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Barley *HVA1* Gene Confers Drought and Salt Tolerance in Transgenic Maize (*Zea Mays* L.)

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Abstract

Drought and salt are two major abiotic stress factors resulting in maize seed and biomass yield losses. In an attempt to produce drought and salt tolerant maize plants, rapidly growing embryogenic calli were produced *in vitro* from maize immature embryos, and bombarded with a gene construct containing the barley *HVA1* (*Hordeum vulgaris* abundant protein) and the Liberty herbicide resistance (*bar*) genes using the Biolistic™ device (gene gun). The bombarded calli were chemically selected in a regeneration medium containing 2.5 mg/L of glufosinate ammonium (the active ingredient of Liberty herbicide), 1 mg/L of benzyl amino purine (BAP). Plantlets were produced four weeks after calli were transferred into the regeneration medium and incubated under fluorescent light condition of 16 hr light∕8 hr dark at 25°C in a growth room. The polymerase chain reaction (PCR) and Southern blot hybridization confirmed the integration, and the reverse transcription PCR (RT-PCR) and Northern blotting confirmed the transcription of the *HVA1* transgene, and herbicide treatment confirmed the expression of the *bar* transgene in upto the fourth generations (T3) of mature plants. The T3 plants that transcribed the *HVA1* showed higher leaf relative water content (RWC), higher leaf and root biomass, and survived complete drought for 15 days while the wild-type non-transgenic control plants died. The T3 plants were also tolerant to 100-300 mM NaCl (especially to 200 mM) while the wild-type control non-transgenic plants showed severe damage under these salt treatments. The research presented here might serve as getting one step closer to developing a drought and salt tolerant maize.

Keywords: *Zea mays*; *HVA1*; Drought; Salinity

Abbreviations: ABA: Abscisic Acid; *Act1*: Actin Rice Promoter; *Pin II*: Potato Inducible Protease II Terminator; BAP: Benzylaminopurine; *35SP*: Cauliflower Mosaic Virus (CaMV) 35S Promoter; *HVA1*: *Hordeum vulgaris* Abundant Protein; IBA: Indole-3-butyric Acid; MS: Murashige and Skoog Media; *Nos*: Nopaline Synthase Terminator; *pBY520*: Plasmid Containing *Bar* and *HVA1* Gene; PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcription-PCR

Introduction

Salt and drought are the two major abiotic stress factors resulting in reduction of maize vegetative biomass and seed yield. Breeding has initially contributed to development of relatively drought and/or salt tolerant maize; however such tolerance has been limited to a minimum level [1]. With advances in molecular and cellular methods, more studies were conducted on finding the reasons behind drought and salt tolerance, and specific genes and transcription factors identified and transferred into the genomes of susceptible crops for drought and/or salt tolerance. For example, indept studies have been performed on the role of abscisic acid (ABA) on regulation of expression of certain genes that are involved with drought and salt tolerance in plants [2-4]. In reality, some of these genes code for proteins that control the closing of the leaf stomata to prevent plant evaporation and to overcome cellular dehydration [5-9]. One of such proteins in barley is called the late embryogenesis abundant 3 (LEA3), encoded by *Hordeum vulgaris* abundant protein (*HVA1*) gene that has been proven to be induced under ABA treatment [2,10]. Several other crops which were genetically transformed with the barley *HVA*1 gene resulted in their increase in vegetative biomass and other symptoms associated crop drought and/ or salt tolerance (Table 1).

The expression of genes in charge of LEA proteins is increased in certain crops under water deficiency in order to prevent the damages to plant cellular and macromolecular structures and to increase ion concentration [18-20].

Drought and salt trigger certain signals resulting in activation of Transcriptional Factors (TFs) involved in drought and salt tolerance. Two groups of TFs include Dehydration Responsive Transcription Factors (*DREB*s) and c-Repeat Binding Factors (*CBFa*) that respectively bind to the Dehydration Response Element (DRE) and c repeat terminal (CRT) cis acting elements [21]. In addition, there are certain other genes called antiporter genes that are associated with production of heat shock proteins (Hsp), some of which are molecular chaperons mediating the exchange between H^+ and Na^+ across cellular membranes and protecting the protein from denaturing, misfolding and aggregating during osmotic stress [21]. For example, the expression of certain bacterial RNA chaperons has been shown to improve drought tolerance and increase yield in maize [22]

According to Serraj and Sinclair [23], drought stress is classified into three stages in which the first stage is when soil moisture is sufficient to maintain maximal stomatal conductance. The second stage includes the inhibition of photosynthesis and leaf growth due to dried soil and failure of stomatal conductance. The third stage is when the water uptake from soil is less than the crop transpirational demand. Transfer of several drought and salt tolerance genes into maize have been previously performed (Table 2).

