlD* gene in transgenic plants

Abebe *et al*. (2003) demonstrated that the expression of the *E. Coli mtlD* gene in third generation transgenic (T2) wheat (*Triticum aestivum* L. cv Bobwhite) resulted in an improved tolerance to drought and salinity, transgenic plants also showed enhanced fresh weight, dry weight and plant height as compared with the wild-type non-transgenic wheat plants. Another report (Karakas *et al*., 1997) indicated that salinity could reduce the growth of non-transgenic tobacco plants by 40%, whereas it did not affect the growth of the *mtlD* transgenic tobacco plants. Other researchers reported that *mtlD* transgenic poplar trees (*Populus tomentosa)* survived up to 40 days in a hydroponic culture containing 75mM NaCl solution, while non-transgenic plants only survived 25mM NaCl. The stomatal conductance and photosynthesis of transgenic poplar were also higher than that of wild-type control plants under the same salt condition treatments (Hu *et al.,* 2005), meaning that stomatal conductance in transgenic plants may have contributed towards reduced transpiration keeping the cellular turgor pressure lower.

Prabhavathi *et al*. (2001) reported that the expression of *mtlD* transgene in the first generation (T0) transgenic eggplant (*Solanum melongena* L.) seeds that were germinated under in vitro salt treatment (MS medium plus 200mM NaCl) condition, resulted in seedlings that grew well, whereas the wild-type control seeds did not germinate under the same conditions. Also, canola (*Brassica napus* L.) transgenic seeds expressing the *mtlD* gene, germinated and survived up to 24 days under in vitro conditions on MS media containing 350 mM of NaCl, whereas the wild-type control seeds failed to germinate (Motallebi and Rahnama, 2011). Motallebi and Rahnama (2011) also reported a high level of accumulation of mannitol in *mtlD* transgenic canola plants as compared to the non-transgenic control plants.

The expression of bacterium *mtlD* gene in transgenic potato plants revealed an increased salt tolerance under both *in vitro* and hydroponic stress conditions. The effect of 100 mM NaCl on potato shoot fresh weight under hydroponic condition was reduced by only 17.3% as compared to 76.5% reduced fresh weight of control non-transgenic plants (Rahnama *et al*., 2011). Maheswari *et al*. (2010) also reported 1.7 to 2.8-fold shoot and root performance enhancement in transgenic sorghum plants expression the *mtlD* gene.

Table 4 represents the effect of expression of bacterial *mtlD* gene for improved salinity and drought tolerance in different plant species.

Table 4: Expression of bacterial mannitol-1-phophate dehydrogenase (*mtlD*) confers biochemical changes resulting in drought and salinity tolerance in different crop species.

Species	**Phenotypes**	**references**
Tobacco	Enhanced plant height and fresh weight under salinity stress conditions	Tarczynski *et al*., 1993
	No contribution to sustained growth under salinity and drought stress.	Karakas *et al.,* 1997
Arabidopsis	Increased germination under salinity stress	Thomas *et al.,* 1995
Rice	Salt tolerance	Huizhong *et al.,* 2000
Wheat	Drought and salinity tolerance of calli and plants	Abebe *et al.*, 2003
Petunia	Chilling tolerance	Chiang *et al.*, 2005
loblolly pine	Accumulation of mannitol and glucitol and high salt tolerance	Tang *et al.*, 2005
Populus tomentosa	Salinity tolerance	Hu *et al.*, 2005
Cotton	Accumulation of amino acids and enhanced salt tolerance	Momtaz *et al.,* 2007
Sorghum	Increased drought and salinity tolerance	Maheswari *et al.,* 2010
Potato	Enhanced salt stress tolerance	Rahnama *et al.*, 2011
Canola	Increased germination under salinity stress	Motallebi and Rahnama. 2011

2.2. GOAL AND OBJECTIVES

The goal of this research is to test the salt tolerance capacity of the bacterial *mtlD* in maize plants.

The specific objectives of the research were:

(i) Transfer the bacterial *mtlD* gene in maize plants.

(ii) Verify their proper expression and stability of transmission of *mtlD* into T0, T1, T2 and T3 plant progenies.

(iii) Evaluate the effects of the *mtlD* transgene on maize seedling development, plant height, and fresh and dry biomass matter as compared to those of the wild-type non-transgenic control plants under salt stress conditions.

(iv) Determine the accumulation of mannitol contents in T3 *mtlD* transgenic versus their wild-type counterpart plants.

2.3. MATERIALS AND METHODS

2.3.1. Gene constructs

The JS101 plasmid (Figure 24) construct was used in this dissertation containing the bacterial mannitol-1-phophate dehydrogenase (*mtlD*) gene regulated by rice actin promoter (*Act1*) and the potato protease II terminator, linked to the *bar* herbicide resistance selectable marker gene regulated by the *35S* promoter and *(Nos)* terminator. This construct was developed by the late Prof. Ray Wu of Cornell University, and obtained via a material transfer agreement between MSU and Cornell University for the purpose of research.

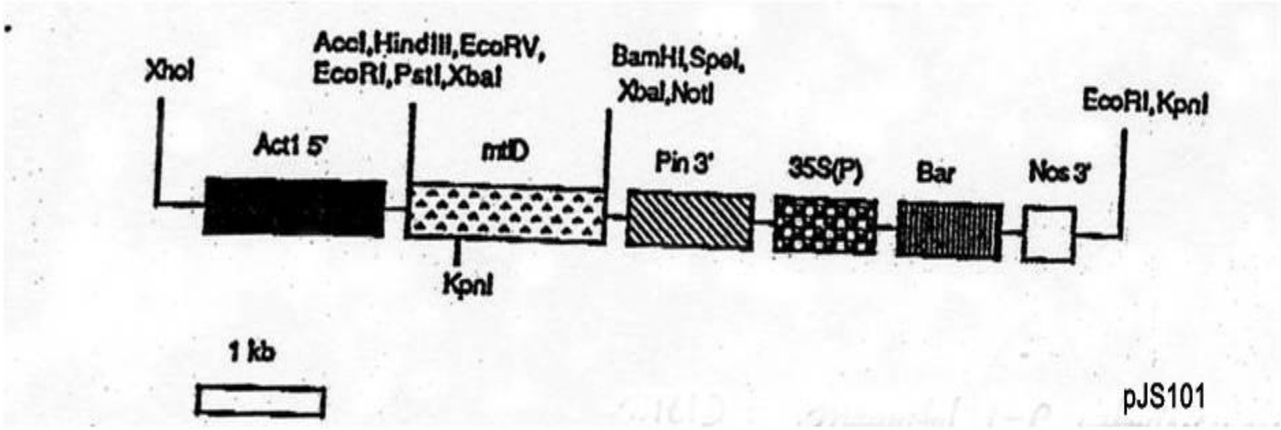


Figure 24: pJS101 plasmid constructs containing a bacterial *mtlD* gene which is driven by Actin Rice Promoter (*Act1*) and potato proteinase inhibitor terminator (Pin); and selectable marker *bar* gene with Cauliflower Mosaic Virus (CaMV) *35S* promoter and nopaline synthase terminator (*Nos*).

The process of maize genetic transformation is as described in the Chapter I of this dissertation.

2.3.2. Conformation of gene integration and expression

The sequences of the primers used in PCR analysis to confirm the *mtlD* transgene are 5’ ATC GGT CGT GGC TTT ATC GG 3’ (forward primer) and 5’ TCG ACA AAG CCA ACG TGT TC 3’ (reverse primer). The PCR program was set at 94^{0}C for 3 min for one cycle; the following 35 cycles of 30 s at 94^{0}C, 30 s at 55.5^{0}C, 45s at 72^{0}C; one cycle at 72^{0}C for 10 min; and the final cycle at 4^{0}C forever. The amplified 431bp segment of the *mtlD* gene was also used as DNA probes for northern and southern blotting.

All the procedures for DNA extraction, RNA extraction, and northern blots were done as described in the chapter I.

2.3.3. Salinity stress tolerance test

A total 40 seeds of T3 of a generation line and a non-transgenic line were sown in the small square plastic pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX). This experiment was replicated in two locations of the same greenhouse. The seedlings were watered daily with normal tap water for two weeks before being salt treated. Salt treatments were performed on the seedlings that were at their 4-leaf stage of growth for 10 days (Figure 25). Plant height, the distance from ground level to the tip of the longest leaf, was measured in cm for the absolute growth rate (AGR) followed the formula: AGR = (h2 -h1)/ (t2 -t1). Where h2 and h1 were final and initial height of plant; t2 and t1 are final and initial days (Singh *et al*., 2011).

Figure 25: T3 and wild-type plants at 4-leaf stage growth before being exposed to four different salinity concentrations.

Thereafter, seedlings were daily treated with equal volume of four different concentrations (0, 100, 200 and 300 mM) of NaCl by gradually increasing 50mM per day to reach the final concentrations within 10 days. The commercial 20-20-20 fertilizer was weekly supplemented into salinity solution for nutritional needs. After 10 days of salt water application, observations were made on shoot fresh weight and shoot and root dry weight. Plants were then watered daily for one week in order to allow them to recover from salt stress injuries.

2.3.4. Mannitol analysis

Carbohydrate extraction, derivatization, and analysis procedures were followed based on protocols devised and updated by Dr. Wayne Loescher and colleagues (2012). Fresh leaves of T3 transgenic and non-transgenic plants were collected 7 days after they were exposed to different salt concentrations. 1 gram of fresh leaf from each sample was ground in liquid nitrogen. The tissue powder was then transferred into 15 ml tube followed by adding 2ml of 95% alcohol, and incubated at 65^{0}C for 30 min. The supernatants were transferred into fresh cultured tubes, and then 2 ml of chloroform was added into each tube and kept overnight at 4^{0}C. Supernatants were transferred into fresh cultured tubes, and the samples were dried out during overnight using the SC 200 Speedvac (Savant, Thermo Fisher Scientific, Asheville, NC, USA). The oximation solution was then added into the dried sample tubes and samples were agitated on the shaker tray for 1 hr to dissolve the sugars. To convert oximes and sugars to trimethylsilyl derivatives, 1.0ml of hexamethyldisilazane and 0.1ml of trifluoroacetic acid were added carefully in the tubes and tubes were left under the laminar flow hood at room temperature for 1 hr. Then, the supernatants were transferred into 2ml clear crimp seal vials (SUPELCO Analytical, Bellefonte, PA, USA, and Cat # 27058). Then, 1.0, 0.5, and 0.1 mg of the mixture of carbohydrates were used as standards. Gas chromatography was performed on a 6890 N Network GC system (Agilent Technologies, Santa Clara, CA, USA).

2.3.5. Analysis of photosynthetic gas exchange

Leaf net photosynthetic rate (µmolCO_2 m^{-2} leaf area s^{-1}), and stomatal conductance (mol$H_2$0 m^{-2} leaf area s^{-1}) were determined on the second-uppermost expanded leaf of plants using the LI-6400XT portable photosynthesis system (LICOR, model LI6400, sr. PSC 2925) as described by Wang *et al*. (2010).

2.4. RESULTS AND DISCUSIONS

2.4.1. Integration of *mtlD* transgene confirmed by PCR and Southern blot analysis, and confirmation of *mtlD* transcription via northern blotting

PCR analysis of T0, T1 and T2 plants confirmed the integration of bacterial *mtlD* transgene in maize plants (Figures 26, 27 and 28). In all progenies, the amplification products showed the expected band size of 431bp in all transformants and in the positive control (JS101 plasmid) whereas as expected, no band was observed in the wild-type control plants.

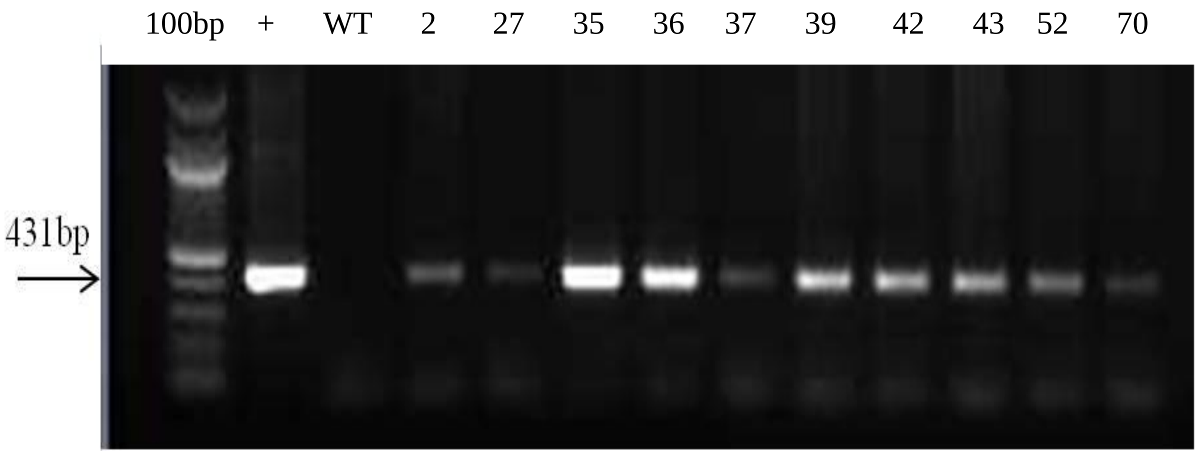


Figure 26: PCR analysis of T0 *mtlD* gene. The expected band size was 431 bp.

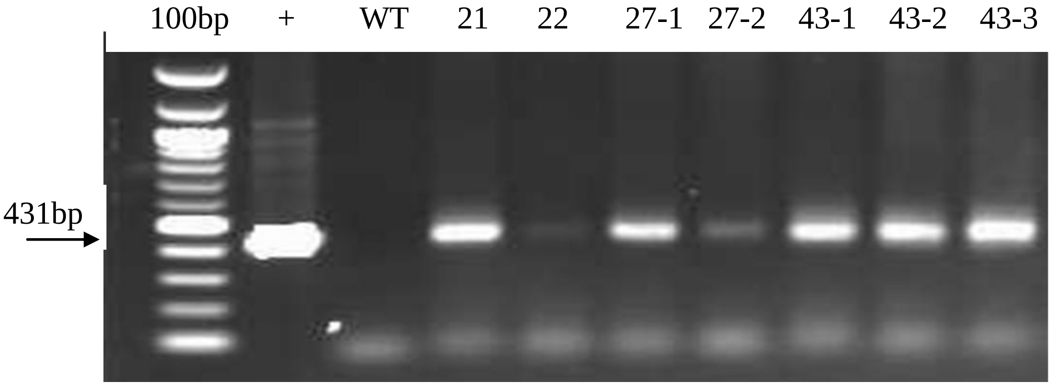


Figure 27: PCR analysis confirmed the *mtlD* transgene in T1 plants, with the expected band size of 431 bp.

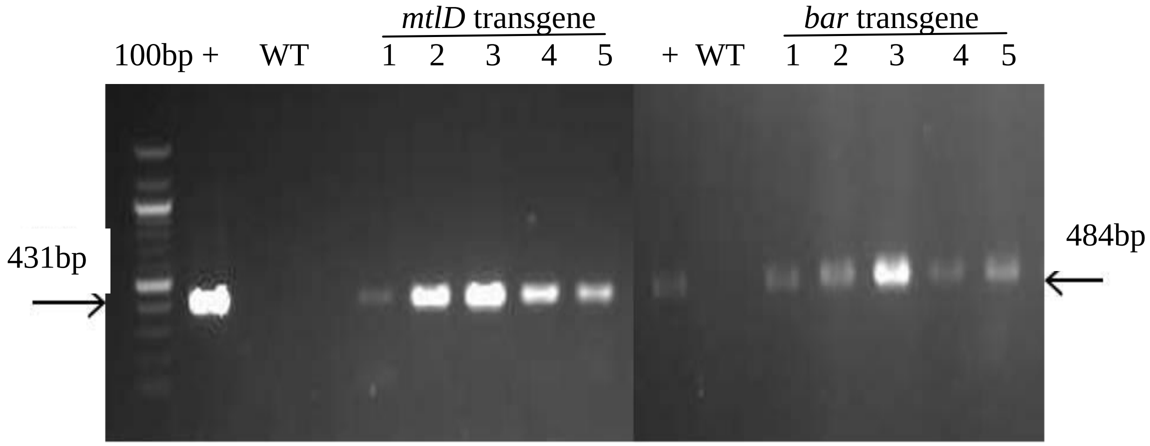


Figure 28: PCR analysis confirmed the stable co-integration of *mtlD* gene and *bar* gene in T2 plants. +: positive DNA from JS101 plasmid; - H20 and wt: wild-type loading as negative control; 1-5: genomic DNA of transgenic plants. The expected band size is 431 bp.

Northern blot hybridization revealed the transcription of transgenes in T1 and T2 plants (Figure 29 and 30). The specific PCR amplification product of *mtlD* sequence was used as a probe for Northern blot hybridization.

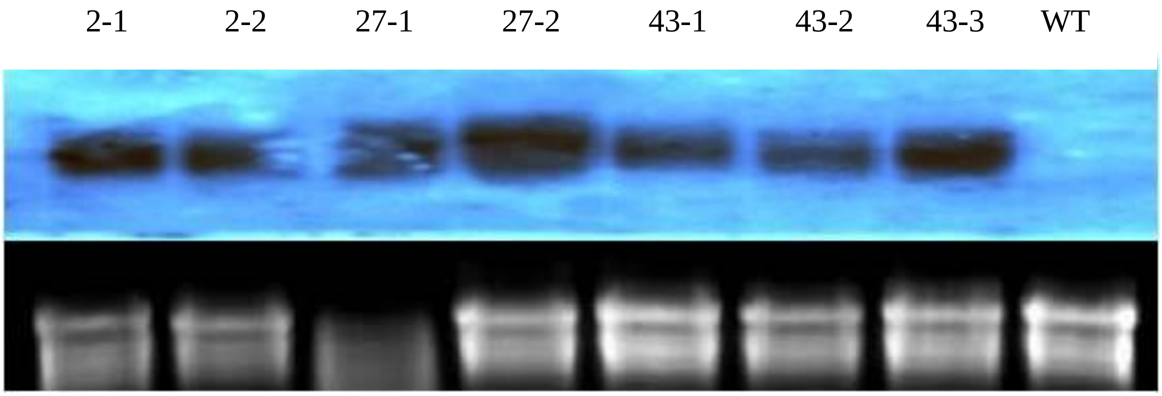


Figure 29: Northern blot hybridization showing the expression of *mtlD* gene in T1 plants.

