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Research Article

Transgene Pyramiding of the *HVA1* and *mtlD* in T3 Maize (*Zea mays* L.) Plants Confers Drought and Salt Tolerance, along with an Increase in Crop Biomass

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The pBY520 containing the *Hordeum vulgare HVA1* regulated by the rice actin promoter (*Act1 5′*) or the JS101 containing the bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) also regulated by rice *Act1 5′* and a combination of these two plasmids were transferred into the maize genome, and their stable expressions were confirmed through fourth generations. Plants transcribing a combination of the *HVA1+mtlD* showed higher leaf relative water content (RWC) and greater plant survival as compared with their single transgene transgenic plants and with their control plants under drought stress. When exposed to various salt concentrations, plants transcribing the *HVA1+mtlD* showed higher fresh and dry shoot and dry root matter as compared with single transgene transgenic plants and with their control plants. Furthermore, the leaves of plants expressing the *mtlD* accumulated higher levels of mannitol. Plants expressing the *HVA1+mtlD* improved plant survival rate under drought stress and enhanced shoot and root biomass under salt stress when compared with single transgene transgenic plants and with their wild-type control plants. The research presented here shows the effectiveness of coexpressing of two heterologous abiotic stress tolerance genes in the maize genome. Future field tests are needed to assure the application of this research.

1. Introductions

In 2007, 60% of the total biotech maize in the United States carried transgenes stacked for both herbicide and insect resistance [1]. Another transgene pyramiding approach has been to express multiple insect and/or disease resistance genes in plants to avoid the possibility that the insect or pathogen develops resistance against the gene products under extreme pressures. For example, transgene stacking delayed the emergence of *Bacillus thuringiensis (Bt)* resistance in insects (biotype) of broccoli [2] and a combination of transgenes improved resistance to a pest and a disease in rice [3].

There are three strategies on how to stack transgenes in plants. The first strategy is cross-breeding of single gene transgenic plants for gene stacking. For example, cross-breeding of different *Bacillus thuringiensis (Bt)* and the phosphinothricin acetyltransferase (*PAT*) herbicide resistant transgenic plants have enhanced corn borer and rootworm resistance along with herbicide tolerance in maize [4]. Wei et al. [5] crossed two transgenic parents, one expressing the *BetA* gene (encoding for choline sulfatase) and the other expressing the H^+-PPase (*TsVP*) gene(encoding the vacuolar H^+ pyrophosphatase of *Thellungiella halophila*) and demonstrated that the combined expression of these transgenes in maize further improved drought tolerance as compared with transgenic plants expressing just one of the two transgenes. This method is time consuming and labor intensive.

The second strategy is called retransformation for transgene stacking. This strategy is to produce a transgenic plant and then retransform it with a second transgene. This method has also been proven to improve traits in several crop species.

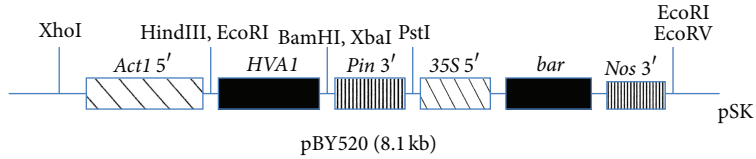

Figure 1: The linear map of pBY520 construct. Rice actin promoter (*Act1*), barley (*Hordeum vulgare*) or *HVA1 LEA3* gene, cauliflower mosaic virus 35S (*35S*) promoter, bar Liberty herbicide resistance gene (*bar*), and nopaline synthase terminator (*Nos 3′*).

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 with nontransgenic controls [6]. Another report [7] on simultaneous retransformation of tobacco with three transgenes (encoding dehydroascorbate reductase (DHAR), copper zinc superoxide dismutase (CuZnSOD), and ascorbate peroxidase (APX)) conferred that the triple transgenic plants showed significantly higher shoot and root dry biomass matter, as well as higher salt tolerance, than those of the single or double-transgene transgenic plants. This system is also labor intensive and time consuming and has the limitation that the two or more transgenes not being linked on the same construct usually segregate separately.