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In our experiment, we transferred of the barley *HVA1* gene into maize genome, confirmed the integration and expression of this transgene in transgenic plants, measured the level of leaf relative water content, photosynthesis, vegetative biomass, and salinity and drought tolerance in transgenic plants versus the wild-type control non-transgenic plants that were grown under the same conditions.

Materials and Methods

Maize explant

Maize immature zygotic embryos were cultured *in vitro* in a medium containing 4 g/L of N6 salt and vitamin, supplemented with 30 g/L sucrose, 2.76 g/L proline and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for production of type II embryogenic calli, and the embryogenic calli were sub-cultured into fresh medium every two weeks.

Transgene construct

Plasmid pBY520 [31], derived from pBluescript II KS (+) (Stratagene, La Jolla, CA) was used for the transformation of maize immature embryos (Figure 1). This construct contains the barley *HVA1* coding sequences driven by 1.3 kb upstream region of the rice Act1 translation codon [32] and terminated by the polyA 3' terminal of potatoes proteinase inhibitor II (*pin II*) gene [33]. This construct also contains the bacterial phosphinothricin acetyl transferase (*bar*) Liberty herbicide resistance selectable marker gene, driven by the cauliflower mosaic virus *35S* promoter, and terminated by the nopaline synthase *nos* 3' region [34].

Transformation of plasmid vectors into *E. coli* competent cells

Plasmid pBY520 was transferred into *E. coli* by heat shocking for 45 second in a water bath at a temperature of 42°C. A total of 2 μl of plasmid DNA was mixed with 50 μl of DH5α *E. coli* competent cells (Sigma Eldrich, St. Louis, MO). The mixture was then immediately placed on ice for 2 min. Then 700 μl of Luria Bertani (LB) medium was added into each tube, and the mixture was incubated for 1 hour at 37°C while shaking at 250 rpm. The cultures were placed on solid LB selection medium containing 100 mg/L ampicillin, and incubated overnight at 37°C. A single selected colony was taken and placed into a glass flask containing 50 ml of LB medium, and the flask was incubated at 37°C while shaking overnight at 250 rpm. The plasmid DNA was isolated and purified using Qiagen plasmid purification kit (Sigma, Northbrook, IL) as per manufacturer's instruction. Purified plasmid was then used for Biolistic™ bombardment of immature embryo-derived calli.

Biolistic™ bombardment of maize immature embryo-derived calli followed by plant growth and development

The best embryogenic calli from 3-5 day old cultures were selected and placed on a 3×3 cm area on N6 medium containing 30 g/L sucrose, 36.4 g/L sorbitol and 36.4 g/L mannitol for four to six hours prior to bombardment. Then, 5-6 μl of the plasmid DNA coated with tungsten particle (M10) was bombarded twice on the selected embryogenic calli as described [35] using the Helium PDS 1000HE device (BioRad, Hercules, CA) with 1100 psi acceleration pressure. The bombarded calli were then cultured on the induction medium for 1 week in a dark incubator at 27°C before they were transferred to selection medium contained 2.5 mg/L of glufosinate ammonium. The cultures were sub-cultured at 2 week intervals. All DNA precipitation and bombardment steps were performed under sterile conditions at room temperature. The selected embryonic calli were transferred to Murashige and Skoog

medium (MS) (Sigma, Northbrook, IL) containing 1 mg/L BAP for regeneration. The regenerated shoots were rooted in magenta boxes on MS medium containing 1 mg/L Indole-3-butyric acid (IBA). Plantlets were transferred into small pots containing BACCO Professional Planting Soil Mix (Royal Lepage Co., Charlottetown, PE, Canada), and the pots were covered using transparent plastic bags for acclimation to the non *in-vitro* conditions. Small (2-3 mm) holes were made daily in each plastic bag to allow air exchange. Then, the plastic covers were removed after 7-10 days and plantlets were transferred to large pots and grown to maturity in a greenhouse.

Confirming the Integration and Expression of the *HVA1* Transgene in Plants

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from young leaf tissues using CTAB method [35]. The primers including the *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 the presence of the *HVA1* in first to fourth generation transgenic (T0-T3) plants. DNA amplifications were performed in a thermo cycler (PerkinElmer/ Applied Biosystem, Forster City, CA). Optimized PCR conditions were 94°C for 3 min for initial denaturation, 35 cycles of 30 s at 94°C, 30 s at 56°C, 45 s at 72°C and a final 10 min extension at 72°C. The PCR product was loaded directly onto a 1% (w/v) agarose gel stained with 2 ul ethidium bromide and visualized under UV light.