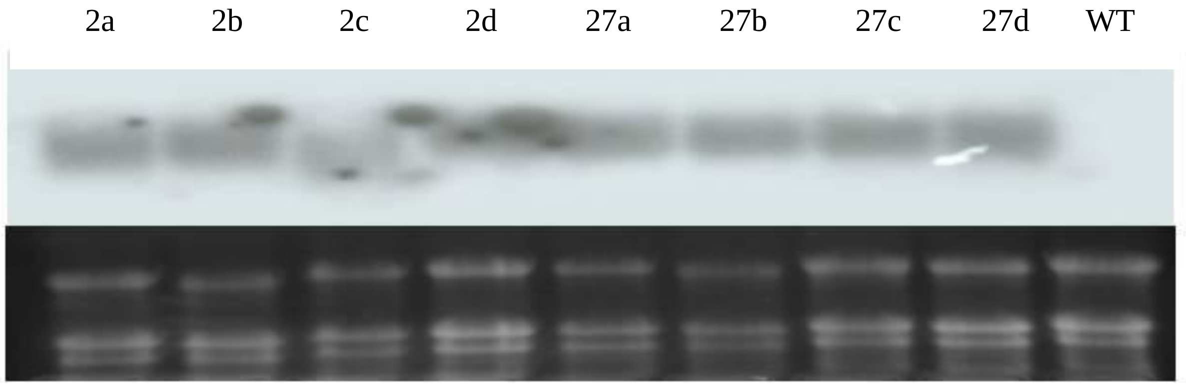


Figure 30: Northern blot hybridization showing the expression of *mtlD* gene in T2 plants

2.4.2. Salt stress tolerance of T3 *mtlD* plants

Under salinity stress conditions, there was a significant reduction in plant height growth rate (cm growth per day) with increasing salinity levels in both T3 and wild-type control plants. However, the wild-type plants were more retarded by salt stress than transgenic plants. At 200 mM NaCl, the average plant growth rate of the wild-type control was 1.5 cm per day and reduced by 1.1 cm per day at 300 mM of NaCl, whereas the corresponding data was 1.6 and 1.4 cm respectively in the transgenic plants. However, there was no significant difference of plant growth rate between transgenic and wild-type plants under different salinity concentrations (Table 5 and Appendix 14).

Table 5: Effect of salt stress treatments on the absolute plant growth rate in T3 *mtlD* plants

Genotype	NaCl treatment	LSMEAN
mtlD	0mM	2.6a
WT	0mM	2.6a
mtlD	100mM	2.0b
WT	100mM	2.0bc
mtlD	200mM	1.6dc
WT	200mM	1.5d
mtlD	300mM	1.4d
WT	300mM	1.1d

LS-means with the same letter are not significantly different.

Figures below represents the shoot fresh weight (Figure 31), shoot dry weight (Figure 32), and root dry weight (Figure 33) 10 days after being treated with different concentration of NaCl.

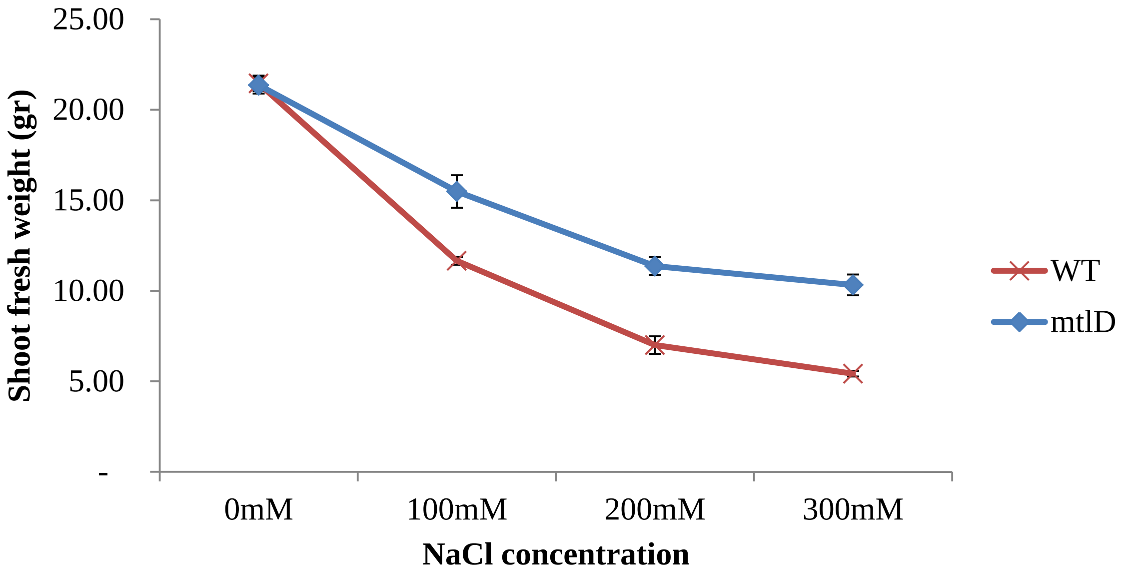


Figure 31: Effect of different salinity conditions on shoot fresh weight of transgenic and non-transgenic control plants. Data are expressed as the means ± SEs (n=3)

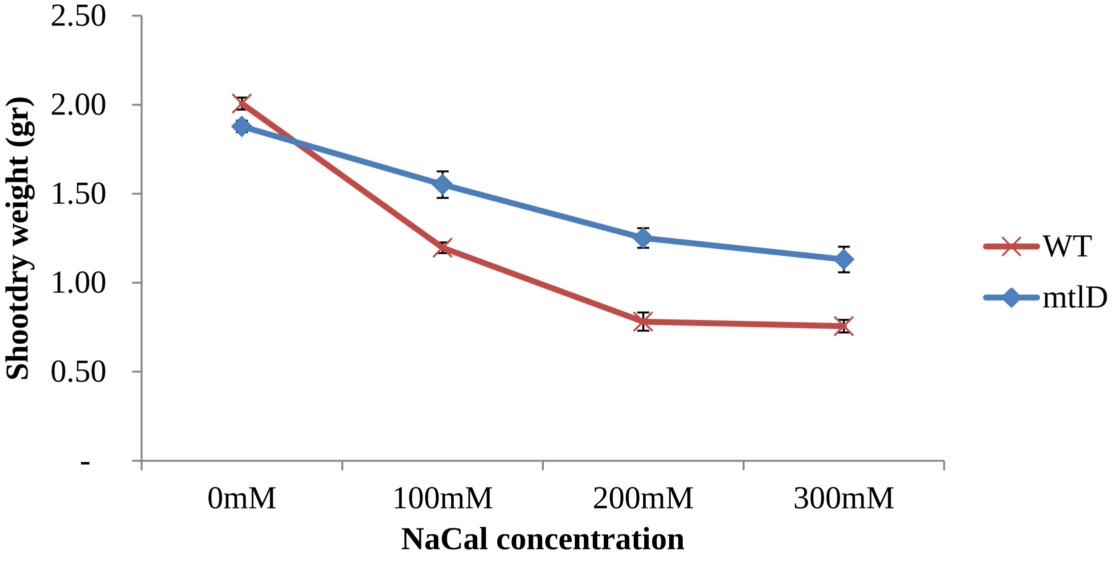


Figure 32*:* Effect of different salinity conditions on shoot dry weight of transgenic and non-transgenic control plants. Data are expressed as the means ± SEs (n=3)

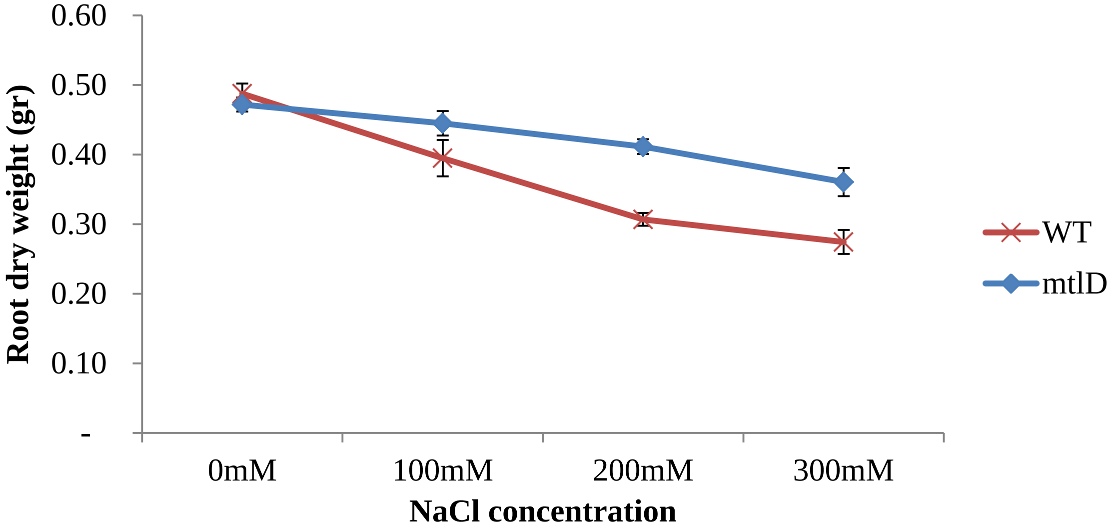


Figure 33: Effect of different salinity conditions on root dry weight of transgenic and non-transgenic control plants. Data are expressed as the means ± SEs (n=3).

Table 6 (below) shows that the salinity stress treatment has affected on the wild-type control seedling growth leading to reduce shoot fresh weight, and shoot and root dry weights. However, the *mtlD* transgenic plants were less affected by salt treatments as compared to the wild-type control plants. At 100mM NaCl treatment, the shoot fresh weight of transgenic plants was reduced by 27%, shoot dry weight by 17%, and root dry weight by 6% as compared to the no salt treated control plants. However, the reduction of shoot fresh weight, shoot, and root dry weights in wild-type was 45%, 40% and 19% respectively.

When exposed to 300mM NaCl, the effect of stress was more severe on wild-type reducing up to 74% of shoot fresh weight, 62% of shoot dry weight, and 44% of root dry weight. The Table 6 also shows that T3 plants exhibited greater salt tolerance at the same salt stress condition than wild-type plants as they reduced shoot fresh weight, shoot dry weight and root dry weight, by 52%, 40%, and 24% for respectively. The effect of salinity stress was also reduced the absolute plant height rate (centimes plant height per day) of both transgenic and wild-type plants. The plant height rate of transgenic and wild-type plants was no significant difference in the non salt stress condition. Overall, the increase in salt concentration has more negative effects on wild-type control plants as compared to the T3 plants.

Table 6: Biomass growth reduction of T3 *mtlD* plants versus the wild-type control plant treated with different NaCl concentrations for 10 days

	Percentage of fresh and dry biomass reduction (%)			
Treatment (NaCl)	**Genotypes**	**Shoot fresh weight**	**Shoot dry weight**	**Root dry weight**
100mM	WT	45.7	40.4b	19.0bc
	mtlD	27.5e	17.4d	5.8d
200mM	WT	67.4b	61.1a	36.8a
	mtlD	46.8	33.4c	12.9cd
300mM	WT	74.7a	62.3a	43.7a
	mtlD	51.7c	39.8b	23.7b

Means within columns followed by the same letter are not significantly different (P<0.05)

Figure 34 (below) represents the T3 *mtlD* versus wild-type control plants after 7 days of water treatment recovery from 10 days of 200mM NaCl salinity treatment.

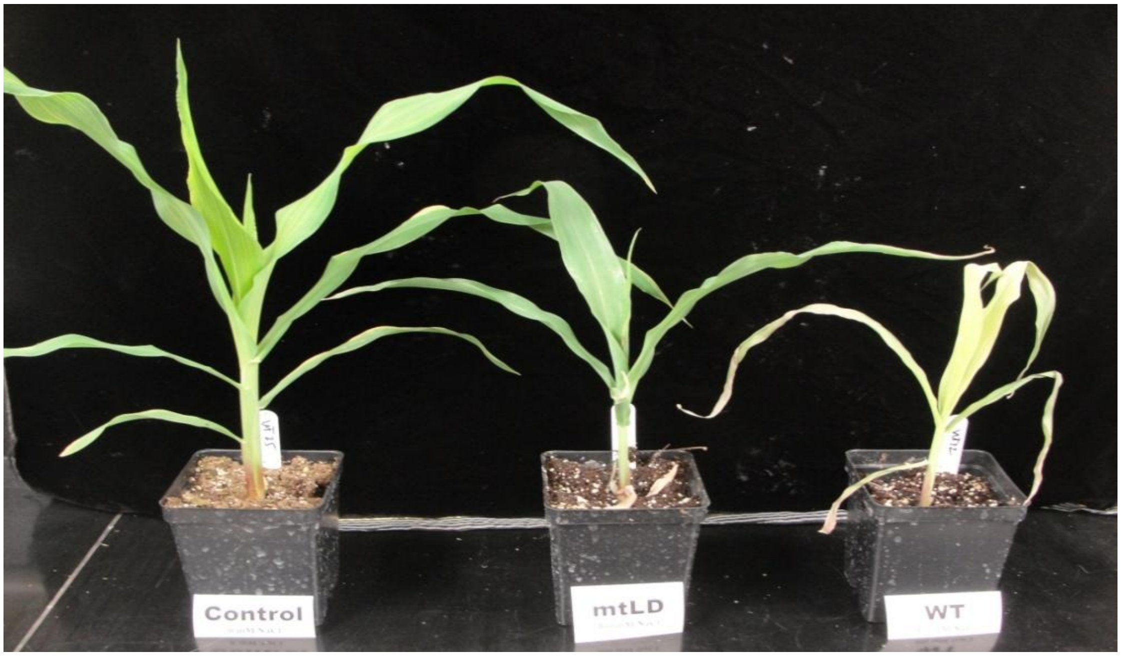


Figure 34: T3 *mtlD* versus wild-type control plants after 7 days of water treatment recovery from 10 days of 200mM NaCl salinity treatment.

The work presented here agrees with the work of other scientists indicating that the expression of *mtlD* gene confers salt tolerance and enhanced fresh and dry biomass of transgenic plants in sorghum (Maheswari *et al.*, 2010) and potato (Rahnama *et al*., 2011). In this study, we did not find any abnormal phenotypes such as dwarfed or stunted growth of transgenic plants that expressed the bacterial *mtlD* transgene as compared to their wild-type control plants under no salt conditions. The same results have been shown in *mtlD* transgenic eggplant (Praghavathi *et al*., 2002)

In the research presented here, salt stress experiments demonstrated significant differences in shoot fresh and dry weight, and root dry weight in transgenic as well as the wild-type control plants under high salinity concentrations. Also the *mtlD* transgene plants showed higher plant height growth rate and less shoot and root reduction under salt stress than wild-type control plants. Similar results were reported that the expression of *mtlD* transgene in transgenic plants resulted in higher root growth as compared to their wild-type control plants under salinity stress treatments in tobacco (Tarczynski *et al.*, 1993), egg plant (Prabhavathi *et al.*, 2002), and wheat (Abebe *et al*., 2003).

The effects of salinity stress on photosynthesis and stomatal conductance of T3 *mtlD* and wild-type control plants are shown in Table 7 and Figure 35. Table 7 shows that the photosynthesis rate and stomatal conductance declined with increased salinity concentrations in wild-type non-transgenic plants and were significantly lower compared to transgenic plants at 100mM NaCl. However, the photosynthesis rate and stomatal conductance were not significantly different in transgenic plants after 7 days of water treatment recovery from 100 and 200mM of NaCl treatment.

Figure 35 shows the effect of salinity on photosynthesis and stomatal conductance in wild-type plants was more severe than those of transgenic plants after 7 days of water treatment recovery from 100mM NaCl. All wild-type plants were dead with shrunken and dried leaves after 7 days of water treatment, not being able to recover from 10 days of 200mM of NaCl treatment. The data presented in table 7 confirm that the *mtlD* transgenic plants exhibited higher photosynthesis and stomatal conductance than wild-type control plants at 100 mM NaCl, agreeing with Hu *et al*. (2005) report indicating that there were higher stomatal conduction, transpiration rates and photosynthetic rates in poplar transgenic plants as compared to their wild-type plants treated with 50 mM NaCl after 21 days of salinity treatment.

Table 7 (below) represents the photosynthesis rate and stomatal conductance of T3 versus wild-type non-transgenic plants 7 days after water treatment recovery from 10 days of different NaCl treatments.

Table 7: Effects of salt stress treatment on T3 plant photosynthesis and stomatal conductance. Values are means ± SEs (n=3)

Genotype/Treatment (NaCl)	**Photosynthesis (μmol CO_2 $m^{-2}s^{-1}$)**	**Stomatal conductance (mol H_20 $m^{-2}s^{-1}$)**
***mtlD* transgenic plants**		
0 mM	23.60 ± 1.18a	0.147 ± 0.014a
100 mM	19.12 ± 0.6b	0.124 ± 0.011a
200 mM	18.06 ± 0.37b	0.113 ± 0.004ab
Wild-type control plants		
0 mM	22.63 ± 1.03a	0.148 ± 0.009a
100 mM	14.90 ± 0.55c	0.086 ± 0.005b
200 mM	-	-

Means within columns followed by the same letter are not significantly different ($P<0.05$)

Figure 35 (below) represents the effect of salt stress treatment on photosynthesis of T3 *mtlD* versus that of the wild-type non-transgenic control plants after 7 days of water treatment recovery regime from 10 days of 100mM NaCl.