The third strategy involves cotransformation for transgene stacking. This strategy is to simultaneously genetically engineer plants with a construct containing multiple transgene cassettes or with a mixture of two to 14 transgene constructs. Using this concept, several researchers [8–14] simultaneously transferred multiple heterologous genes into different plant species. This is the most promising strategy because it is less labor intensive and less time consuming than the first two.

The research presented here utilizes third strategy to simultaneously cotransfer the barley (*Hordeum vulgare*) *HVA1* and the bacterial mannitol 1 phosphate dehydrogenase (*mtlD*) genes into the maize genome. The *HVA1* encodes for one of the LEA proteins [15]. Several teams have reported that the expression of *HVA1* gene in other plants resulted in drought and/or salinity tolerance and has helped in understanding of the mechanisms of plant drought and/or salinity tolerance [16–25].

Abiotic stress tolerant plants often have the capability to synthesize and accumulate certain compatible solutes or osmoprotectants in their cells to balance the osmotic pressure inside of their cells to match that of the environment outside of their cells [26] and therefore to prevent cellular dehydration. Mannitol is one of the plant osmoprotectants. An early report by Sheveleva et al. [27] indicated that an increase in mannitol accumulation in tobacco (*Nicotiana tobacum*) and in chickpea (*Cicer arietinum* L.) tissues caused accumulation of proline, an amino acid needed to balance carbon nitrogen under stress conditions, resulting in improved yield [28]. It has also been reported that expression of certain osmoprotectant pathways improves tolerance to water deficit in wheat (*Triticum aestivum*), soybean (*Glycine max*) [29, 30], and rice (*Oryza sativa*) [31]. The mannitol dehydrogenase (MTLD) enzyme, encoded by the bacterial *mtlD* gene, is the key enzyme in mannitol metabolism, reversibly converting fructose-6-phosphate to mannitol-1-phosphate. The *mtlD* gene has been transferred to several crop species, resulting in certain cases in enhanced plant height, fresh and dry biomass weight, increase in salinity and/or drought tolerance, and often in accumulation of mannitol [32–42]. Research on transfer of bacterial RNA chaperones performed to induce abiotic stress tolerance in maize is among other promising research areas [43].

To date, there has been no report on the coexpression of a combination of the *HVA1* and *mtlD* transgenes in maize plants. Here, we report on the coexpression of such combination of transgenes in maize genome and compare their effects on plant biomass yield and salt and/or drought tolerance with HVA1 and mtlD single transgene transgenic plants and with their wild-type nontransgenic control plants.

2. Materials and Methods

2.1. Explant. Maize Hi II immature zygotic embryo-derived calli were maintained in an embryo induction medium containing 4 g/L of N6 base salt and vitamins, supplemented with 30 g/L sucrose, 2.76 g/L proline, and 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Type II embryogenic calli were transferred to fresh medium every two weeks for their faster growth and proliferations.

2.2. Transgene Constructs. Plasmids, pBY520 (Figure 1) and pJS101 (Figure 2), were used in this research. The pBY520 contains the *HVA1* coding sequences driven by the rice *Act1* promoter [44] and terminated by the potato protease inhibitor II (*pin II*) 3′ region [45]. It also contains the bacterial phosphinothricin acetyl transferase (*bar*) structural gene as a selectable marker. In this construct, the *bar* coding sequences are driven by the cauliflower mosaic virus (*35S*) promoter and terminated by nopaline synthase (Nos) 3′ region.

The JS101 contains the bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) gene regulated by the rice actin promoter (*Act1*) and the potato protease inhibitor II terminator. This cassette is also linked to the *bar* herbicide resistance selectable marker gene regulated by the *35S* promoter and *Nos* 3′ region.