Southern blot hybridization analyses

Southern blot hybridization analyses were performed to determine the stability of transgenic integration in maize progenies and the copy number of *HVA1* and *bar* in transgenic plants. A total of 20 μg of maize genomic DNAs of each PCR positive line and the wild-type control plants were digested with *Hind* III or *Bam* HI enzymes in a proper buffer containing 500 mM Tris-HCl (pH 6.5) and 1 mM EDTA, in a total volume of 250 μl. Each mixture was incubated overnight at 37°C. The digested DNA was precipitated with 70% ethanol at -20°C overnight, pelleted by centrifugation at 13,000 rpm for 10 min, allowed to dry under a laminar flow hood at room temperature and then the DNA was re-dissolved in sterile distilled water before electrophoresis. The digested DNA was electrophoresed at 70 V on 0.8% agarose gel. After electrophoresis, the DNA was transferred from the gel into a Hybond-N⁺ membrane (Amersham-Pharmacia Biotech, Piscataway, New Jersey) 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, Irvine, CA), and labeled with α-[^{32}P]-dCTP using Random Prime labeling kit (GE Healthcare, Pittsburgh, PA) according to the manufacturer's instructions. Membranes were hybridized at 68°C overnight with the radio-labeled probe and auto-radio graphed on premium autoradiography film (Hyblot CL, Denville, Scientific INC, E3018) at -80°C overnight.

Reverse transcription-PCR (RT-PCR)

Plants that showed the integration of the *HVA1* and *bar* were used in RT-PCR analysis. A total of 200 mg of young leaf tissue were grinded into liquid nitrogen, then 1ml Trizol Reagent (Invitrogen, Carlsbad, CA) and 0.2 ml chloroform were added to the mix, and the mixture was vortexed for a 3-5 seconds. The tubes were placed into a centrifuge and spun at maximum speed for 15 min at 4°C. Its aqueous phase was

then transferred into fresh tubes, and 0.5 ml of cold isopropanol was added to the mixture. Samples were incubated at -20^{0}C for 1 hour and centrifuged at maximum speed for 10 min at 4°C. The supernatant was discarded, leaving the RNA pellet. The pellets were then washed with 700 μl of 70% ethanol and then spun in a centrifuge at 12,000 rpm for 5 min at temperature of 4°C. The RNA pellets were dried out under a laminar flow hood at room temperature and the RNA was dissolved in RNase-free water and quantified using a spectrophotometer. A total of 2 μg RNA was used for cDNA synthesis using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The same PCR primers and conditions were used as for the *HVA1* PCR analysis, as described above.

Northern blot analyses

Northern blot analyses were performed using Random Prime labeling kit (GE Healthcare, Pittsburgh, PA) as described in Southern blot analyses to confirm the *HVA1* transcription of transgenic plants. A total of 20 μg of RNA per sample was loaded onto a 1.2% (w/v) agarose-formaldehyde denaturing gel as described by Sambrook et al. [36] and then the RNA was transferred to a Hybond-N$^+$ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker at an energy level of 1200 J. The same Southern blot DNA α-[^{32}P]-dCTP labeled probe was used for detection of the *HVA1* transcripts.

Herbicide tolerance test

Five different concentrations (200, 250, 300, 350, and 400 mg/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 as leaf painting using a cotton swab to determine the lowest concentration that could kill maize leaf. Then, 350 mg/L of glufosinate ammonium solution was used and applied to leaves using brush to find the segregation ratios of transgenic progenies. The tips of young leaves of 3-leaf stage seedlings were selected and painted as performed before [37]. The leaf injury caused by herbicide application was recorded one week after painting treatment.

Studies of mendelian inheritance

The 350 mg/L concentration of commercial Liberty herbicide, containing 18.2% glufosinate ammonium was used in order to determine whether the transgene segregation followed the Mendelian inheritance. Because the *HVA* and the *bar* genes were translationally linked and proven to integrate into the maize genome at 100%, the experiment used the *bar* gene here was assumed correct for the segregation of the *HVA* transgene in maize progenies.

Drought tolerance test

A total of 30 seeds from the fourth generation (T3) transgenic line and a non-transgenic line were planted in 15 cm diameter pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX) under greenhouse conditions of 29°C and 25°C corresponding to day and night temperatures; 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) and percentage of plant survival was determined 7 days after re-watering.