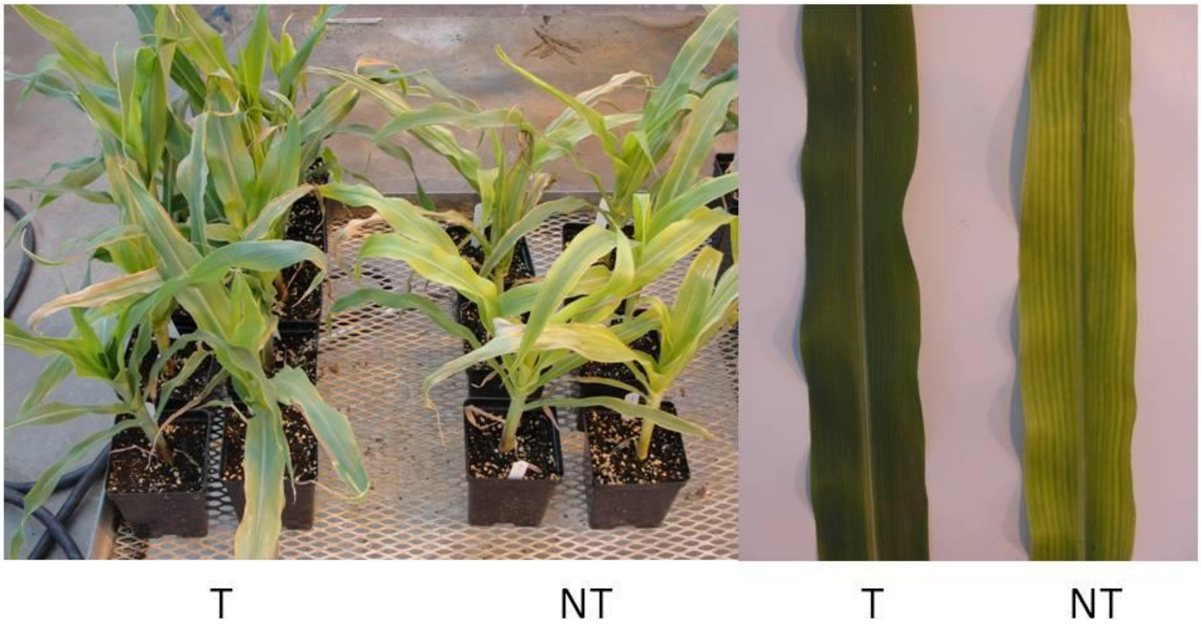


Figure 35: Effect of salt stress treatment on photosynthesis of T3 *mtlD* versus that of the wild-type non-transgenic control plants after 7 days of water treatment recovery regime from 10 days of 100mM NaCl. T: T3, NT: non-transgenic control plants.

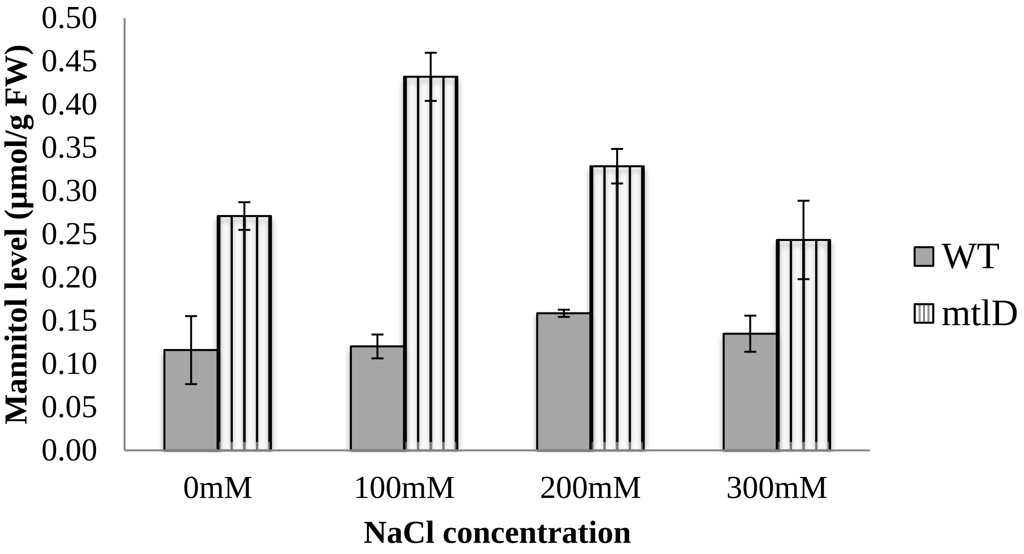


Figure 36: Accumulation levels of mannitol in leaves of *mtlD* transgene plants treated 7 days with different concentration of NaCl.

Mannitol (or sorbitol) accumulation: The results from the Figure 36 indicate that there was an increase in detectable mannitol in transgenic plants. Mannitol has increased significantly in transgenic plants under 100mM and 200mM of NaCl stress conditions as compared to wild-type control plants (Appendix 21). Under no salt stress condition (0mM), mannitol also accumulated significantly higher in transgenic plants as compared to wild-type non-transgenic plants. There was no difference of mannitol accumulation in wild-type control when increased concentrations of NaCl (Figure 36, Appendix 21).

The accumulation of mannitol in transgenic plants usually acts as osmotic adjustment compound. In this study, it was found that T3 plants showed mannitol accumulation; however the wild-type control plants also had a low level of mannitol. The presence of mannitol in wild-type control plants are under study by Professor Wayne Loescher to see whether the wild-type control and T3 plants have shown "sorbitol" accumulation rather than mannitol accumulation because the wild-type control maize plants should not contain mannitol. Since sorbitol is sometimes found in maize tissues (Carey *et al.,* 1982; Shaw *et al.*, 1984), confirmation of these results awaits a mass spectrometric analysis of the samples to see whether the results shown here were for sorbitol or for mannitol

Hu *et al*. (2005) reported the accumulation of mannitol of 0.003-0.035mg/g fresh weight could improve salt tolerance in transgenic poplar plants. Similarly, mannitol content was 0.04mg/g dry weight in transgenic canola (Motallebi and Rahnama 2011), and 0.8-2.3µmol/g fresh weight in *Arabidopsis* (Zhifang and Loescher 2005). Their results demonstrated that the small amount of mannitol accumulated in the transgenic plants were not enough to act as osmolyte and suggested that the present of mannitol may indirectly enhance osmotic adjustment and salt tolerance in transgenic plants. Another possible reason is that the integration and expression of a transgene accumulating mannitol in transgenic plants may activate and express other stress inducible genes conferring drought and salt tolerance. Several trehalose synthesized genes, ABA receptor genes, and raffinose genes that known to reduce the oxidative damage in plants under salt stress were up-regulated under salt stress in the mannose-6-Photphatse (*M6PR*) transgenic *Arabidopsis* accumulating mannitol (Chan *et al.,* 2011).

In general, because mannitol has stress-protective functions in plants (Rahnama *et al.*, 2010) it is possible that drought and salt tolerance of transgenic maize in this study is partially due to mannitol accumulation in them. In a report presented by Abebe *et al*. (2003) shows the effect of drought stress on the mannitol content of transgenic leaves is shown to be 150% higher than in the wild-type control plants, but this experiment was not repeated. This report also indicated that transgenic wheat plants expressing the *mtlD* transgene showed significantly different shoot dry weight, plant height, and leaf length compared to non-transgenic control under salt conditions.

CHAPTER III

COEXPRESSION OF A COMBINATION OF *HVA1* AND *MTLD* TRANSGENES CONFERS DROUGHT AND SALT TOLERANCE IN TRANSGENIC MAIZE PLANTS

3.1. LITERATURE REVIEWS

3.1.1. Introduction

Previous two chapters of this dissertation consecutively described the effect of barley *HVA1* and the bacterial *mtlD* on drought and/or salt tolerance in maize. This chapter follows the concept that “stacking” or “pyramiding” of these two stress tolerance transgenes might prove more effective than each of the above two transgenes alone on drought and/or salt tolerance in maize.

Gene stacking has been commercially performed for improving a combination of different traits such as insect and herbicide resistance. The transfer of a combination of multiple transgenes or transgene stacking to improve multiple traits at commercial level has become one of the promising approaches in plant genetic engineering and biotechnology in the last decade. Therefore in 2007, 40% of Biotech crops in the US carried multiple transgenes. Furthermore, the 60% of the total biotech maize Biotech crop in the US carried transgenes stacked for herbicide and insect resistance (James, 2007).

Another concept is the use of a transcription factor as a transgene that can activate a multiple number of naturally existing genes in plants resulting in improvement of a trait such as salt or drought tolerance (Su *et al*. 2010). This concept will be described more in Chapter IV where this candidate transferred sorghum drought tolerance transcription factor into the maize genome.

3.1.2. Strategies for transgene stacking via breeding of transgenic plants

One strategy in gene stacking is to combine two or more transgenes into a crop species via the intercrossing of two parental transgenic lines each containing one transgene, followed by the selection of an ideal trait until all desirable transgenes are assembled into the progeny. Gene stacking for insect, disease and weed resistance has been used widely to improve maize, cotton, and tobacco. For example, cross-bred combination of different *Bacillus thuringiensis* (*Bt)* genes along with phosphinothricin *acetyltransferase* (*PAT)* herbicide resistance gene have enhanced corn borer and rootworm resistance along with herbicide tolerance in maize (Jain and Brar, 2010).

By breeding of two different transgenic tobacco plants, one expressing the *betA* gene encoding choline dehydrogenase and the other expressing a vacuolar Na^+/H^+ antiporter *(AtNHX1)*gene, enhanced salt tolerance of double transgene plants as compared to transgenic plants that expressed only *betA* or *AtNHX1* gene (Duan *et al*., 2009) was demonstrated. More specifically, at 150mM NaCl stress condition, seeds of T0 plants expressing both of these genes showed a significantly higher germination rate when compared to plants that expressed only *betA* or *AtNHX1*. Also, the two-transgene stacked transgenic seedlings performed much better than the wild-type non-transgenic control seedlings under 342 mM NaCl stress for 10 days, displaying no injury symptoms. From these results, Duan *et al* (2009) recommended that the stacking of transgenic tobacco via breeding might be a better approach than developing transgenic plants expressing a single salt tolerance transgene.

Similarly, Wei *et al*. (2011) cross bred two maize transgenic parents, one expressing the *BetA* gene and the other expressing the H^+-PPase (*TsVP)* gene *(*encoding the vacuolar H^+ pyrophosphaatase of *Thellungiella halophila*)*,* and demonstrated that the expression of both transgenes in pyramided plants could further improve drought tolerance as compared to transgenic plants expressing just one of the two transgenes. In the second experiment, after 21 days of withholding water, the pyramided plants showed less cell membrane damage, higher amount of soluble sugars, proline content, greater growth of root system and higher biomass as compared with single *BetA* or *TsVP* transgenic plants, and non-transgenic control plants. These maize pyramided transgenic plants also showed significant differences in phenotypic performance including greater tassel and silk synchronization after drought stress. Similarly, percentage of pollen viability in pyramided plants was much higher as compared to single transgenic plants and the wild-type control plants. They concluded that a number of different pathways might be involved in drought tolerance as the reason why their transgene-stacked plants performed much better than transgenic plants expressing each of the two transgenes, meaning that each transgene might be able to go through one pathway.

3.1.3. Strategies for transgene stacking via re-transformation of transgenic plants

The method of cross breeding of two more transgenic plants to stack genes is relatively labor intensive and time consuming. Another gene stacking strategy is called re-transformation for transgene stacking. This strategy is to produce a transgenic plant and then re-transform it with a second transgene. This method has also been proven to improve traits in several crop species. For instance, the independent expression of either glyoxalase I (*glyI*) or glyoxalase II (*glyII*) gene in transgenic tobacco was reported to enhance salinity tolerance as compared to their non-transgenic control plants (Singla-Pareek *et al.*, 2003). However, when transgenic plant expressing the *glyI* was re-transformed with the *glyII* gene, the double transformants (*glyII* + *glyI*) were found to be show higher level of salt tolerance. This report indicates that under 400mM and 800mM NaCl treatment for 5 days, the treated leaf discs of double transgenic plants (*glyII* + *glyI*) showed highly significant chlorophyll content in comparison with each of the single transgene transgenic plants, as well as, those of non-transgenic control plants. Similarly, the double-transgene seedlings showed lowest injury symptoms at 400mM NaCl concentration as compared to all other plants. When tested under the same (400mM) salinity conditions, the seedlings and leaves of single gene transformants (*glyII* or *glyI)* showed no injury, but the wild-type seedlings showed severe injury and died after 25 days.

The simultaneous re-transformation of tobacco with three transgenes (encoding dehydroascorbate reductase -DHAR, copper zinc superoxide dismutase -CuZnSOD, and ascorbate peroxidase -APX) was reported to confer highly salt tolerance in the transgenic tobacco (Lee *et al*., 2007). The shoot and root dry weight of triple-transgene transgenic plants showed significant salt tolerance than that of single or double-transgene transgenic plants after 10 days of 100mM NaCl treatment.

Moreover, transgene stacking via re-transformation is reported to delay the emergence of *Bt* resistance in insects of broccoli (Cao *et al*., 2002) and delay the tolerance of both pest and disease in rice (Datta *et al.*, 2002).

With all advantages of transgene stacking approach via re-transformation, this method has the limitation that the two or more transgenes are not linked on the same construct, and therefore they will segregate apart from each other in crop progenies. Re-transformation is also labor intensive and time consuming.

3.1.3. Strategies for transgene stacking via co-transformation of transgenic plants

Co-transformation is to genetically engineer plants at once with a mixture of two or more transgene constructs, each construct containing a specific transgene regulated under specific regulatory sequences. The ratios of mixing of constructs vary, but usually are 1:1 for two constructs or 1:1:1 for three constructs.

Co-transformation can be performed via *Agrobacterium* or the gene gun technique. The co-transformation approach is also less time consuming and less labor intensive than that of re-transformation approach. The *Agrobacterium* system can also be used in co-transformation when multiple gene cassettes are placed in one binary vector for transformation. Using this concept, Zhu *et al*. (2012) transferred an *Agrobacterium* binary vector containing three linked broad-spectrum potato disease resistance genes in potato, and found that 23 putative transgenic plants contained all three genes. Those triple-gene transformants were reported tolerant to all three selected isolates of *Phytophthora infestans*.

Tang *et al*. (2005) co-transferred a mixture of two *Agrobacterium* into loblolly pine, one *Agrobacterium* containing the gene encoding mannitol-1-photphate dehydrogenase (*mtlD)* and the other containing the glucitol-6-photphate dehydrogenase (*GutD*). These researchers demonstrated the simultaneous integration and expression of these two genes in transgenic plants resulting in an increased accumulation of both mannitol and glucose. The transgenic plants also maintained higher survival rates when grown under 85mM and 120mM NaCl as compared to their wild-type control plants.

When a single expression vector construct containing six linked expression cassettes (cassettes including two selective marker genes *pat* and *als,* two reporter genes *LUC* and *GUS*, and two defensin genes *RsAFP2* and *DmAMP1*) were transformed into *Arabidopsis* via *Agrobacterium,* all six genes were integrated into plants (Goderis *et al*. 2002) because all six genes were linked together. Also, Su *et al*. (2011) delivered four different constructs containing five different genes (*SacB*, *JERF36, vgb*, and *BtCry3A +OC-I*) into poplar plants and confirmed that the stacking of all five transgenes in two poplar transgenic lines D5-20 and D5-21 resulted in their tolerance to drought, salinity, water logging, and insects, respectively.

Transgenic rice plants co-transformed with two *Suaeda salsa* genes encoding Glutathione S-transferase (GST) and Catalase (CAT1) showed significantly increased salt tolerance (200 mM NaCl for 10 days in greenhouse) as compared to non-transgenic rice plants (Zhao and Zhang 2006). Catalase and glutathione S-transferase are known to play critical roles in plant defense mechanisms and in the detoxification of xenobiotics (Anderson *et al*., 1998).

Chen *et al*. (1998) co-bombarded a mixture of 14 plasmids into embryogenic rice tissues, and regenerated plants. The results showed that using the PCR method to confirm transgene integration, over 70% of the T0 plants were found to contain from two to eight transgenes, 17% of T0 plants contained more than 10 transgene, and 2.4% contained 13 transgenes. The stability of transgenes integration and co-expression in transgenic rice plants were also inherited through three generations (T2). This multiple transgene transformation method sounds to be a promising strategy for gene stacking via co-bombardment. Similarly, Agrawal *et al*. (2005) simultaneously co-transformed five minimal cassettes (without vector backbone) into rice genome via the gene gun method and found all transgenic plants had integrated two of the transgenes, but only 16% of had integrated all five transgenes, likely because genes were not linked in one construct. Transgenic plants also showed the stability of co-integration and co-expression of those transgenes in the subsequent generations

The gene gun particle bombardment is considered a superior method for multiple gene co-transformations over the *Agrobacterium* mediated transformation system because one can simply mix constructs rather than placing of several gene cassettes on one binary vector. However, in case of the gene gun method, the size of the construct must be relatively small (less than 10 kb), otherwise, it might break during bombardment.

Due to its potential advantages, this candidate chose the co-transformation method of transgene pyramiding in maize via gene gun bombardment for his studies of drought and salt tolerance.

3.2. RESEARCH GOAL AND OBJECTIVES

The goal of this research is to stack the *HVA1* and *mtlD* transgenes to increase the transgenic plant drought and/or salt tolerance as compared to single transgene (*HVA1* or *mtlD*) transgenic plants.

The specific objectives of this research are listed below:

(i) Co-bombard the barley *HVA1* and the bacterial *mtlD* gene constructs in a 1:1 ratio into maize genome.

(ii) Verify the proper expression and stability of transgenes transmission into 2nd (T1) 3rd (T2) and 4th (T3) progenies.

(iii) Evaluate the effects of the *HVA1-mtlD* transgene stacking on maize seedling development, plant height, and fresh and dry biomass matter as compared to single *HVA1* or *mtlD* transgenic plants, and as compared to those of the wild-type non-transgenic control plants under drought and salt stress conditions.

(iv) Determine the accumulation of mannitol and other soluble sugar contents in T3 (*HVA1-mtlD*) transgenic versus single *HVA1* or *mtlD* transgenic and the wild-type plants.

3.3 MATERIALS AND METHODS

Two DNA plasmids, pBY520 containing the barley *HVA1* gene (Figure 1) and JS101 containing the bacterial *mtlD* gene (Figure 24) were co-bombarded with the ratio of 1:1 into immature maize embryogenic calli as described in chapter I. Then, transgenic plants were selfed and the T3 of *HVA1-mtlD* transgenic plants (line#51-1) were selected for drought and salt tolerance as compared to single transgene *HVA1* transgenic plants (line #161) and *mtlD* transgenic plants (line#27-1). The drought tolerance test and salt tolerance test in this chapter were followed as described in chapter I and II, respectfully.

3.4. RESULTS AND DISCUSSIONS

3.3.1. Confirmation of integration of the stacked *HVA1-mtlD* transgenes via PCR analysis, and their transcription via northern blot hybridization

Three lines developed via co-transformation bombardment contained both the *HVA1* and *mtlD* transgenes. The T0 of these plants were selfed producing T1 progeny plants. Figure 37 (below) represents the co-integration of both *HVA1* and *mtlD* transgenes in T1 plants.

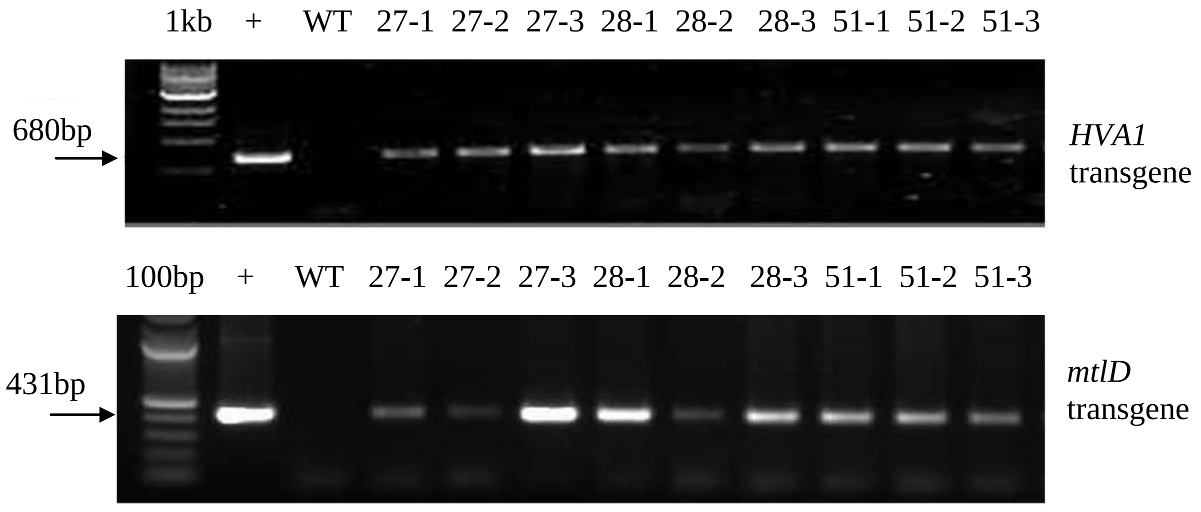


Figure 37: PCR analysis of co-integration of *HVA1* and *mtlD* genes in T1 maize lines.

Figure 38 and 39 (below) represent the co-transcription of the *HVA1* (Figure 38) and *mtlD* (Figure 39) transgenes in the same plants.

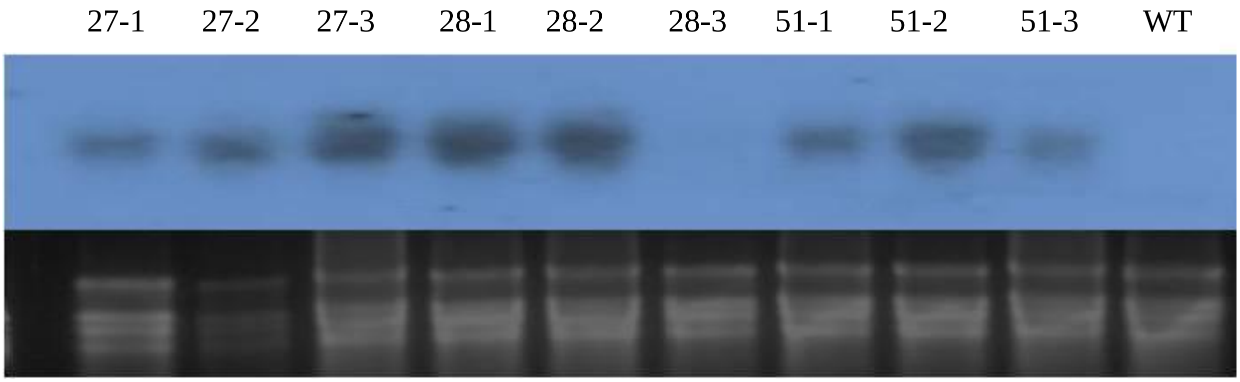


Figure 38: Northern blot of *HVA1* transgenes in T1 *HVA1-mtlD* plants using *HVA1* probe

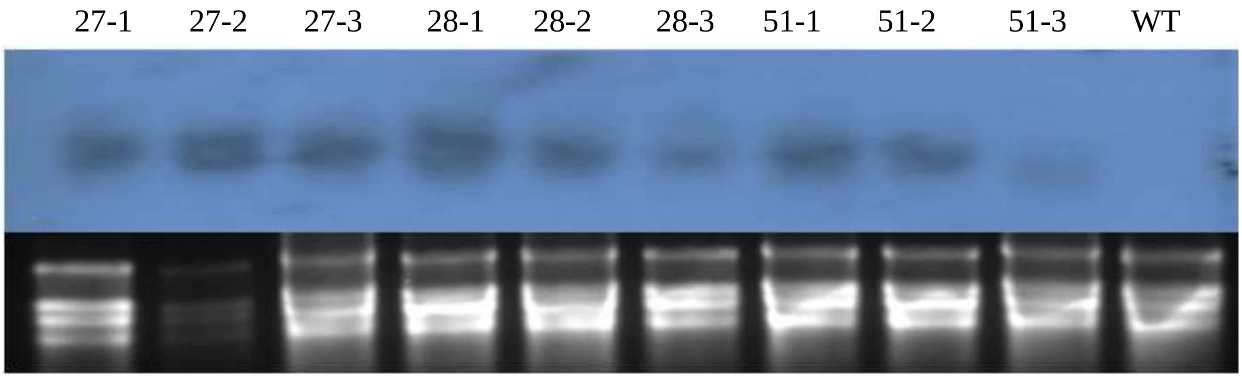


Figure 39*:* Northern blot hybridization of *mtlD* transgene in T1 of *HVA1-mtlD* transgenes plants using *mtlD* probe

Figures 40 and 41 represent the co-transcription of *HVA1* and *mtlD* transgenes in the same T2 plants.

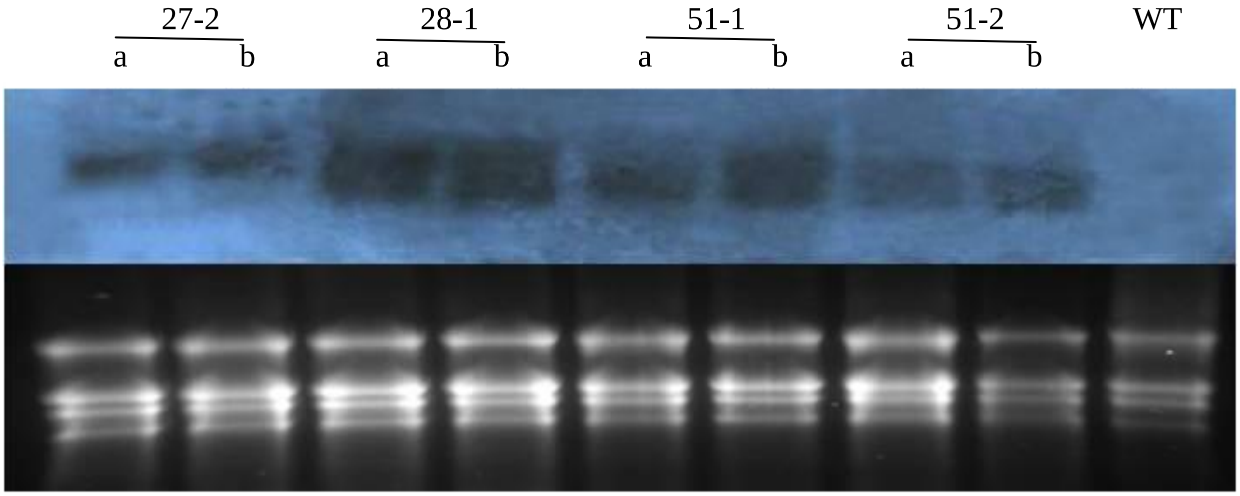


Figure 40: Northern blot hybridization of *HVA1* in *HVA1-mtlD* T2 plants using *HVA1* probe

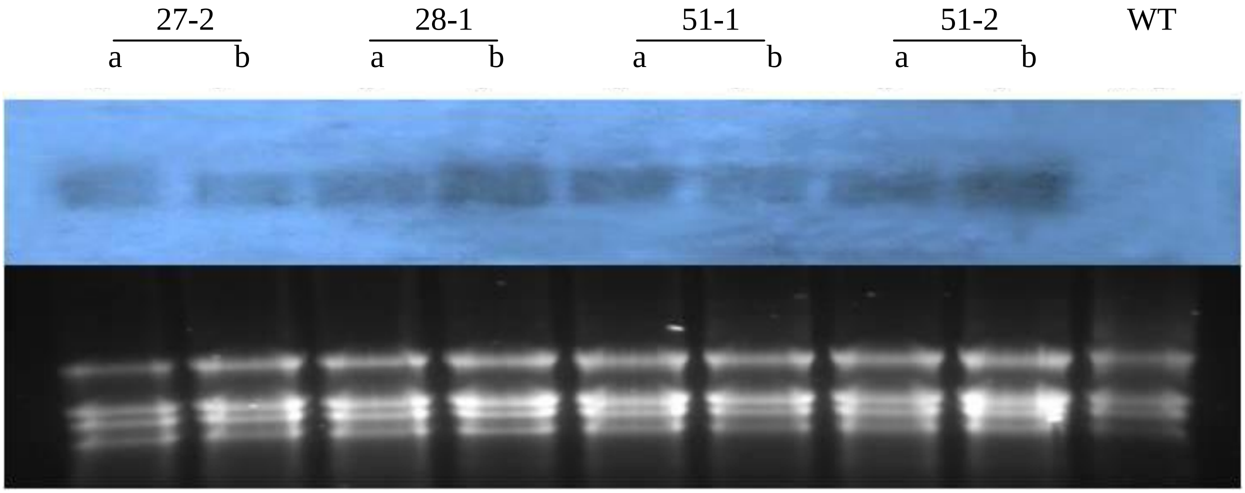


Figure 41: Northern blot hybridization of *mtlD* transgene in *HVA1-mtlD* in T2 plants using *mtlD* probe

Figure 42 (below) shows the integration of both transgenes in T3 generation plants by PCR analysis.

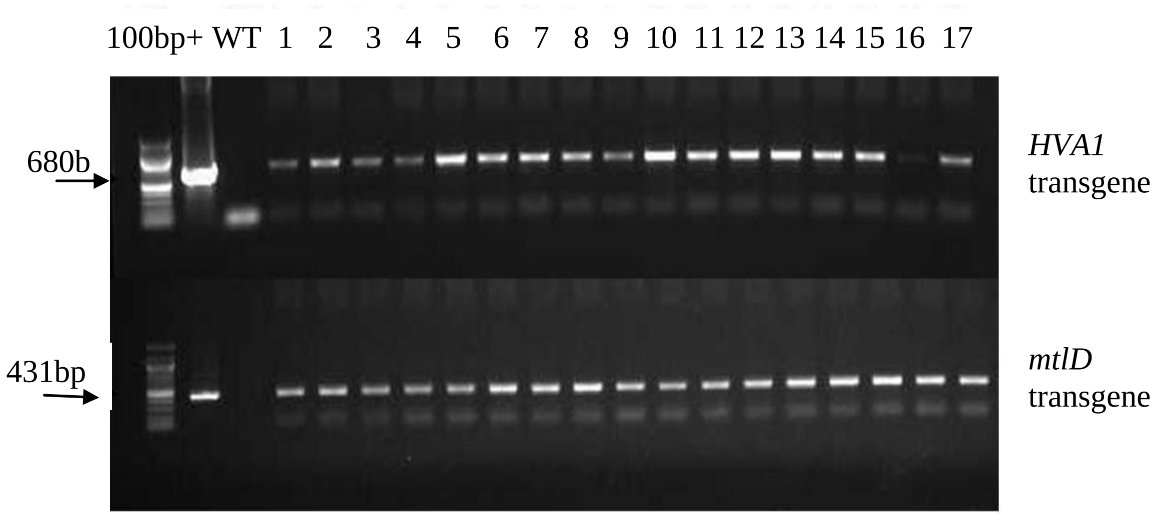


Figure 42: PCR analysis confirming the stable integration of both *HVA1* and *mtlD* transgenes in T3 plants (line #51-1).

The above molecular methods confirm the co-integration and co-transcription of the *HVA1* along with *mtlD* in plants that were co-transformed via the 1:1 ratio co-bombardment of the *HVA1* and *mtlD* transgene constructs.

The above results agree with Agrawal *et al*. (2005) who could obtain co-integration of up to two mixed (1:1 ratio) bombarded transgenes in plants. Co transformation methods that show co-integration occur more likely when both transgenes are integrated in the same chromosomal position, and that therefore may be inherited together in the progeny (Halpin, 2005). It is rare in co-bombardment, but chemical selection of both transgenes can assist in both transgenes transmissions into the progenies. In this case, occasionally, multiple transgenes from multiple plasmid co-transformations can co-integrate and inherit stably in the subsequent progenies (Halpin *et al*., 2001).

3.3.2. Drought stress tolerance in T3 transgenic maize plants that were co-transformed with two constructs

Four week old plants of T3 stacked transgenic and single transgenic and wild-type plants were used for drought tolerance test (Figure 43). Figure 44 shows the percentage of leaf relative water content (RWC) after 10 and 15 days of water withholding of transgenic plants versus wild-type non-transgenic control with no water with-holding.

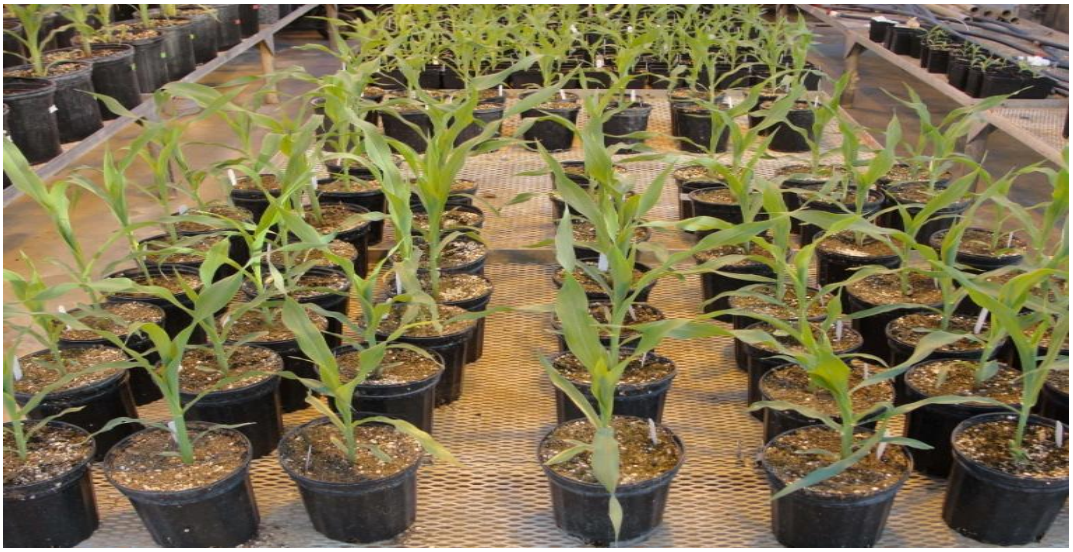


Figure 43: T3 transgenic and wild-type plants at four week old plants before exposing to water withholding.

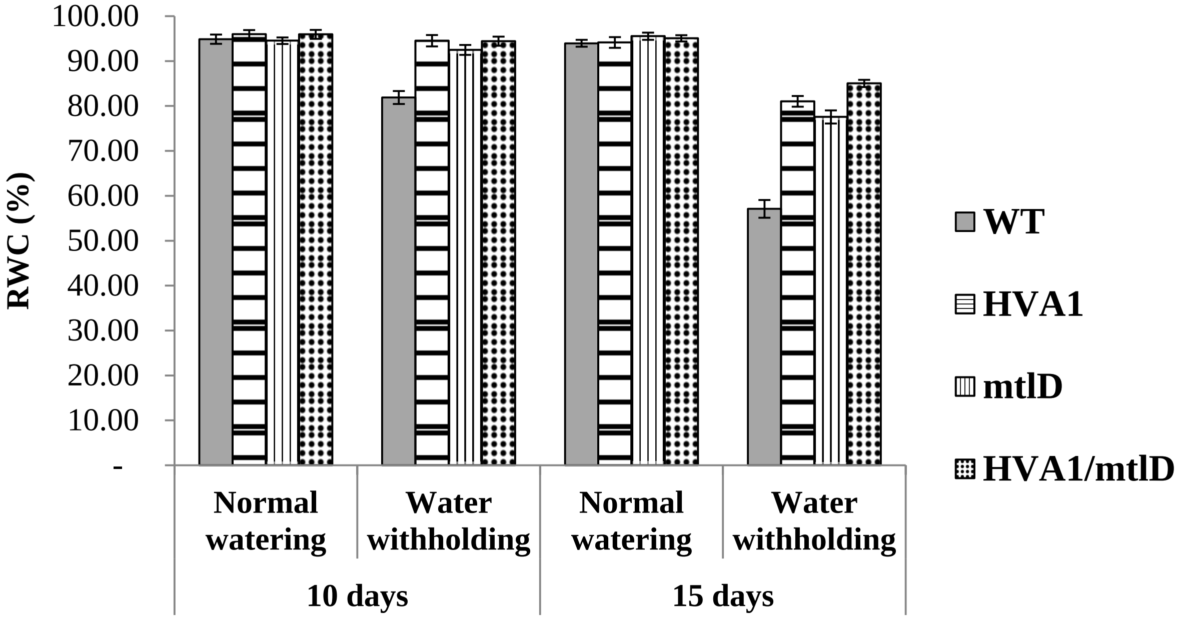


Figure 44: Percentage of leaf relative water content (% RWC) of transgenic lines after 10 and 15 days of water deficit (withholding). Bars are mean ± SE of three samples.