Leaf relative water content (RWC) test

The leaf samples from transgenic and non-transgenic plants were taken to determine the 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 8 AM, their mid-ribs were cut and discarded and the rest of the leaf tissues 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 4 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°C overnight, placed in a desiccator 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 [37-39]:

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

Salt tolerance stress test

A total of 40 seeds of T3 of a transgenic line and a non-transgenic line were sown in small round clay pots containing BACCTO High Porosity Professional Planting Mix. This experiment was replicated in two locations of the same greenhouse for result accuracy. Transgenic seeds were allowed to germinate under 15 mg/L of glufosinate ammonium. The surviving seedlings were PCR tested, then daily treated with equal volume of four different concentrations (0, 100, 200 and 300 mM) of NaCl by gradually increasing 50 mM per day to reach their final concentrations in 10 days. The commercial water soluble Peter 20-20-20 fertilizer (Peter, Salem, OR) was supplemented into salinity solution for nutritional needs. Seedling samples were randomly selected for data recording 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°C for two days and weighed to determine shoot and root dry weights.

Statistical analysis

All data were collected and statistically analyzed using a completely randomized design (CRD) experiment 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.
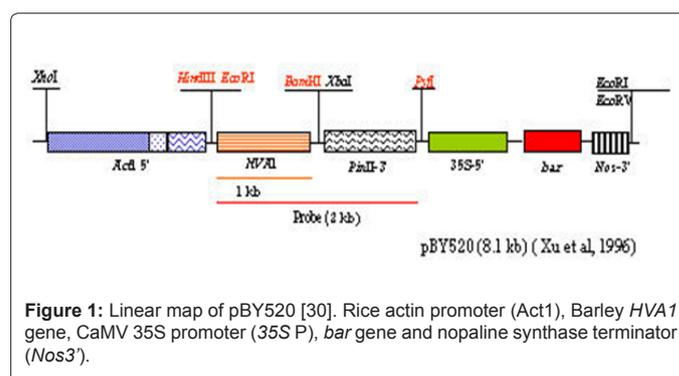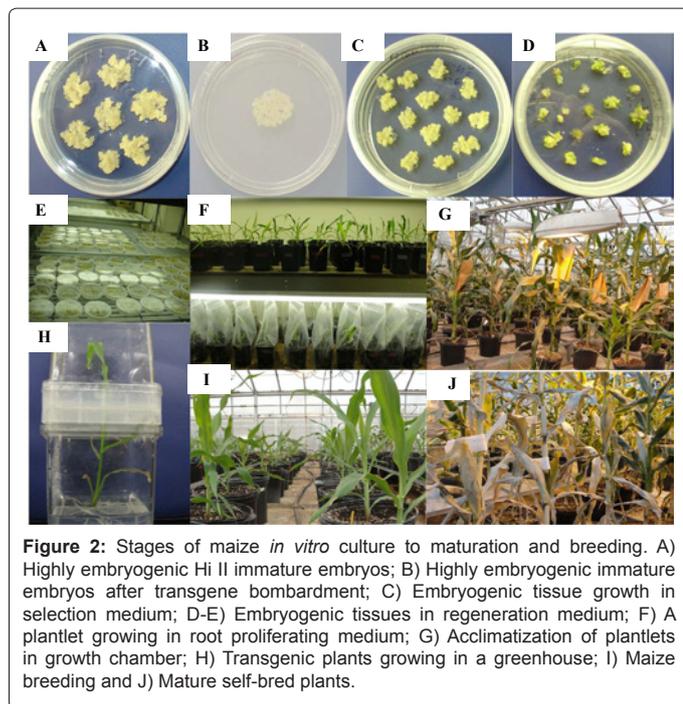

Figure 1: Linear map of pBY520 [30]. Rice actin promoter (Act1), Barley *HVA1* gene, CaMV 35S promoter (*35S* P), *bar* gene and nopaline synthase terminator (*Nos3'*).

Figure 2: Stages of maize *in vitro* culture to maturation and breeding. A) Highly embryogenic Hi II immature embryos; B) Highly embryogenic immature embryos after transgene bombardment; C) Embryogenic tissue growth in selection medium; D-E) Embryogenic tissues in regeneration medium; F) A plantlet growing in root proliferating medium; G) Acclimatization of plantlets in growth chamber; H) Transgenic plants growing in a greenhouse; I) Maize breeding and J) Mature self-bred plants.

Results and Discussions

In-vitro regeneration, transformation and breeding of transgenic plants
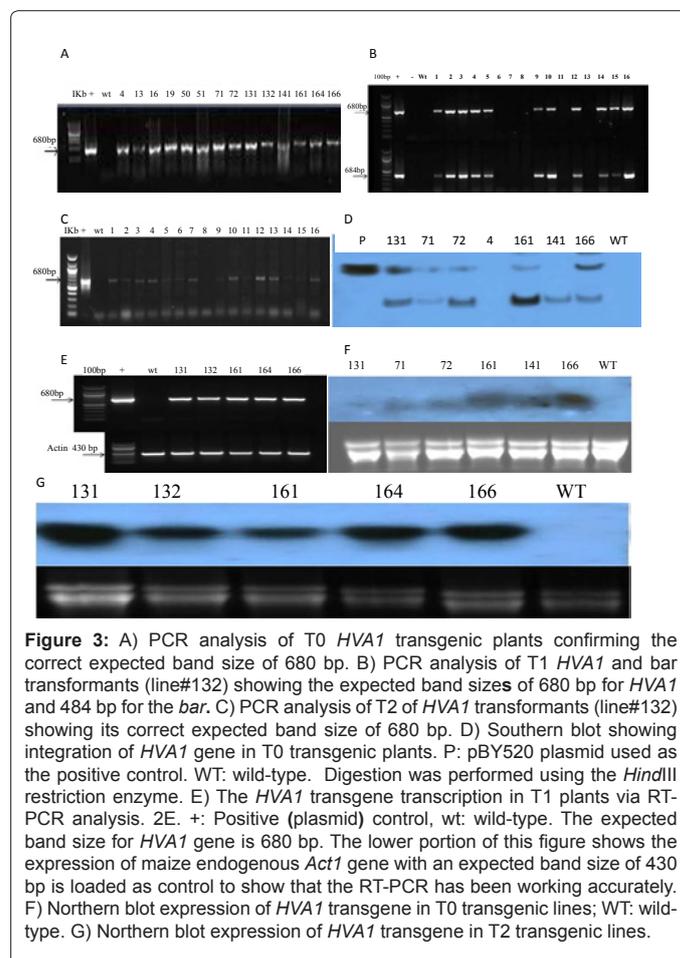
Results of maize *in vitro* culture and plant regeneration, followed by self pollination are shown on figure 2. Embryogenic calli were produced from maize immature embryos (Figure 2A). These calli were used in the transformation experiments using Biolistic™ device. The bombarded calli grew well in the N6 osmotic medium at 25°C in complete darkness for the first two weeks after bombardment (Figure 2B). Selection of herbicide resistant cultures took place in 8-10 months after the bombarded calli was selected on the culture medium containing 2.5 mg/L of glufosinate ammonium. These calli proliferated rapidly and displayed somatic embryos in form of fast growing whitish granular sectors. There were also some non-proliferating and partially necrotic mother calli (brown sectors) which were removed at each subculture. Four weeks after transferred into the regeneration medium containing 1 mg/L of BAP and incubated under fluorescent light condition of 16 hr light/8 hr dark and 25°C growth room, somatic embryos quickly regenerated into shootlets with small roots. Four weeks after they were transferred into root proliferating medium containing the MS plus 1mg/L of IBA, plantlets developed large primary and secondary roots. The acclimated plantlets transferred to 30 cm diameter soil pots and placed in our maize greenhouses grew well and mostly produced seeds after their self hand pollinations (Figure 2F-J).

The research presented here agrees with a couple of research groups [40,41] that using 2 µg of DNA plasmid mixed with 1 µm particle size, 6 cm distance between the loaded gene construct and target, and two shots on each specimen with pressure of 1100 Psi are the optimum conditions for maize genetic transformation using the Biolistic™ device. The research presented here also agrees with two other research groups that bombarded calli transferred from the N6 medium to MS medium regenerated within four weeks, and probably the purpose of

N6 osmotic treatment was to minimize the damages of the cell walls caused by bombardment [21,41,42].