The Figure 44 results show that after 10 days of water withholding, there was no significant differences between the leaf RWC of all three sets of transgenic plants (*HVA1*, *mtlD*, and *HVA1-mtlD*), whereas, the leaf RWC of wild-type plants reduced by 81.9%. After 15 days of water withholding, the leaf RWC in *HVA1-mtlD* line was 85% higher than in individual *HVA1* transgenic plants (81%) and *mtlD* transgenic plants (77.6%) and signifiantly higher as compared to wild-type control plants (57.1%) . Non-transgenic control plants with no water with-holding showed no reduction in leaf RWC (Appendix22).

Table 8 represents the effect of drought tolerance on survival of T3 plants versus their wild-type control plants.

Table 8: Effect of drought tolerance on the percentage of survival of T3 lines versus wild-type control plants that were exposed to water deficit for 15 days followed by 7 days of recovery from water deficit.

Genotypes	**Total number of plants**	**Number of plant survival**	**Percentage (%)**
HVA1	21	11	52
mtlD	20	9	45
HVA1-mtlD	21	14	67
WT	20	7	35

All three sets of transgenic lines showed quick recovery after watering and a higher percentage of plants survived as compared to their wild-type non-transgenic control plants that were grown under 15 days of water deficit followed by 7 days of watering. Table 8 shows that the *HVA1-mtlD* line showed the highest percentage of survival (67%), followed by *HVA1* single transgene transgenic plants (52%) and then the *mtlD* transgenic plants (45%) while their wild-type non-transgenic plants showing only 35% survival.

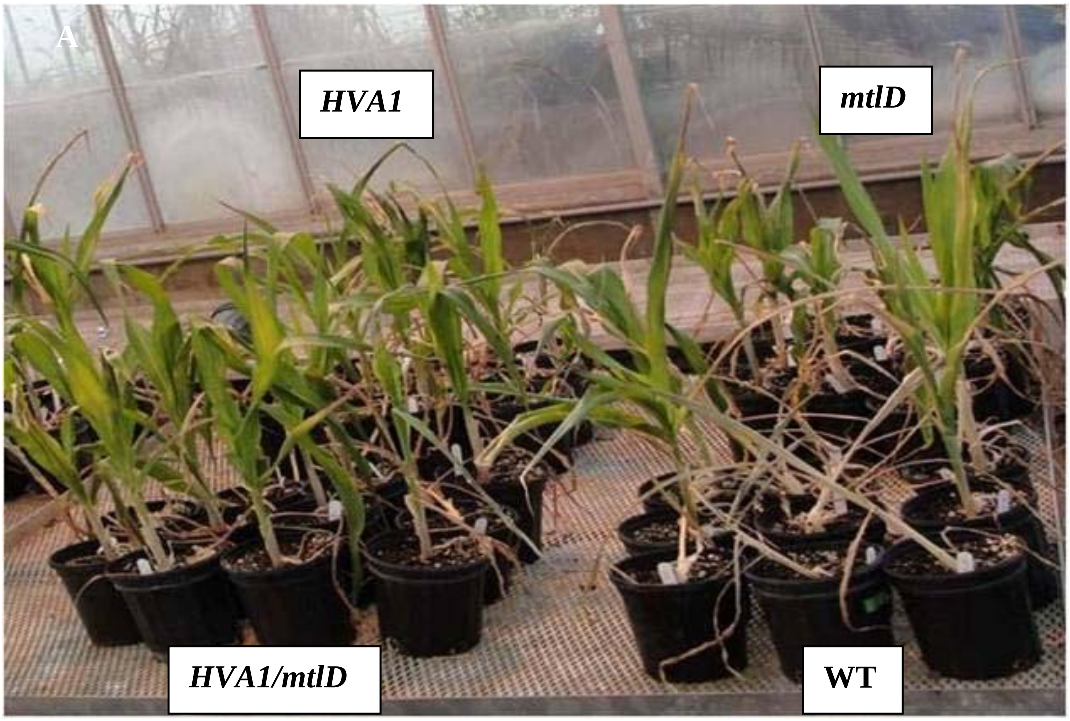


Figure 45: Effect of 15 days of drought stress followed by 7 days of water treatment recovery on the survival of *HVA1-mtlD* transgenic plants (lower left) as compared with the *HVA1* (upper left) and *mtlD* transgenic plants (upper right), and wild-type non-transgenic plants.

Because the wild-type control plant did survive 15 days of water stress after they were watered for 7 days, another experiment was conducted to identify the critical threshold for wild-type plants (number of drought stress needed to kill the control plants). In this experiment, when transgenic and wild-type plants were exposed to 20 days (instead of 15 days) of water withholding followed by 7 days of water recovery, all wild-type non- transgenic plants died with severe symptoms of drought stress (Figure 46), whereas, the transgenic plants including *HVA1*, *mtlD* and *HVA1-mtlD* survived the water stress treatment with at quick recovery just after 2-3 days after watering. The *HVA1-mtlD* plants showed a faster recovery as compared with the single transgene plants. After 20 days of water deficit stress followed by 7 days of water recovery, the percentage of plant survivals were respectively 45.8%, 41% and 33% for the transgene stacked *HVA1-mtlD*, single transgene *HVA1* and *mtlD* transgenic plants.

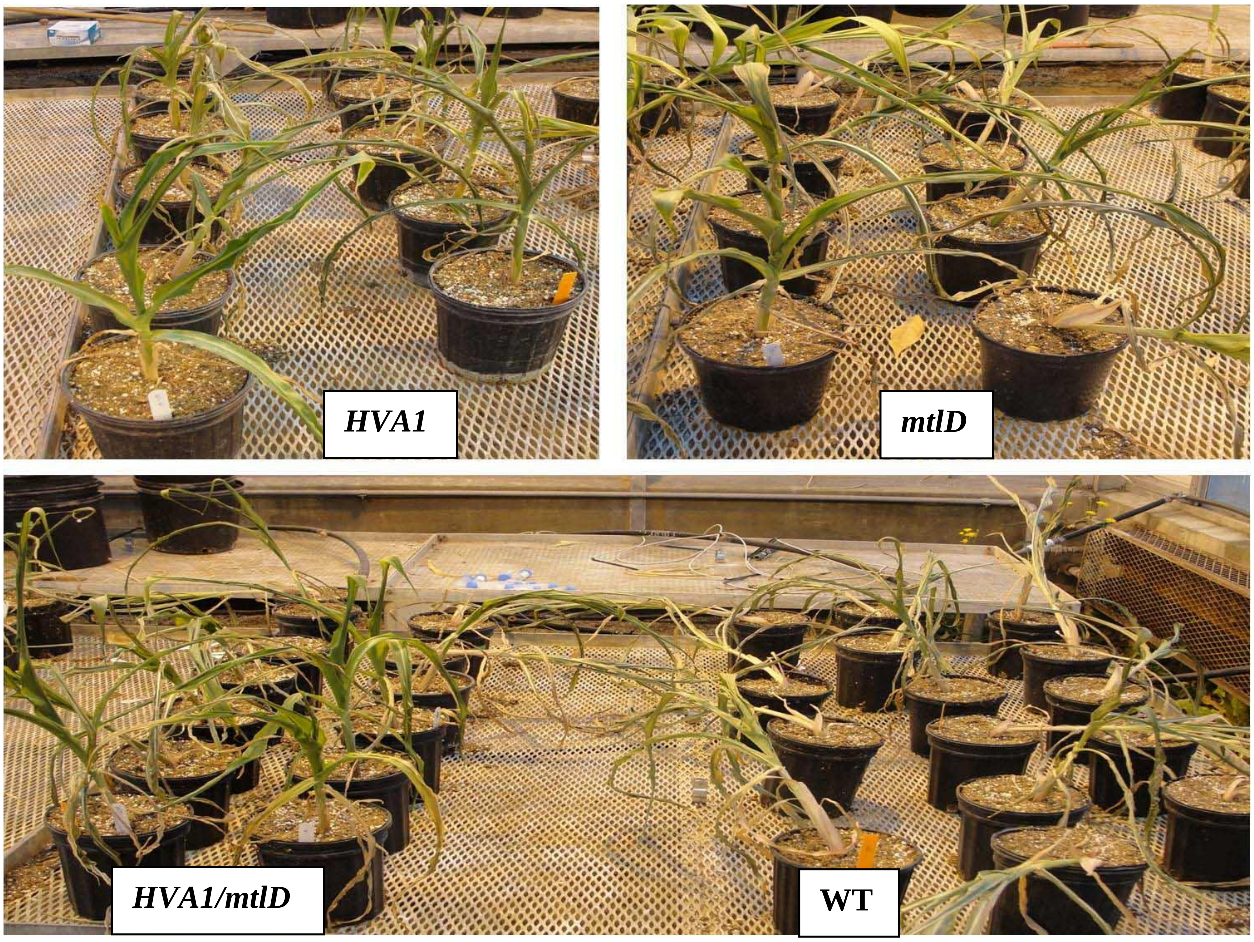


Figure 46: Effect of 20 days of drought stress on the growth of *HVA1*, *mtlD*, and *HVA1- mtlD* transgenic plants as compared with the wild-type non-transgenic plants.

When plants are exposed to severe water stress, they result in reduced RWC and closed stomata. Such conditions also tend to be associated with a decrease in the accumulation of abscisic acid (ABA) and/or sugar contents in plants (Yordanov *et al*., 2003). Therefore, the leaf RWC is considered an idea symptom for water retention capacity of a tissue. It has been reported that when the leaf RWC is reduced below the critical threshhold (below 0.3 g H2O g–1 DW), there is inefficient water left for preferential hydrations (Crowe *et al*., 1990). As such, the wild-type plants of the present study were more affected by water stress and died (in case of 20 days of drought stress followed by 7 days of water recovery).

The present work agrees with Su *et al.* (2011) work in transgenic poplar, and Wei *et al*. (2011) work in transgenic maize that shows that the two-transgene stacked in a plant can further improve drought tolerance as compared to the drought tolerance in transgenic plants expressing only one transgene.

3.3.3. Salinity stress tolerance in T3 transgenic plants

Table 9 (below) shows that the effects of salinity stress treatment increased as the level of salinity concentrations increased from 100mM NaCl to 300mM NaCl, resulting in reduced shoot length, shoot fresh weight, and shoot and root dry weights of transgenic plants (*HVA1*, *mtlD*, and *HVA1-mtlD*) versus wild-type control plants. In this experiment, the wild-type control plants were more affected by salt treatments as compared to all three sets of transgenic plants (Table 9 and Appendix 23-26).

At 100mM NaCl, the transgene stacked plants showed least damage by salt stress treatment as compared to single transgene *HVA1* or *mtlD* plants, and the wild-type control plants. The percentage reduction of shoot fresh weight, and shoot and root dry weights of the transgene stacking (*HVA1-mtlD*) plants were 13%, 18.4%, and 21.4%, respectively. While they were 32.1% and 14.8%, and 25.0% for *HVA1* transgenic plants and 25.9%, 27.0%, and 21.9% for the *mtlD* transgenic plants, respectively (Table 9 and Appendix 28-30).

Table 9: Effects of salt treatment on the percentage reduction of shoot length, shoot fresh weight, and shoot and root dry weight of each set of three transgenic plants (*HVA1*, *mtlD*, and *HVA1-mtlD*) affected by different concentrations of salt treatments. Number in this table represent the percentage of reduced shoot length, shoot fresh weight, or shoot and root dry weight as compared to those of transgenic and wild-type plants at 0mM NaCl (non salt treatment).

	Percentage reduction (%) of			
	Shoot length	**Shoot fresh weight**	**Shoot dry weight**	**Root dry weight**
HVA1				
100mM	7.6f	32.1f	14.8h	25.0defg
200mM	17.0d	43.3e	49.2d	25.1def
300mM	24.0c	66.4c	58.5b	43.2b
mtlD				
100mM	8.0f	25.9g	27.0g	21.9efg
200mM	11.3e	51.3d	35.7f	27.9de
300mM	16.7d	65.6c	50.9dc	30.1cd
HVA1/mtlD				
100mM	10.3ef	13.0h	18.4h	21.4fg
200mM	17.6d	50.3d	42.1e	18.6g
300mM	29.7b	67.7bc	54.7bc	34.6c
WT				
100mM	15.3d	45.6e	40.3ef	30.2cd
200mM	22.6c	70.0b	57.0b	44.4b
300mM	37.7a	76.3a	73.3a	51.9a

Means within columns followed by the same letter are not significantly different ($P<0.05$)

At high salinity concentration, there were no significant difference in reduction of shoot fresh and dry weight between the transgene stacking (*HVA1-mtlD*) and single transgene *HVA1* or *mtlD* transgenic plants. Root of the transgenic plants (*HVA1-mtlD* and *mtlD*) accumulating mannitol showed less affected by salt stress displaying low percentage reduction in root dry weight relative to *HVA1* transgenic and wild-type plants.

Under differential salt concentrations, the *HVA1-mtlD* plants showed more shoot length reduction as compared to *HVA1* or *mtlD* transgenic plants, but showed lower shoot reduction than the wild-type control plants.

Figure 47 (below) compares the effect of 10 days of salt treatments on the growth of T3 of *HVA1*, *mtlD* and *HVA1-mtlD* plants versus their wild-type control plants.

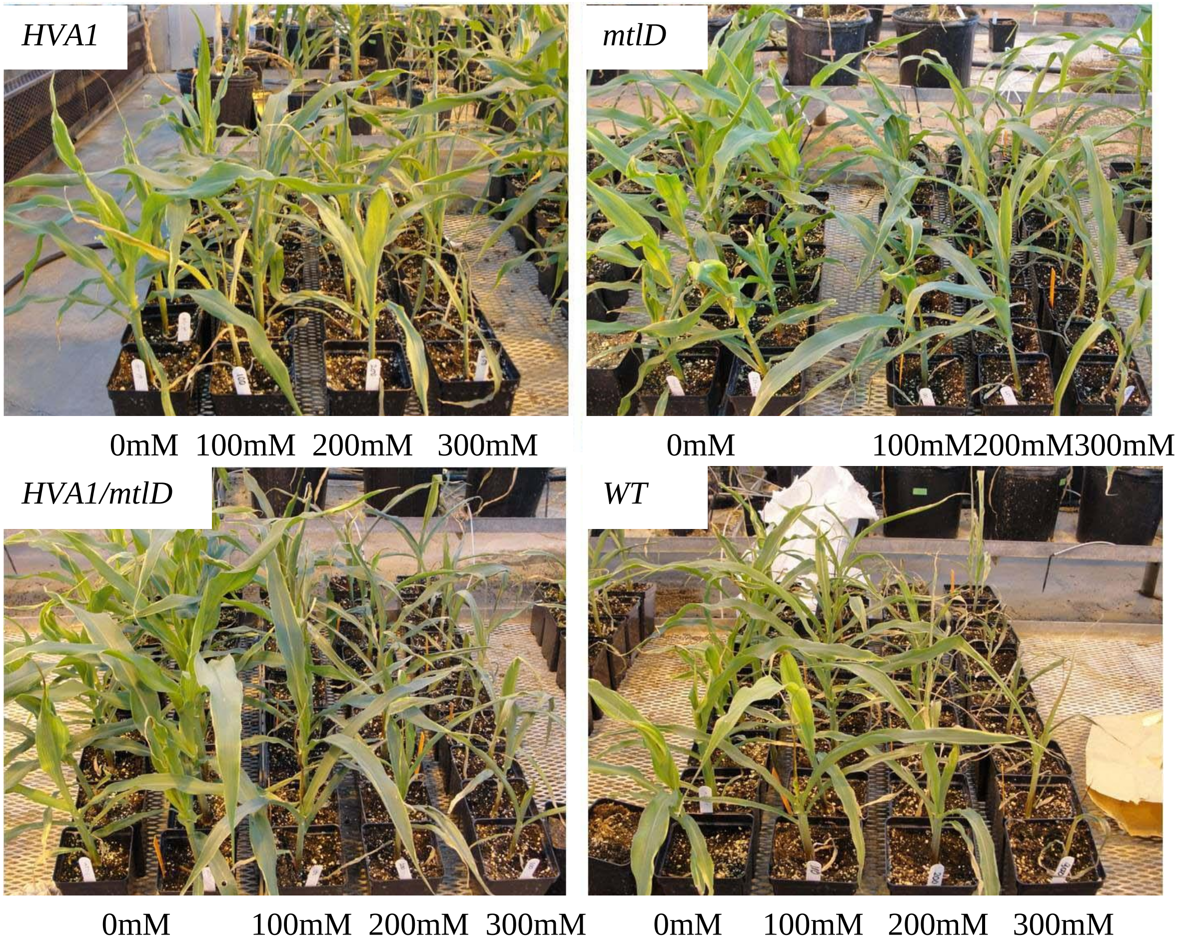


Figure 47: Comparisons of the effect of 10 days of salt treatments on the growth of T3 of *HVA1*, *mtlD* and *HVA1-mtlD* plants versus their wild-type control plants.

These results are in agreement with the previous researches which indicated that the expression of multiple resistance genes showed better shoot and root growth performance as compared to their wild-type control poplar (Su *et al*., 2011), tobacco (Duan *et al*., 2009), and loblolly pine (*Pinus taeda* L.) (Tang *et al*., 2005).

3.3.4. Accumulation of mannitol and other soluble sugar contents after 7 days exposing to different salinity concentrations.

Table 10 (below) represents data collected on the effect of different salt concentrations on sucrose, fructose, glucose, and mannitol contents of transgenic versus wild-type plants.

Table 10: Effects of different salt concentrations on mannitol sucrose, glucose, fructose and inositol contents in transgenic and wild-type plants treated with 0, 100, 200, or 300mM NaCl.

NaCl	**Mannitol** (μmol/g FW)	**Glucose** (μmol/g FW)	**Sucrose** (μmol/g FW)	**Fructose** (μmol/g FW)	**Inositol** (μmol/g FW)
mtlD transgene					
0mM	0.26 ± 0.04	5.66 ± 0.54	33.3 ± 3.4	4.15 ± 1.00	0.61 ± 0.04
100mM	0.43 ± 0.05	8.54 ± 1.00	45.2 ± 1.8	2.49 ± 0.17	1.02 ± 0.01
200mM	0.56 ± 0.05	5.52 ± 0.23	42.8 ± 1.3	4.34 ± 0.17	1.22 ± 0.03
300mM	0.35 ± 0.02	7.11 ± 0.20	49.7 ± 6.0	5.94 ± 0.10	1.24 ± 0.21
HVA1-mtlD transgene					
0mM	0.32 ± 0.09	7.55 ± 2.12	35.5 ± 5.3	4.70 ± 1.90	0.65 ± 0.16
100mM	0.37 ± 0.06	9.04 ± 0.99	50.9 ± 3.5	8.68 ± 0.39	0.99 ± 0.01
200mM	0.52 ± 0.13	9.41 ± 0.68	45.3 ± 3.4	7.56 ± 0.45	1.37 ± 0.19
300mM	0.55 ± 0.18	7.20 ± 0.48	48.1 ± 4.8	6.31 ± 0.15	1.55 ± 0.14
Wild-type					
0mM	0.18 ± 0.08	3.85 ± 0.39	30.3 ± 0.6	1.83 ± 0.01	0.46 ± 0.06
100mM	0.21 ± 0.04	9.19 ± 2.11	41.0 ± 2.0	2.74 ± 0.46	0.75 ± 0.05
200mM	0.19 ± 0.02	10.65 ± 1.24	55.6 ± 6.3	4.78 ± 0.40	1.12 ± 0.04
300mM	0.14 ± 0.02	6.92 ± 1.80	36.0 ± 2.2	2.95 ± 0.45	0.76 ± 0.01

Mannitol (or sorbitol) accumulation: The results from the Table 10 indicate that there was an increase in detectable mannitol in transgenic plants. Since sorbitol is sometimes found in maize tissues (Carey *et al*., 1982; Shaw *et al.,* 1984), confirmation of these results awaits a mass spectrometric analysis of the samples to see whether the results shown were for sorbitol or for mannitol. Whether mannitol or/and sorbitol, it has increased under salt stress conditions, especially under 200mM of NaCl (Table 10 and Appendix 31).

Similar to chapter II there were various literature reports that the expression of *mtlD* gene in transgenic plants conferred the accumulation of mannitol and improved drought and salt tolerance in different plant species such as potato (Rahnama *et al*., 2011); sorghum (Maheswari *et al*., 2010); wheat (Abebe *et al*., 2003); canola (Motallebi and Rahnama 2011); *Arabidopsis* (Zhifang and Loescher 2005). However, the reasons for the drought and salt tolerance of transgenic plants accumulating small amounts of mannitol are unclear. The results demonstrated that the small amount of mannitol accumulated in the transgenic plants but significant difference that in wild-type non-transgenic plants could be enhanced osmotic adjustment and resulted in improved salt tolerance in transgenic plants.

Glucose accumulation: Glucose is increased in wild-type plants under salt stress of 100-300mM NaCl, especially under 200nm NaCl. However no significant difference was found in glucose accumulation between wild-type and the transgenic plants under salt treatment (Appendix 32).

Sucrose accumulation: Similar to glucose, sucrose accumulated more in wild-type plants that were stressed under 100-300mM NaCl, especially those under 200mM NaCl. Also, transgenic plants under 200 mM of NaCl stress showed less sucrose accumulation as compared to their wild-type control, but higher sucrose accumulation under 100mM and 300 mM of NaCl (Appendix 33).

Fructose accumulation: Fructose accumulation increased in the wild-type control plants under salt stress, especially under 200mM of NaCl. Transgenic plants expressing a combination of *HVA1-mtlD* showed significantly higher fructose accumulation under salt stress as compared to their wild-type control plants, especially under 100mM of NaCl (Appendix 34).

Inositol accumulation: The wild-type inositol accumulation under no salt stress was lower than inositol accumulations in all wild-type plants subject to salt stress, but inositol accumulation in wild-type plants increased due to salt stress, especially under 200 mM of NaCl. There was no significant difference in inositol accumulation of transgenic plants expressing a combination of *HVA1-mtlD* transgenes and *mtlD* transgenic plants alone (Appendix 35). In this research, interestingly, levels of inositol in both *mtlD* and *HVA1-mtlD* transgenic plants were 1.5-2 folds than that in wild-type plants. Similarly, high level of inositol was reported in barley leaf cultivar that was tolerant to high Na^+ accumulation compared to barley salt sensitive control plants and the high amount of inositol was suggested to involve in cellular protection against salinity (Widodo *et al*., 2009).

In Chapter 1, the integration and expression of single transgene *HVA1* conferred drought and salt tolerance in transgenic maize plants. In Chapter 2, the *mtlD* transgenic maize plants showed tolerant to salt tolerance (not sufficient seeds to test for drought tolerance). From the results in this chapter, we initially conclude that the transgene stacking *HVA1-mtlD* showed greater leaf RWC, higher percentage of plants survival under drought, and higher fresh and dry biomass weight at 100mM NaCl as compared to single transgene *HVA1* and *mtlD* and the wild-type non-transgenic control plants. However the transgene stacked plants showed reduced shoot length and were more affected by high salt concentrations than those of single transgene transgenic and the wild-type control plants.

CHAPTER IV

SORGHUM TRANSCRIPTION FACTOR DREB2 TRANSGENIC MAIZE PLANTS FOR DROUGHT AND SALT TOLERANCE

4.1. LITERATURE REVIEW

In plants, stress signaling pathways lead to activation of specific drought tolerance transcription factors (TFs). TFs play a role at a higher level of the stress response gene cascade and a single TF can turn on a large number of downstream drought tolerance-related genes.

DNA microarrays used to study patterns of gene expression in response to drought tolerant genotypes show modulation of a large number of genes coding for TFs (Hayano-Kanashiro *et al*. 2009). Drought stress induced TFs are activated at the transcriptional or at the protein level by the transduced drought signal. The TFs act as master switches in triggering a network of expression of the stress response genes.

The promoters of stress response genes are known to have several types of *cis*-elements called drought responsive elements (DRE). DRE binds to the TFs of the same family or different families (Srivastav *et al*., 2010).

TFs are divided into two groups, ethylene response factors (ERFs) that are induced under biotic stress conditions, and drought response element binding (DREB) proteins that bind to promoters of genes induced under abiotic stress conditions. Expression profiles of the genes under different stress situations indicate that DREB1 genes are expressed mainly under cold stress whereas DREB2 genes are expressed under dehydration due to cell osmosis. DREB2A expression is highly inducible by high salinity and drought stress rather than cold, and the DREB2A protein requires posttranscriptional modification for its activity/stability (Qin *et al*., 2011). Overproduction of a constitutively active form of DREB2A (DREB2A-CA) protein in plants led to an increase in the expression of a number of genes responsive to water deficit stress, and enhanced drought stress tolerance in transgenic plants (Sakuma *et al*. 2006).

Similarly, Maruyama *et al*. (2009) reported that the overexpression of DREB1A and DREB2A conferred cold and/or drought tolerance in transgenic *Arabidopsis* plants. Moreover, only DREB1A overexpression was reported to enhance the accumulation of many metabolites in transgenic plants and resulted in an improved tolerance of both cold and drought stress. Whereas transgenic plants expressing DREB2A had strong capability of drought tolerance but produced less tolerance under cold stress.

Dubouzet *et al*. (2003) found that overexpression of the DREB1A/CBF3 in transgenic *Arabidopsis* plants led to the activation of many target genes, and an increase in drought and cold tolerance. Xu *et al.* (2009) also reported that the HvDREB1expression up-regulated RD29A gene and improved salt tolerance. The expression of DREB1 or OsDREB1 was reported to increase the content of osmoprotectants and different soluble sugars and confer abiotic stress tolerance in the transgenic rice and *Arabidopsis* (Ito *et al.*, 2006, Yamaguchi-Shinozaki & Shinozaki, 2006). Wang and Dong (2009) demonstrated the accumulation of ZmDBP3 protein, one of the CBF/DREB from maize, activating C-repeat/DREs in the promoter regions of genes and improving tolerant to drought and cold stress in transgenic plants.

DREB2A apparently had dual functions in modulating the expression of different sets of downstream genes under both heat and water deficit stress. Over-expression of DREB2C in plants significantly enhanced their thermotolerance (Lim *et al*., 2007). Additionally, DREB2A and DREB2C are known to interact with AREB/ABF proteins suggesting that they may function cooperatively to activate the transcription of ABA-responsive genes (Lee *et al.*, 2010).

The expression of a transcription factor, DREB2 gene from sorghum driven by a stress-inducible *Arabidopsis rd29* promoter showed better seed set in transgenic rice resulting in an increased number of pentacles relative to wild-type plants under drought stress (Bihani *et al.*, 2011). The overexpressing of rice DREB1G, rice DREB2B, or rice DREB1E was also reported in transgenic rice to confer drought tolerance (Chen et al., 2008). In this experiment, transgenic plants expressing DREB1G or DREB2B had a much higher percentage of plant survival rate after 15 days without watering, followed by 10 days re-watering as compared to wild-type control plants. The expression of DREB1E in transgenic rice plants showed mediated levels in response to drought stress displaying lower plant survival rate as compared to the DREB1G or DREB2B transgenic plants, but significantly higher than wild-type under the same water stress.

The overexpression of soybean DREB2 was demonstrated to regulate the expression of stress inducible genes and caused an increase tolerance to drought and high salinity in transgenic *Arabidopsis* (Chen *et al.*, 2007). Moreover, the transgenic tobacco expressing this soybean DREB2 gene was reported to accumulate large amount of proline in transgenic tobacco plants. This research confirmed the function of soybean DREB2 for drought and salt tolerance in plants

Transgenic wheat and barley plants expressing TaDREB2 and TaDREB3 genes showed significantly higher plant survival under drought stress as compared to their wild-type control. Also the expression of these transcription factors in transgenic wheat and barley activated various numbers of other genes that are known to protect cell damage against stress conditions (Morran *et al*., 2011). Qiu *et al*. (2007) found that the overexpression of the DREB2-type transcription factor, ZmDREB2A from maize resulted in an activation of various stress inducible genes regarding to LEA and heat shock proteins in transgenic *Arabidopsis* plants. Also the transgenic plants expressing ZmDREB2A driven by inducible or constitutive promoters showed significantly higher percentage of survival compared to wild-type plants when plants exposed to 10 days of withholding water, or 45^{0}C for 1hr.

This candidate transferred the sorghum DREB2 regulated by *Arabidopsis thaliana* drought inducible promoter, rd29 into the maize genome. Sorghum is the major food crop of sub-Saharan Africa. Like maize, sorghum is also a unique plant feedstock in that all its above ground growth components (in the form of stem sap sugar, biomass or grain starch) can be converted into biofuel. However when compared to maize, sorghum is highly tolerant to many abiotic stresses including drought, heat, and salinity. Therefore, it is hoped that the transfer of sorghum DREB2 regulated by the rd29A drought inducible promoter will result in production of a maize genotype that will resist salt and drought.

The goal and specific objectives of this research are shown below.

4.2. GOALS AND OBJECTIVES:

The goal of this research is to study the drought and tolerance of maize plants expressing the sorghum DREB2. The specific objectives of this project are listed below:

(i) Using a gene construct containing the sorghum drought tolerance transcription factor, DREB2 regulated by *Arabidopsis* rd29 drought inducible promoter in maize genetic transformation.

(ii) Genetically engineering maize plants using the above construct and a construct containing the *bar* herbicide resistance gene.

(iii) Confirming the sorghum DREB2 transgene integration via PCR analysis.

4.3. MATERIALS AND METHODS

4.3.1. Gene construct

The gene construct was provided to our laboratory under Material Transfer Agreement between MSU and the University of Pune, Pune, India.

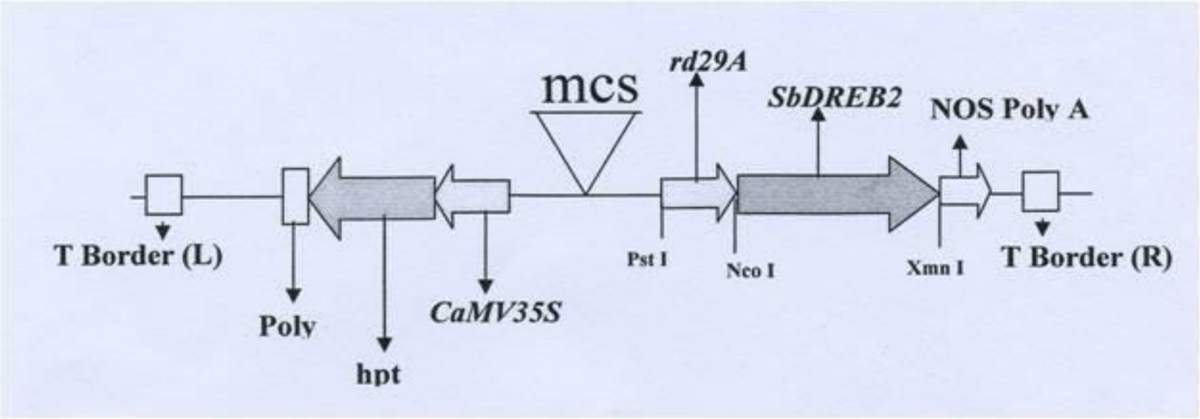


Figure 48: pCAMBIA1301 plasmid containing the sorghum transcription factor SbDREB2 regulated by Arabidopsis inducible promoter (*rd29A*), and nos terminator, cauliflower mosaic virus *35S* promoter and Hygromycin phosphotransferase (*hpt*) gene.

A second construct (pBY520; Figure 1) was chosen to be co-transferred into maize along with the pCAMBIA1301 construct containing the sorghum DREB2 gene.

Maize genetic transformation was performed using a 1:1 mixture of pC1301and pBY520 co-bombardment via the gene gun as described in Chapter 1.

4.3.2. Conformation of SbDREB2 transgene via PCR analysis

Specific sequence primers for *hpt* gene, 5`-AAAGCCTGAACTCACCGC-3` (forward primer) and 5`-GCTTTCCACTATCGGCGA-3` (reverse primer), were used to detect the *hpt* gene in T0 plant generation. DNA amplifications were performed with optimized PCR conditions 94^0C for 4 min for initial denaturation, 35 cycles of 45s at 94^0C, 30 s at 57^0C, 1min at 72^0C and a final 10 min extension at 72^0C.

4.4. RESULTS AND DISCUSSIONS

In this experiment, 48 transgenic lines were regenerated in the first regeneration (T0). Because maize also contains DREB2 gene, the candidate used the hygromycin resistance selection marker *(hpt)* gene to confirm the integration of sorghum DREB2 as the *hpt* and DREB2 cassettes were linked in the plasmid (Figure 53 below). ,

Therefore, PCR analysis was carried out to identify the integration of *hpt* gene in DREB2 maize plants. The results from the Figure 53 show that twelve out of 48 T0 putatively transgenic maize plants were transgenic.

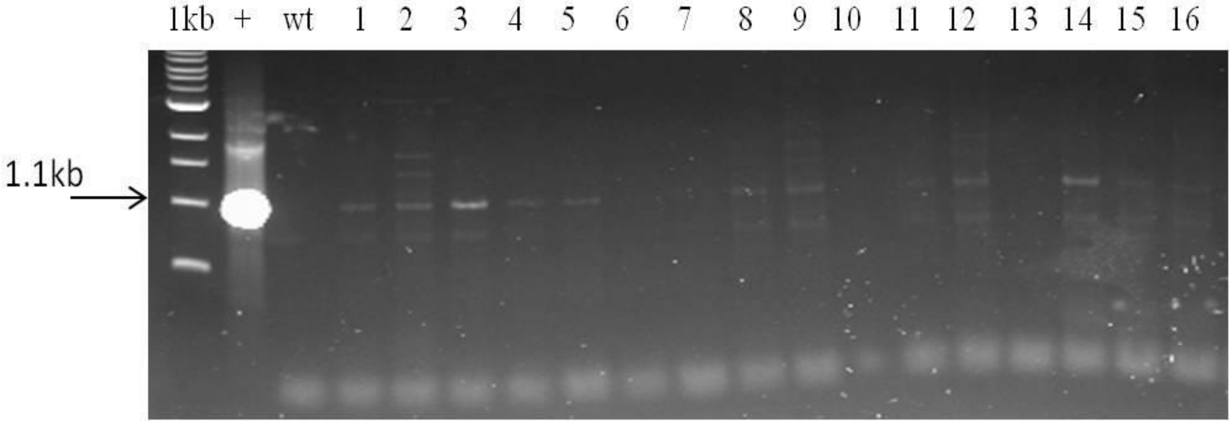


Figure 49*:* PCR analysis confirming the integration of *hpt* gene in the first generation (T0) transgenes plants.

The initial result of the PCR analysis confirms the integration of *hpt* gene T0 generation. This candidate assumes the co-existence of two transgene cassettes linked together on one plasmid. A similar work was in reported in the various literatures for stable integration of two transgenes at 100% frequency (Maqbool *et al*., 2002; Oraby *et al.*, 2005; Kingdom *et al*., 2012).

More research on molecular and physiological analysis of progenies of these DREB2 transgenic plants are needed to be completed by another researcher in our laboratory.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

The Biolistic bombardment method used in these studies to transfer single abiotic stress tolerance transgenes and their combination into maize immature embryo cell lines followed by regeneration of mature transgenic plants is a relatively fast technique for maize transformation (Frame *et al.*, 2000; Aulinger *et al*., 2003, Sticklen and Oraby, 2005). However the Hi II (i.e. a hybrid between A188 and B73) maize chosen in these studies was not easy to work with because the hybrid segregates and the progenies vary; but there is no maize inbred line that could be easily transformed. The candidate concludes that the Hi II maize chosen was an ideal choice because unlike all inbred lines, it was relatively easy to genetically transform, and because it is non-proprietary maize.

Studies have shown that the use of gene bombardment for maize genetic transformation can result in multiple integrated copies of a transgene in plants, and multiple copies of a transgene could result in silencing of the transgene (Pawlowski *et al.,* 1996; Kohli *et al*., 1998; Dai *et al*., 2001). However, this candidate selected transgenic plants that contained no more than 2-3 copies of each transgenes. Furthermore, Northern blotting and the response to salt and drought tolerance of transgenic plants proved that transgenes were not silenced. Therefore, here it is concluded that the use of the gene gun for genetic transformation of maize was a good choice, because in maize, this method is far more efficient as compared to the *Agrobacterium* method of genetic transformation of maize.