Confirmation of the integration of *HVA1* and *bar* transgenes via PCR and southern blot analysis

The PCR results confirmed the integration of *HVA1* and *bar* transgenes in T0 to T2 generations (Figure 3A-D). Furthermore, the PCR results confirmed the co-integration of *HVA1* and *bar* genes in T0-T2 progenies at 100%. The 100% co-integration of *HVA1* and *bar* genes in transgenic plants is due to the transcriptional linking of the *HVA1* and the *bar* gene cassettes in the same construct (Figure 1). Similar observations were reported by Maqbool et al. [12], Oraby et al. [15] and Kwapata et al. [43] who confirmed stable 100% co-integration of two linked genes in the subsequent transgenic progenies of sorghum and dry bean, respectively. Southern blot hybridization confirmed (Figure 3D) the integration of *HVA1* transgene in T0 plants. Southern blot analyses results showed the integration of three HVA1 transgene copies, except for the transgenic line 141 that shows one inserted transgene copy (Figure 3D). Although the T0 line #4 plant showed the integration of *HVA1* transgene using the PCR technology, Southern blot did not confirm the same because this transgenic plant might have been chimerically transformed (the piece taken for PCR analysis did carry the transgene, but not the piece taken for Southern blotting). We observed that some of the transgenic plants contained more than three

Figure 3: A) PCR analysis of T0 *HVA1* transgenic plants confirming the correct expected band size of 680 bp. B) PCR analysis of T1 *HVA1* and bar transformants (line#132) showing the expected band sizes of 680 bp for *HVA1* and 484 bp for the *bar*. C) PCR analysis of T2 of *HVA1* transformants (line#132) showing its correct expected band size of 680 bp. D) Southern blot showing integration of *HVA1* gene in T0 transgenic plants. P: pBY520 plasmid used as the positive control. WT: wild-type. Digestion was performed using the *Hind*III restriction enzyme. E) The *HVA1* transgene transcription in T1 plants via RT-PCR analysis. 2E. +: Positive (plasmid) control, wt: wild-type. The expected band size for *HVA1* gene is 680 bp. The lower portion of this figure shows the expression of maize endogenous *Act1* gene with an expected band size of 430 bp is loaded as control to show that the RT-PCR has been working accurately. F) Northern blot expression of *HVA1* transgene in T0 transgenic lines; WT: wild-type. G) Northern blot expression of *HVA1* transgene in T2 transgenic lines.

Host plant	Results	Reference
Wheat	Increased biomass and water use efficiency under stress	[11]
Oat	Delayed wilting under drought stress	[12]
Rice	Drought and salinity tolerance	[13]
Rice	Dehydration avoidance and cell membrane stability	[14]
Oat	Salinity tolerance and increased yield	[15]
Wheat	Improved yield under drought conditions in the field	[16]
Mulberry	Salinity and drought resistance	[17]

Table 1: Expression of barley *HVA1* gene in different transgenic crop species.

Gene	Enzyme involved/role	Symptoms	Reference
CspA, CspB	Molecular chaperones	Drought tolerance in yield increase under field conditions	[22]
NPK1	Mitogen-activated protein kinase	Improved photosynthesis and drought tolerance	[24]
ZmACS6	Ethylene synthesis	Non-functional mutant expressed drought induced senescence	[25]
betA	Choline dehydrogenase for glycine betaine synthesis	Drought resistance at seedling Drought tolerance and yield increase	[26]
NFYB2	Transcription factor regulating other genes	Drought resistance	[27]
BADH-1	Glycine betaine production	Salinity tolerance	[28]
GDH	Glutamate dehydrogenase	Drought resistance	[30]

Table 2: A summary of genes previously transferred into maize genome for development of drought and/or salinity tolerance.

copies of the *HVA1*; however we discarded those plants because other studies had shown that multiple transgenes can result in rearrangement of the transgenes and transgene silencing [44-46]

Confirmation of *HVA1* transcription via RT-PCR and northern blot analysis

The RT-PCR analysis confirmed the transcription of *HVA1* transgene in plants (3E), and the same is confirmed by Northern blot analyses of T0 and T2 plants (Figures 3F-3G).

Confirmation of herbicide resistance of transgenic plants

The kill curve experiment showed that the minimum concentration of glufosinate anominum that killed the leaf tissues in the wild-type plants was 350 mg/L. Therefore, this concentration was used to determine the Mendelian inheritance of the *bar* transgene. Results of the Chi-square test showed that the bar gene segregation did not follow Mendelian inheritance. This might be because most transgenic plants tested had integrated 2-3 transgene inserts, and therefore the multiple copies of transgene insertion may have caused the transgene not to follow the Mendelian segregation. Furthermore, not every seed was strong and healthy to be able to germinate possibly due to our greenhouse conditions.

Confirmation of drought tolerance in T3 plants

The results of symptoms of drought tolerance of PCR positive T3 plants are shown on figure 4. 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 4A.

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., wilted leaves, burned at the leaf edge and tip, and the upper leaves turned yellow after 15 days of water withholding. The wild-types showed much 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 4B shows that the leaf RWC measured in wild-type leaves was decreased to 81.7% whereas leaf RWC in transgenic plants leaves maintained water 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 of 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 4B represents the survival rate of transgenic versus wild-type control plant after 7 days of water recovery treatment. Figure 4B shows that when plants were re-watered for 7 days after 15 days of water withholding, transgenic plants showed a quick (2-3 days) recovery and performed high percentage of survival (65% recovery) compared to wild-type (30% recovery).

Maize is very susceptible to drought. It 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 it 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
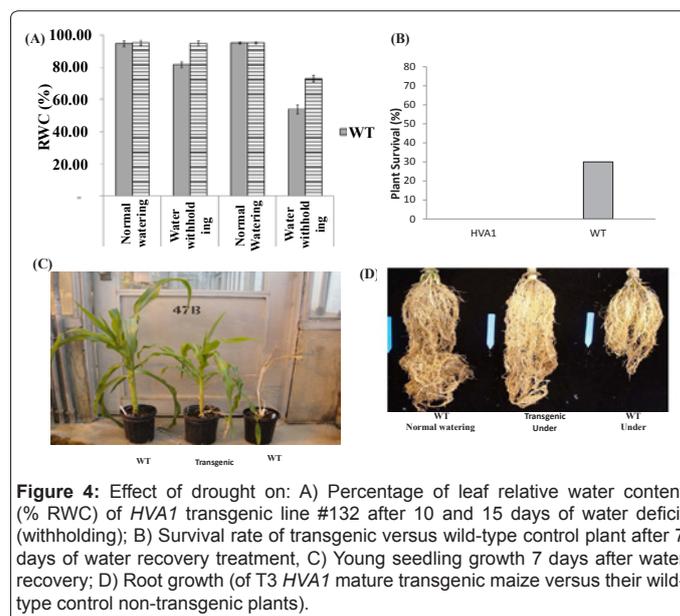

Figure 4: Effect of drought on: A) Percentage of leaf relative water content (% RWC) of *HVA1* transgenic line #132 after 10 and 15 days of water deficit (withholding); B) Survival rate of transgenic versus wild-type control plant after 7 days of water recovery treatment, C) Young seedling growth 7 days after water recovery; D) Root growth (of T3 *HVA1* mature transgenic maize versus their wild-type control non-transgenic plants).

in 5 to 10% yield losses [47]. The left photo in figure 4C represents the effect of drought on a young transgenic versus wild-type control plant of the same age.

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 ABA found in drought tolerant lines as compared with drought sensitive lines [48]. ABA is known to regulate stomata opening and other responses resulting in tolerance of plants to drought [8]. In the results presented here, it is possible that accumulation of ABA may have played a role as *HVA1* is shown to increase ABA accumulation in other plants [49]. Also, the expression of *HVA1* gene encoding the Barley LEA3 *HVA1* protein in this study is in agreement with 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 [14,31].

The level of salt tolerance on T3 plants

The T3 and the wild-type plants showed normal development up to one week after being exposed to 100 mM NaCl, but wild-type plant leaves started showing major injury symptom after being treated with initial NaCl concentration of 50 mM. After increasing the NaCl concentration to 200 mM, the wild-type non-transgenic plants were severely affected as compared to those treated with 100 mM. At 300 mM NaCl concentration, both transgenic and wild-type plants showed severe leaf injury after one week of salt treatment. Figure 5 displays the results of the effect of different salt concentrations on T3 versus non-transgenic control plants on shoot and root length, and shoot and root fresh and dry weight biomasses. Figure 5 shows the results of symptoms of salinity tolerance of the T3 plants.

Table 3 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 200 mM, percentage of shoot and root length reduction in wild-type plants increased significantly to 55.7%