Maize genetic transformation studies presented in this dissertation demonstrated that under greenhouse conditions, transgenic maize plants expressing the *HVA1*, *mtlD*, or a combination of *HVA1-mtlD* transgenes showed improved drought and salt tolerance as compared to their wild-type non-transgenic plant counterparts. Witnessing the drought of 2012 and that of

2008 in the Corn Belt, corn growers do need a quick technology to protect their crop from the unpredicted drought.

The barley *HVA1* gene alone in maize genome conferred drought and salt tolerance in T3 plants, agreeing with other researchers who tested the same gene in few other transgenic crops (Xu *et al.,* 1996; Detvisitsakun *et al*., 2001; Babu *et al*., 2004; Fu *et al*., 2007). As the result of their tolerance to these two abiotic stresses, the *HVA1* transgenic lines contained a higher leaf relative water content as compared to their control non-transgenic plants, due to possible certain protein accumulation in transgenic plants protecting of plant cell membranes (Babu *et al.,* 2004, Lal *et al.,* 2008, Checker *et al*., 2012).

It is important to note that the maize explants used for genetic engineering is a hybrid (A188 x B73). This hybrid was used in this dissertation research because maize genetic engineering is very genotype-specific, and this hybrid it highly regenerable and transformable. Although the use of Hi II maize has the benefit of its transformability, it has a major down side, i.e. its selfed individuals are heterogeneous populations of selfed lines that have been derived from the Hi II hybrid.

The preliminary results from Figure 36 of the second chapter of this dissertation and Table 10 of the third chapter (*HVA1-mtlD* transgenic plants) of this dissertation indicate that there was an increase in detectable mannitol in transgenic plants. Mannitol has increased significantly in transgenic plants under 100mM and 200mM of NaCl stress conditions as compared to the wild-type control plants. Under no-salt stress condition (0mM), mannitol also accumulated significantly higher in transgenic plants as compared to the wild-type non-transgenic plants. The mannitol accumulation in both *mtlD* and the *HVA-mtlD* transgenic plants were similar because *HVA1* should not contribute towards mannitol accumulation. The only discrepancy in the mannitol accumulation results is the fact that mannitol also accumulated in wild-type control plants, even thought maize plants are not thought to contain mannitol. As per Prof. Wayne Loescher (MSU Professor of Horticulture Department), sorbitol accumulation may have been a factor as gas chromatograph does not easily distinguish mannitol from sorbitol. Studies are in progress in Dr. Loescher's laboratory to see the possible contributions of sorbitol in these studies.

The bacterial *mtlD* gene in maize genome conferred salt tolerance in T3 plants, resulting in accumulation of a small amount of mannitol (and/or sorbitol) in transgenic plants. The small amount of mannitol (and/or sorbitol) may have been the reason for the enhancement in osmotic adjustment and salt tolerance in transgenic plants (Hu *et al.*, 2005, Motallebi and Rahnama 2011). The combination of the two transgenes (*HVA1-mtlD*) improved drought and salt tolerance as compared to plants that were expressing only one transgene. However this increase was not accumulative or doubled, probably because plant cells have a limit in their accumulative osmoprotectants. Similarly, a high level of inositol was shown in barley leaf cultivar that was tolerant to high Na^+ accumulation as compared to barley salt sensitive control plants, suggesting that inositol is involved in cellular protection against salinity (Widodo *et al.*, 2009).

The results showed that mannitol, sucrose, fructose and inositol increased significantly in transgenic plants under salt stress conditions especially at 100mM NaCl as compared to wild-type control plants. In contrary, glucose was not different in plants under salt stress.

This candidate concludes that the method of "co-transformation" of the two abiotic stress tolerance transgenes (*HVA1-mtlD)* used was an ideal choice. The method used is ideal because it is a less time consuming and less labor intensive method as compared to the "re-transformation" method, especially that the method used resulted in co-integration of both transgenes in crop progenies, similar to the work reported by Drouglas and Halpin (2010).

Overall, the research results presented here met its promised goals and objectives of being able to transform maize plants with two single and a combination of the two abiotic stress tolerance transgenes resulting in an increase in stability of transgenes integration and expressions in up to fourth progenies, and conferring an increase in fresh and dry vegetative biomass as results of the salt and/or drought tolerance of the transgenic plants.

The candidate also developped transgenic maize plants that show the integration of the sorghum DREB2 transgene regulated by the *Arabidopsis thaiana rd29A* drought inducible promoter. This work will be completed by another researcher in our laboratory.

More studies are needed on progenies of the *HVA1*, *mtlD*, *HVA1-mtlD*, and DREB2 transgenic maize plants. The contradicting studies of mannitol versus sorbitol in these studies needs to be further studied by Sticklen's team in the near future.

APPENDICES

APPENDIX 1: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF WATER STRESS ON LEAF RELATIVE WATER CONTENT (%) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Type I SS	Mean Square	F Value	Pr > F
day	1	1784.86	1784.86	646.33	<.0001
ge	1	812.6302	812.6302	294.27	<.0001
tr	1	4448.675	4448.675	1610.94	<.0001
Day*ge*tr	4	2712.983	678.2456	245.6	<.0001

R-Square	Coeff Var	Root MSE	Mean
0.988808	1.942808	1.661789	85.53542

Day	Genotype	Treatment	LSMEAN	LSMEAN Number
10day	*HVA1*	Control	95.4	1
10day	*HVA1*	stress	94.9	2
10day	WT	Control	94.6	3
10day	WT	stress	81.7	4
15day	*HVA1*	Control	95.2	5
15day	*HVA1*	stress	73.2	6
15day	WT	Control	95.5	7
15day	WT	stress	53.9	8

LS-means with the same letter are not significantly different.

	LSMEAN	Day	genotype	treatment	LSMEAN Number
A	95.5	15day	WT	Control	7
A	95.4	10day	*HVA1*	Control	1
A	95.2	15day	*HVA1*	Control	5
A	94.9	10day	*HVA1*	stress	2
A	94.6	10day	WT	Control	3
B	81.7	10day	WT	stress	4
C	73.2	15day	*HVA1*	stress	6
D	53.9	15day	WT	stress	8

APPENDIX 2: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT LENGTH (CM) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1335.368	267.0736	41.72	<.0001
Error	30	192.0417	6.401389		
Corrected Total	35	1527.41			

R-Square	Coeff Var	Root MSE	Mean
0.87427	11.42113	2.530097	22.15278

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	31.3	1
HVA1	100mM	23.3	2
HVA1	200mM	20.5	3
WT	0mM	26.5	4
WT	100mM	19.5	5
WT	200mM	11.8	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	31.3	*HVA1*	0mM	1
B	26.5	WT	0mM	4
C	23.3	*HVA1*	100mM	2
CD	20.5	*HVA1*	200mM	3
D	19.5	WT	100mM	5
E	11.8	WT	200mM	6

APPENDIX 3: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON ROOT LENGTH (CM) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1251.917	250.3833	14.5	<.0001
Error	30	518.0833	17.26944		
Corrected Total	35	1770			

R-Square	Coeff Var	Root MSE	Mean
0.707298	14.08696	4.155652	29.5

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	36.7	1
HVA1	100mM	29.3	2
HVA1	200mM	29.5	3
WT	0mM	34.9	4
WT	100mM	28.3	5
WT	200mM	18.3	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	36.7	*HVA1*	0mM	1
A	34.9	WT	0mM	4
B	29.5	*HVA1*	200mM	3
B	29.3	*HVA1*	100mM	2
B	28.3	WT	100mM	5
C	18.3	WT	200mM	6

APPENDIX 4: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT FRESH WEIGHT (GR) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	14.89774	2.979548	33.57	<.0001
Error	30	2.662934	0.088764		
Corrected Total	35	17.56067			

R-Square	Coeff Var	Root MSE	Mean
0.848358	18.72231	0.297934	1.59133

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	2.3	1
HVA1	100mM	2.0	2
HVA1	200mM	1.4	3
WT	0mM	2.2	4
WT	100mM	1.3	5
WT	200mM	0.4	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	2.3	*HVA1*	0mM	1
AB	2.2	WT	0mM	4
B	2.0	*HVA1*	100mM	2
C	1.4	*HVA1*	200mM	3
C	1.3	WT	100mM	5
D	0.4	WT	200mM	6

APPENDIX 5: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT DRY WEIGHT (GR) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.107536	0.021507	9	<.0001
Error	30	0.071716	0.002391		
Corrected Total	35	0.179252			

R-Square	Coeff Var	Root MSE	Mean
0.599914	31.42871	0.048893	0.155569

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	0.218	1
HVA1	100mM	0.169	2
HVA1	200mM	0.154	3
WT	0mM	0.214	4
WT	100mM	0.118	5
WT	200mM	0.061	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	0.218	*HVA1*	0mM	1
A	0.214	WT	0mM	4
AB	0.169	*HVA1*	100mM	2
B	0.154	*HVA1*	200mM	3
BC	0.118	WT	100mM	5
C	0.061	WT	200mM	6

APPENDIX 6: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON ROOT FRESH WEIGHT (GR) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	12.85837	2.571674	34.63	<.0001
Error	30	2.227612	0.074254		
Corrected Total	35	15.08598			

R-Square	Coeff Var	Root MSE	Mean
0.852339	20.58165	0.272495	1.323972

genotype	treatment	LSMEAN	LSMEAN Number
HVA1	0mM	2.114	1
HVA1	100mM	1.691	2
HVA1	200mM	1.077	3
WT	0mM	1.759	4
WT	100mM	0.990	5
WT	200mM	0.313	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	2.114	HVA1	0mM	1
B	1.759	WT	0mM	4
B	1.691	HVA1	100mM	2
C	1.077	HVA1	200mM	3
C	0.990	WT	100mM	5
D	0.313	WT	200mM	6

APPENDIX 7: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON ROOT DRY WEIGHT (GR) OF *HVA1* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.059751	0.01195	53.73	<.0001
Error	30	0.006672	0.000222		
Corrected Total	35	0.066423			

R-Square	Coeff Var	Root MSE	Mean
0.899554	19.97061	0.014913	0.074675

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	0.131533	1
HVA1	100mM	0.0724	2
HVA1	200mM	0.048917	3
WT	0mM	0.124033	4
WT	100mM	0.052383	5
WT	200mM	0.018783	6

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	0.13153	*HVA1*	0mM	1
A	0.12403	WT	0mM	4
B	0.07240	*HVA1*	100mM	2
C	0.05238	WT	100mM	5
C	0.04892	*HVA1*	200mM	3
D	0.01878	WT	200mM	6

APPENDIX 8: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT LENGTH REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1180.832	393.6106	17.86	0.0088
Error	4	88.1657	22.04143		
Corrected total	7	1268.997			

R-Square	Coeff Var	Root MSE	Mean
0.988808	1.942808	1.661789	85.53542

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Ge	1	242.8808000	242.8808000	11.02	0.0294
Tr	1	735.7448000	735.7448000	33.38	0.0045
Ge*Tr	1	202.2060500	202.2060500	9.17	0.0388

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	55.7	WT	200mM	4
B	34.6	HVA1	200mM	2
B	26.4	WT	100mM	3
B	25.5	HVA1	100mM	1

APPENDIX 9: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON ROOT LENGTH REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1200.44	400.1465	26.27	0.0043
Error	4	60.93145	15.23286		
Corrected total	7	1261.371			

R-Square	Coeff Var	Root MSE	Mean
0.951694	14.71485	3.90293	26.52375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	366.7986	366.7986	24.08	0.008
tr	1	405.1281	405.1281	26.6	0.0067
ge*tr	1	428.5128	428.5128	28.13	0.0061

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	47.7	WT	200mM	4
B	20.0	HVA1	100mM	1
B	19.6	HVA1	200mM	2
B	18.9	WT	100mM	3

APPENDIX 10: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT FRESH WEIGHT REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	4273.612	1424.537	12.19	0.0176
Error	4	467.541	116.8853		
Corrected total	7	4741.153			

R-Square	Coeff Var	Root MSE	Mean
0.901387	24.6048	10.81135	43.94

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	2235.13	2235.13	19.12	0.0119
tr	1	1935.664	1935.664	16.56	0.0152
ge*tr	1	102.8178	102.8178	0.88	0.4014

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	79.8	WT	200mM	4
B	41.5	WT	100mM	3
B	39.2	HVA1	200mM	2
B	15.3	HVA1	100mM	1

APPENDIX 11: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT DRY WEIGHT REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2865.522	955.1742	114.43	0.0002
Error	4	33.3877	8.346925		
Corrected total	7	2898.91			

R-Square	Coeff Var	Root MSE	Mean
0.988483	6.867783	2.889105	42.0675

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	2098.224	2098.224	251.38	<.0001
tr	1	570.8821	570.8821	68.39	0.0012
ge*tr	1	196.4162	196.4162	23.53	0.0083

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	71.7	WT	200mM	4
B	44.9	WT	100mM	3
C	29.4	HVA1	200mM	2
C	22.4	HVA1	100mM	1

APPENDIX 12: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON ROOT FRESH WEIGHT REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3939.993	1313.331	3444.8	<.0001
Error	4	1.525	0.38125		
Corrected total	7	3941.518			

R-Square	Coeff Var	Root MSE	Mean
0.999613	1.266573	0.617454	48.75

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	1615.393	1615.393	4237.1	<.0001
tr	1	2279.475	2279.475	5978.95	<.0001
ge*tr	1	45.125	45.125	118.36	0.0004

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	82.2	WT	200mM	4
B	49.0	HVA1	200mM	2
C	43.7	WT	100mM	3
D	20.0	HVA1	100mM	1

APPENDIX 13: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON ROOT DRY WEIGHT REDUCTION (%) OF T3 *HVA1* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1661.67	553.8901	86.52	0.0004
Error	4	25.60785	6.401963		
Corrected total	7	1687.278			

R-Square	Coeff Var	Root MSE	Mean
0.984823	4.041627	2.53021	62.60375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	611.6253	611.6253	95.54	0.0006
tr	1	1008.23	1008.23	157.49	0.0002
ge*tr	1	41.81551	41.81551	6.53	0.0629

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	84.9	WT	200mM	4
B	62.8	HVA1	200mM	2
B	57.8	WT	100mM	3
C	44.9	HVA1	100mM	1

APPENDIX 14: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON PLANT HEIGHT GROWTH RATE (CM/DAY) OF *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	15.47793	2.211132	12.34	<.0001
Error	48	8.602657	0.179222		
Corrected Total	55	24.08058			

R-Square	Coeff Var	Root MSE	Mean
0.642755	22.84609	0.423346	1.853036

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	2.6	*mtlD*	0mM	5
A	2.6	WT	0mM	1
B	2.0	*mtlD*	100mM	6
BC	2.0	WT	100mM	2
DC	1.6	*mtlD*	200mM	7
D	1.5	WT	200mM	3
D	1.4	*mtlD*	300mM	8
D	1.1	WT	300mM	4

APPENDIX 15: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT FRESH WEIGHT (GR) OF *MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1515.671	216.5245	98.67	<.0001
Error	40	87.7801	2.194503		
Corrected Total	47	1603.451			

R-Square	Coeff Var	Root MSE	Mean
0.945256	11.38666	1.481385	13.00983

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	21.460	WT	0mM	1
A	21.357	*mtlD*	0mM	5
B	15.488	*mtlD*	100mM	6
C	11.658	WT	100mM	2
C	11.364	*mtlD*	200mM	7
C	10.327	*mtlD*	300mM	8
D	7.004	WT	200mM	3
D	5.421	WT	300mM	4

APPENDIX 16: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT DRY WEIGHT (GR) OF *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	8.998253	1.285465	67.66	<.0001
Error	40	0.759958	0.018999		
Corrected Total	47	9.75821			

R-Square	Coeff Var	Root MSE	Mean
0.922121	10.45091	0.137837	1.318896

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
WT	0mM	2.006	1
WT	100mM	1.197	2
WT	200mM	0.782	3
WT	300mM	0.756	4
mtlD	0mM	1.878	5
mtlD	100mM	1.551	6
mtlD	200mM	1.251	7
mtlD	300mM	1.130	8

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	2.006	WT	0mM	1
A	1.878	*mtlD*	0mM	5
B	1.551	*mtlD*	100mM	6
C	1.251	*mtlD*	200mM	7
C	1.197	WT	100mM	2
C	1.130	*mtlD*	300mM	8
D	0.782	WT	200mM	3
D	0.756	WT	300mM	4

APPENDIX 17: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON ROOT DRY WEIGHT (GR) OF *MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.245573	0.035082	13.34	<.0001
Error	40	0.105184	0.00263		
Corrected Total	47	0.350756			

R-Square	Coeff Var	Root MSE	Mean
0.700123	13.01168	0.05128	0.394104

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
WT	0mM	0.488	1
WT	100mM	0.395	2
WT	200mM	0.307	3
WT	300mM	0.274	4
mtlD	0mM	0.472	5
mtlD	100mM	0.445	6
mtlD	200mM	0.412	7
mtlD	300mM	0.361	8

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	0.488	WT	0mM	1
A	0.472	*mtlD*	0mM	5
AB	0.445	*mtlD*	100mM	6
BC	0.412	*mtlD*	200mM	7
BC	0.395	WT	100mM	2
CD	0.361	*mtlD*	300mM	8
DE	0.307	WT	200mM	3
E	0.274	WT	300mM	4

APPENDIX 18: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT FRESH WEIGHT REDUCTION (%) OF T3 *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2840.657	568.1313	108.42	<.0001
Error	6	31.44	5.24		
Corrected total	11	2872.097			

R-Square	Coeff Var	Root MSE	Mean
0.989053	4.378268	2.289105	52.28333

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	74.7	WT	300mM	6
B	67.4	WT	200mM	5
C	51.7	*mtlD*	300mM	3
CD	46.8	*mtlD*	200mM	2
D	45.7	WT	100mM	4
E	27.5	*mtlD*	100mM	1

APPENDIX 19: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT DRY WEIGHT REDUCTION (%) OF T3 *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2923.388	584.6775	425.99	<.0001
Error	6	8.235	1.3725		
Corrected total	11	2931.623			

R-Square	Coeff Var	Root MSE	Mean
0.997191	2.76469	1.171537	42.375

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	1	1783.641	1783.641	1299.56	<.0001
tr	2	1123.145	561.5725	409.16	<.0001
ge*tr	2	16.60167	8.300833	6.05	0.0365