and 47.7%, respectively 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 100 mM and up to 80% and 72% at 200 mM NaCl concentration. Salt stress also reduced by 85% of root dry weight in wild-type compared to 63% reduction in transgenic plants at 200 mM 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.
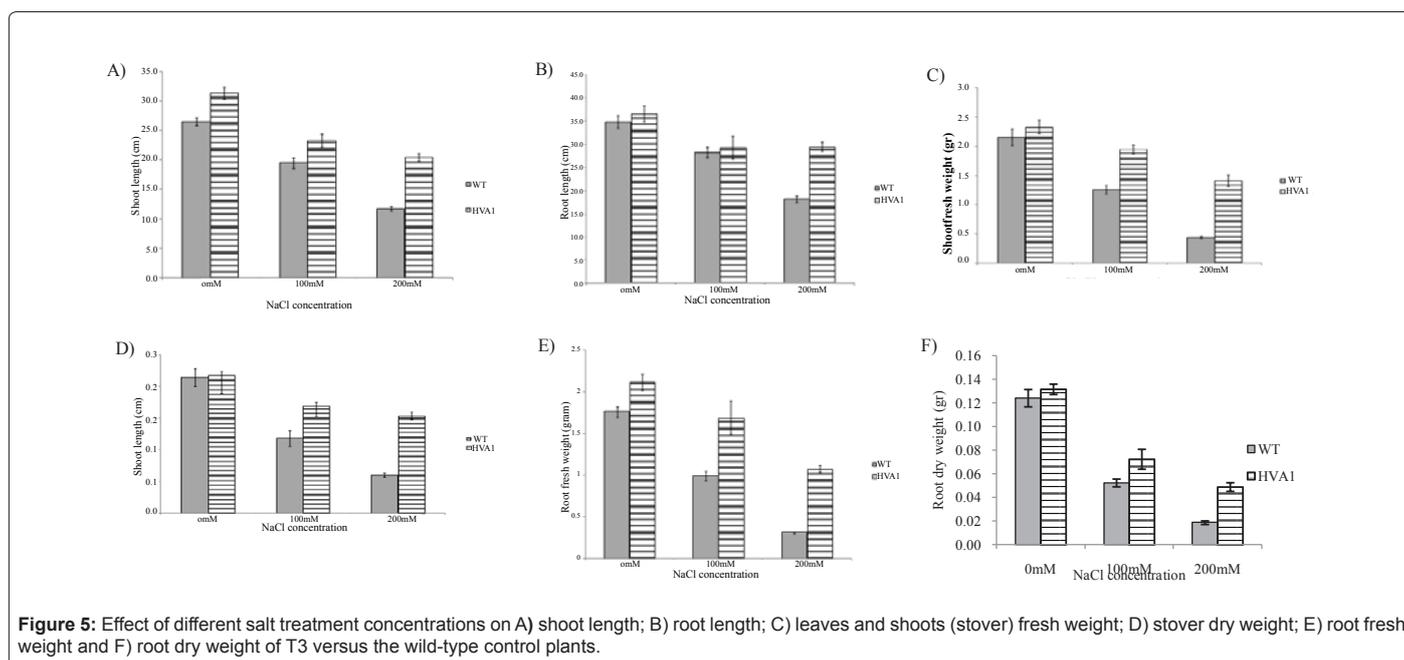
In general, the growth of seedlings and fresh and dry biomass of transgenic plants were less affected by different NaCl levels as compared to their wild-type control plants.

In Turkey test, the mean values with the same letter (such as a and a or b and b, etc.) are not significantly different from each other, but mean values with different letters (such as a and b or b and c) are significantly different (P<0.05).

Shoot and root length, and shoot and root fresh and dry 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.

The results of additional studies performed using 3-leaf stage plants exposed to four different NaCl concentrations (0, 100, 200, 300 mM) for 6 days (after all treatments reached to the final NaCl concentration) are presented in table 4. A

Table 3 shows that the number of leaves per plants in the wild-type was significantly less as compared to that in *HVA1* transgenic plants under NaCl stress. Under 300 mM NaCl level, the youngest leaves (the fourth leaves) of the wild-type control plants were rolled and dead, whereas the fourth leaves in transgenic plants were healthy and extended normally and grew into normal leaves. The effect of NaCl stress resulted

Figure 5: Effect of different salt treatment concentrations on A) shoot length; B) root length; C) leaves and shoots (stover) fresh weight; D) stover dry weight; E) root fresh weight and F) root dry weight of T3 versus the wild-type control plants.

Salt concentration	100 mM		200 mM	
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

Table 3: Reduction in shoot and root length and their biomass affected by salt treatments.

	Number of leaf (leaf/plant)			
NaCl levels	0 mM	100 mM	200 mM	300 mM
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 4: Effect of salinity stress on number of leaves per plant after 6 days of NaCl treatment.

in decreased number of leaves per plant in wild-type plants from 3.3 to 3.0 at 100 mM and 300 mM 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 100 mM and 300 mM (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. 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 200 mM NaCl [15]. Similar findings were reported by Xu et al. [30] in transgenic rice and wheat 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 [11,16], rice [13,14], and oat [12,15].

The research presented here might be one step closer to developing of a maize genotype that can tolerate the unpredictable drought, and salinity in parts of the world that farmers suffer from losses of these two abiotic stress factors.

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