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	62.3	WT	300mM	3
A	61.1	WT	200mM	2
B	40.4	WT	100mM	1
B	39.8	*mtlD*	300mM	6
C	33.4	*mtlD*	200mM	5
D	17.4	*mtlD*	100mM	4

APPENDIX 20: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON ROOT DRY WEIGHT REDUCTION (%) OF T3 *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2070.29	414.058	44.16	0.0001
Error	6	56.26	9.376667		
Corrected total	11	2126.55			

R-Square	Coeff Var	Root MSE	Mean
0.973544	12.94771	3.062134	23.65

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	43.8	WT	300mM	3
A	36.8	WT	200mM	2
B	23.7	*mtlD*	300mM	6
BC	19.1	WT	100mM	1
CD	12.9	*mtlD*	200mM	5
D	5.8	*mtlD*	100mM	4

APPENDIX 21: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON MANNITOL ACCUMULATION (μMOL/GR FW) OF *MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.182546	0.026078	9.09	0.0029
Error	8	0.02295	0.002869		
Corrected Total	15	0.205495			

R-Square	Coeff Var	Root MSE	Mean
0.888321	23.73199	0.05356	0.225688

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
WT	0mM	0.116	1
WT	100mM	0.121	2
WT	200mM	0.159	3
WT	300mM	0.135	4
mtlD	0mM	0.271	5
mtlD	100mM	0.432	6
mtlD	200mM	0.329	7
mtlD	300mM	0.244	8

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	0.432	*mtlD*	100mM	6
AB	0.329	*mtlD*	200mM	7
BC	0.271	*mtlD*	0mM	5
BCD	0.244	*mtlD*	300mM	8
CDE	0.159	WT	200mM	3
DE	0.135	WT	300mM	4
DE	0.121	WT	100mM	2
E	0.116	WT	0mM	1

APPENDIX 22: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF WATER STRESS ON LEAF RELATIVE WATER CONTENTS (%) OF *HVA1, MTLD* AND *HAV1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	1	542.5888	542.5888	105.74	<.0001
treatment	1	1161.226	1161.226	226.29	<.0001
genotype	3	555.383	185.1277	36.08	<.0001
time*treatme*genotyp	10	1041.022	104.1022	20.29	<.0001

Day	Treatment	Genotype	LSMEAN	LSMEAN Number
10day	control	*HVA1*	95.6	1
10day	control	*HVA1/mtlD*	95.9	2
10day	control	WT	94.9	3
10day	control	*mtlD*	95.4	4
10day	stress	*HVA1*	94.5	5
10day	stress	*HVA1/mtlD*	94.3	6
10day	stress	WT	81.9	7
10day	stress	*mtlD*	92.7	8
15day	control	*HVA1*	94.1	9
15day	control	*HVA1/mtlD*	95.1	10
15day	control	WT	94.0	11
15day	control	*mtlD*	95.6	12
15day	stress	*HVA1*	81.0	13
15day	stress	*HVA1/mtlD*	85.0	14
15day	stress	WT	57.1	15
15day	stress	*mtlD*	77.6	16

APPENDIX 22 (Cont'd):

LS-means with the same letter are not significantly different.

	LSMEAN	Day	Treatment	Genotype	LSMEAN Number
A	95.9	10day	control	*HVA1/mtlD*	2
A	95.6	10day	control	*HVA1*	1
A	95.6	15day	control	*mtlD*	12
A	95.4	10day	control	*mtlD*	4
A	95.1	15day	control	*HVA1/mtlD*	10
A	94.9	10day	control	WT	3
A	94.5	10day	stress	*HVA1*	5
A	94.3	10day	stress	*HVA1/mtlD*	6
A	94.1	15day	control	*HVA1*	9
A	94.0	15day	control	WT	11
A	92.7	10day	stress	*mtlD*	8
B	85.0	15day	stress	*HVA1/mtlD*	14
BC	81.9	10day	stress	WT	7
BC	81.0	15day	stress	*HVA1*	13
C	77.6	15day	stress	*mtlD*	16
D	57.1	15day	stress	WT	15

APPENDIX 23: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT LENGTH (CM) OF *HVA1*, *MTLD* AND *HAV1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	3631.581	242.1054	39.54	<.0001
Error	80	489.7917	6.122396		
Corrected Total	95	4121.372			

R-Square	Coeff Var	Root MSE	Mean
0.881158	5.365045	2.474348	46.11979

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	52.3	1
HVA1	100mM	48.3	2
HVA1	200mM	43.4	3
HVA1	300mM	39.8	4
HVA1/mtlD	0mM	56.7	5
HVA1/mtlD	100mM	50.8	6
HVA1/mtlD	200mM	46.7	7
HVA1/mtlD	300mM	39.8	8
WT	0mM	51.3	9
WT	100mM	43.4	10
WT	200mM	39.7	11
WT	300mM	31.9	12
mtlD	0mM	53.3	13
mtlD	100mM	49.0	14
mtlD	200mM	47.3	15
mtlD	300mM	44.3	16

APPENDIX 24: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT FRESH WEIGHT (GR) OF *HVA1, MTLD* AND *HAV1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	3956.901	263.7934	81.35	<.0001
Error	80	259.4063	3.242578		
Corrected Total	95	4216.307			

R-Square	Coeff Var	Root MSE	Mean
0.938475	12.84632	1.800716	14.01736

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	23.90	1
HVA1	100mM	16.22	2
HVA1	200mM	13.56	3
HVA1	300mM	8.02	4
HVA1/mtlD	0mM	23.00	5
HVA1/mtlD	100mM	20.02	6
HVA1/mtlD	200mM	11.43	7
HVA1/mtlD	300mM	7.44	8
WT	0mM	18.59	9
WT	100mM	10.12	10
WT	200mM	5.57	11
WT	300mM	4.40	12
mtlD	0mM	24.10	13
mtlD	100mM	17.86	14
mtlD	200mM	11.74	15
mtlD	300mM	8.30	16

APPENDIX 25: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SHOOT DRY WEIGHT (GR) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	29.55438	1.970292	27.86	<.0001
Error	80	5.65773	0.070722		
Corrected Total	95	35.21211			

R-Square	Coeff Var	Root MSE	Mean
0.839324	18.49436	0.265935	1.437927

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	1.923	1
HVA1	100mM	1.639	2
HVA1	200mM	0.977	3
HVA1	300mM	0.798	4
HVA1/mtlD	0mM	2.472	5
HVA1/mtlD	100mM	2.017	6
HVA1/mtlD	200mM	1.432	7
HVA1/mtlD	300mM	1.121	8
WT	0mM	1.775	9
WT	100mM	1.060	10
WT	200mM	0.763	11
WT	300mM	0.473	12
mtlD	0mM	2.290	13
mtlD	100mM	1.672	14
mtlD	200mM	1.471	15
mtlD	300mM	1.125	16

APPENDIX 26: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON ROOT DRY WEIGHT (GR) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	2.158422	0.143895	10.19	<.0001
Error	80	1.129157	0.014114		
Corrected Total	95	3.287579			

R-Square	Coeff Var	Root MSE	Mean
0.656539	22.40445	0.118804	0.530271

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1	0mM	0.888	1
HVA1	100mM	0.665	2
HVA1	200mM	0.665	3
HVA1	300mM	0.504	4
HVA1/mtlD	0mM	0.708	5
HVA1/mtlD	100mM	0.556	6
HVA1/mtlD	200mM	0.576	7
HVA1/mtlD	300mM	0.462	8
WT	0mM	0.596	9
WT	100mM	0.416	10
WT	200mM	0.331	11
WT	300mM	0.287	12
mtlD	0mM	0.572	13
mtlD	100mM	0.447	14
mtlD	200mM	0.412	15
mtlD	300mM	0.399	16

APPENDIX 27: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT LENGTH REDUCTION (%) OF *HVA1, MTLD, HVA1-MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1811.129	164.6481	101.51	<.0001
Error	12	19.46375	1.621979		
Corrected total	23	1830.593			

R-Square	Coeff Var	Root MSE	Mean
0.989368	7.013209	1.273569	18.15958

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	3	554.6645	184.8882	113.99	<.0001
tr	2	1134.543	567.2715	349.74	<.0001
ge*tr	6	121.9214	20.32023	12.53	0.0001

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	37.7	WT	300mM	9
B	29.7	HVA1/*mtlD*	300mM	6
C	24.0	HVA1	300mM	3
C	22.6	WT	200mM	8
D	17.6	HVA1/*mtlD*	200mM	5
D	17.0	HVA1	200mM	2
D	16.7	*mtlD*	300mM	12
D	15.3	WT	100mM	7
E	11.3	*mtlD*	200mM	11
EF	10.3	HVA1/*mtlD*	100mM	4
F	8.0	*mtlD*	100mM	10
F	7.6	HVA1	100mM	1

APPENDIX 28: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT FRESH WEIGHT REDUCTION (%) OF *HVA1, MTLD, HVA1-MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	8504.324	773.1203	613.78	<.0001
Error	12	15.1153	1.259608		
Corrected total	23	8519.439			

R-Square	Coeff Var	Root MSE	Mean
0.998226	2.217372	1.122323	50.615

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	3	1485.574	495.1913	393.13	<.0001
tr	2	6468.952	3234.476	2567.84	<.0001
ge*tr	6	549.7974	91.63291	72.75	<.0001

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	76.3	WT	300mM	9
B	70.0	WT	200mM	8
BC	67.7	HVA1/*mtlD*	300mM	6
C	66.4	HVA1	300mM	3
C	65.6	*mtlD*	300mM	12
D	51.3	*mtlD*	200mM	11
D	50.3	HVA1/*mtlD*	200mM	5
E	45.6	WT	100mM	7
E	43.3	HVA1	200mM	2
F	32.1	HVA1	100mM	1
G	25.9	*mtlD*	100mM	10
H	12.97	HVA1/*mtlD*	100mM	4

APPENDIX 29: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON SHOOT DRY WEIGHT REDUCTION (%) OF *HVA1, MTLD, HVA1-MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	6620.224	601.8386	134.73	<.0001
Error	12	53.60215	4.466846		
Corrected total	23	6673.826			

R-Square	Coeff Var	Root MSE	Mean
0.991968	4.862187	2.113491	43.46792

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ge	3	1459.617	486.5388	108.92	<.0001
tr	2	4768.923	2384.462	533.81	<.0001
ge*tr	6	391.6841	65.28069	14.61	<.0001

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	73.3	WT	300mM	9
B	58.5	HVA1	300mM	3
B	57.0	WT	200mM	8
BC	54.7	HVA1/*mtlD*	300mM	6
DC	50.9	*mtlD*	300mM	12
D	49.2	HVA1	200mM	2
E	42.1	HVA1/*mtlD*	200mM	5
EF	40.3	WT	100mM	7
F	35.7	*mtlD*	200mM	11
G	27.0	*mtlD*	100mM	10
H	18.4	HVA1/*mtlD*	100mM	4
H	14.8	HVA1	100mM	1

APPENDIX 30: ANOVA, LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF SALT TREATMENT ON ROOT DRY WEIGHT REDUCTION (%) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC AND WILD-TYPE PLANTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	2375.953	215.9957	24.65	<.0001
Error	12	105.1521	8.762675		
Corrected total	23	2481.105			

R-Square	Coeff Var	Root MSE	Mean
0.957619	9.490296	2.960182	31.19167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
genotype	3	1085.153	361.7176	41.28	<.0001
treatment	2	999.4293	499.7146	57.03	<.0001
genotype*treatment	6	291.371	48.56184	5.54	0.0058

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment	LSMEAN Number
A	51.9	WT	300mM	9
B	44.4	WT	200mM	8
B	43.2	HVA1	300mM	3
C	34.6	HVA1/*mtlD*	300mM	6
CD	30.2	WT	100mM	7
CD	30.1	*mtlD*	300mM	12
ED	27.9	*mtlD*	200mM	11
DEF	25.1	HVA1	200mM	2
FDGE	25.0	HVA1	100mM	1
FGE	21.9	*mtlD*	100mM	10
FG	21.4	HVA1/*mtlD*	100mM	4
G	18.6	HVA1/*mtlD*	200mM	5

APPENDIX 31: LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON MANNITOL CONTENTS (µMOL/GR FW) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1/mtlD	0mM	0.33	1
HVA1/mtlD	100mM	0.37	2
HVA1/mtlD	200mM	0.52	3
HVA1/mtlD	300mM	0.55	4
WT	0mM	0.18	5
WT	100mM	0.21	6
WT	200mM	0.19	7
WT	300mM	0.14	8
mtlD	0mM	0.26	9
mtlD	100mM	0.43	10
mtlD	200mM	0.56	11
mtlD	300mM	0.35	12

LS-means with the same letter are not significantly different.

	LSMEAN	Genotype	Treatment	LSMEAN Number
A	0.56	*mtlD*	200mM	11
AB	0.55	*HVA1-mtlD*	300mM	4
ABC	0.52	*HVA1-mtlD*	200mM	3
ABCD	0.43	*mtlD*	100mM	10
ABCD	0.37	*HVA1-mtlD*	100mM	2
ABCD	0.35	*mtlD*	300mM	12
ABCD	0.33	*HVA1-mtlD*	0mM	1
ABCD	0.26	*mtlD*	0mM	9
BCD	0.21	WT	100mM	6
CD	0.19	WT	200mM	7
CD	0.18	WT	0mM	5
D	0.14	WT	300mM	8

APPENDIX 32: LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON GLUCOSE CONTENT (µMOL/GR FW) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1/*mtlD*	0mM	7.55	1
HVA1/*mtlD*	100mM	9.04	2
HVA1/*mtlD*	200mM	9.41	3
HVA1/*mtlD*	300mM	7.20	4
WT	0mM	3.85	5
WT	100mM	9.19	6
WT	200mM	10.65	7
WT	300mM	6.92	8
mtlD	0mM	5.66	9
mtlD	100mM	8.54	10
mtlD	200mM	5.52	11
mtlD	300mM	7.11	12

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment
A	10.65	WT	200mM
A	9.41	*HVA1/mtlD*	200mM
A	9.19	WT	100mM
A	9.04	*HVA1/mtlD*	100mM
AB	8.54	*mtlD*	100mM
AB	7.55	*HVA1/mtlD*	0mM
AB	7.20	*HVA1/mtlD*	300mm
AB	7.11	*mtlD*	300mM
AB	6.92	WT	300mM
AB	5.66	*mtlD*	0mM
AB	5.52	*/mtlD*	200mM
B	3.85	WT	0mM

APPENDIX 33: LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON SUCROSE CONTENTS (µMOL/GR FW) OF *HVA1*, *MTLD*, *HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1/mtlD	0mM	35.51	1
HVA1/mtlD	100mM	50.87	2
HVA1/mtlD	200mM	45.26	3
HVA1/mtlD	300mM	48.13	4
WT	0mM	30.31	5
WT	100mM	40.97	6
WT	200mM	55.62	7
WT	300mM	36.02	8
mtlD	0mM	33.30	9
mtlD	100mm	45.21	10
mtlD	200mm	42.80	11
mtlD	300mM	49.67	12

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment
A	55.62	WT	200mM
AB	50.87	*HVA1/mtlD*	100mM
ABC	49.67	*mtlD*	300mM
ABC	48.13	*HVA1/mtlD*	300mM
ABCD	45.26	*HVA1/mtlD*	200mM
ABCD	45.21	*mtlD*	100mM
ABCD	42.80	*mtlD*	200mM
ABCD	40.97	WT	100mM
BCD	36.02	WT	300mM
BCD	35.51	*HVA1/mtlD*	0mM
CD	33.30	*mtlD*	0mM
D	30.31	WT	0mM

APPENDIX 34: LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON FRUCTOSE CONTENTS (µMOL/GR FW) OF *HVA1, MTLD, HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1/mtlD	0mM	4.70	1
HVA1/mtlD	100mM	8.68	2
HVA1/mtlD	200mM	7.56	3
HVA1/mtlD	300mM	6.31	4
WT	0mM	1.83	5
WT	100mM	2.74	6
WT	200mM	4.78	7
WT	300mM	2.95	8
mtlD	0mM	4.15	9
mtlD	100mM	2.49	10
mtlD	200mM	4.34	11
mtlD	300mM	5.94	12

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment
A	8.68	*HVA1/mtlD*	100mM
AB	7.56	*HVA1/mtlD*	200mM
ABC	6.31	*HVA1/mtlD*	300mM
ABC	5.94	*mtlD*	300mM
BCD	4.78	WT	200mM
BCD	4.70	*HVA1/mtlD*	0mM
CD	4.34	*mtlD*	200mM
CD	4.15	*mtlD*	0mM
D	2.95	WT	300mM
D	2.74	WT	100mM
D	2.49	*mtlD*	100mM
D	1.83	WT	0mM

APPENDIX 35: LEAST SQUARES MEAN AND T COMPARISON FOR THE EFFECT OF DIFFERENT SALINITY CONCENTRATIONS ON INOSITOL CONTENTS (μMOL/GR FW) OF *HVA1, MTLD, HVA1-MTLD* TRANSGENIC VERSUS WILD-TYPE PLANTS

Genotype	NaCl treatment	LSMEAN	LSMEAN Number
HVA1/*mtlD*	0mM	0.65	1
HVA1/*mtlD*	100mM	0.99	2
HVA1/*mtlD*	200mM	1.37	3
HVA1/*mtlD*	300mM	1.55	4
WT	0mM	0.46	5
WT	100mM	0.75	6
WT	200mM	1.12	7
WT	300mM	0.76	8
mtlD	0mM	0.61	9
mtlD	100mM	1.02	10
mtlD	200mM	1.22	11
mtlD	300mM	1.24	12

LS-means with the same letter are not significantly different.

	LSMEAN	genotype	treatment
A	1.55	*HVA1/mtlD*	300mM
AB	1.37	*HVA1/mtlD*	200mM
ABC	1.24	*mtlD*	300mM
ABC	1.22	*mtlD*	200mM
ABCD	1.12	WT	200mM
ABCD	1.02	*mtlD*	100mM
BCDE	0.99	*HVA1/mtlD*	100mM
CDE	0.76	WT	300mM
CDE	0.75	WT	100mM
ED	0.65	*HVA1/mtlD*	0mM
ED	0.61	*mtlD*	0mM
E	0.46	WT	0mM

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