2.3. Genetic Transformation and Chemical Selection. The embryogenic type II friable calli were bombarded twice with 6 μL of each plasmid or with a 1 : 1 ratio of both plasmids containing DNA coated tungsten particles (M10) under a laminar

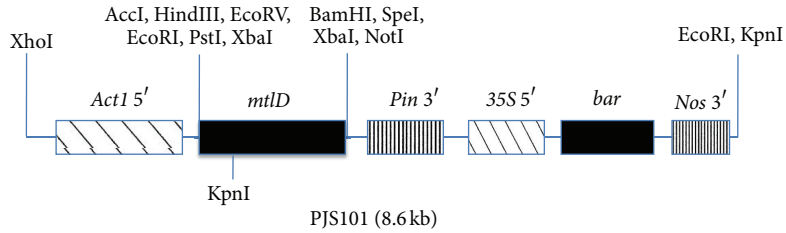

Figure 2: The linear map of JS101 construct. The bacterial *mtlD* gene driven by rice actin promoter (*Act1 5′*) and potato protease inhibitorII terminator (*pinII′*), and the *bar* selectable marker gene regulated by the cauliflower mosaic virus (*35S*) promoter and *Nos 3′* region.

flow hood (Envirco Corp, Albuquerque, NM) as described by Frame et al. [46] using the Helium PDS 1000HE device (Biotechnology Systems Division, Wilmington, DE) with a 1100 psi disc. Bombarded calli were cultured on the induction medium containing N6 medium [47] supplemented with 2.7 g/L proline and 30 g/L sucrose for one week while maintained in the dark at 27°C before they were transferred onto Murashige and Skoog (MS) medium [48] containing 1 mg/L 6-benzylaminopurine (BAP). The regenerated shoots were rooted on MS medium containing 1 mg/L indole-3-butyric acid (IBA) and 2.5 mg/L glufosinate ammonium (the active ingredient of the Liberty herbicide). *In vitro* cultures were subcultured at two-week intervals. Plantlets were transferred into small pots containing BACCO Professional Planting Soil Mix (Royal Lepage Co., Charlottetown, PE, Canada), and the plantlets were transferred into large pots and grown to maturity in greenhouses.

2.4. Molecular Analysis. Plant genomic DNA was isolated from young leaf tissues using the CTAB (cetyltrimethylammonium bromide) method. PCR primers for *HVA1* were 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). These primers were used to detect *HVA1* gene for T0–T3 plant regenerations. DNA amplifications were performed in a thermocycler (PerkinElmer/Applied Biosystems, Forster City, CA). The sequences of the primers used in PCR analysis to confirm the *mtlD* transgene were 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°C for 3 min for one cycle; the following 35 cycles of 30 s at 94°C, 30 s at 55.5°C, and 45 s at 72°C; one cycle at 72°C for 10 min; and the final cycle at 4°C.

Northern blot analysis was performed to measure the relative levels of transcription of each transgene, using Random Prime labeling kit (GE Healthcare). The DNA α-[^{32}P]-dCTP labeled probe was used for detection of transcripts.

Due to the fact that there were too many PCR positive plants to test for transgenes transcriptions, reverse transcription PCR (RT-PCR) was also performed to detect transgenes transcriptions in most plants. PCR positive plants of *HVA1* gene were used in northern blotting or in RT-PCR analysis. For RT-PCR, a sample of 200 mg young leaf tissues was ground 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°C. An aqueous phase was then transferred into fresh tubes and 0.5 mL of cold isopropanol was added. Samples were incubated at −20°C for 1 hour and centrifuged at maximum speed for 10 min at 4°C. The supernatant was discarded leaving the RNA pellet. This was washed with 700 μL of 70% ethanol and then spun in a centrifuge at 12,000 prm for 5 min at temperature of 4°C. The RNA pellets were dried at room temperature and dissolved in RNAase-free water and quantified using a spectrophotometer. Two μg of the obtained RNA was used for cDNA synthesis as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). The same primers and conditions were used for the *HVA1* PCR amplification as described above.

2.5. Drought Tolerance Tests. A total of 30 seeds from the fourth (T3) generation of the RT-PCR positive transgenic lines along with seeds of the wild-type nontransgenic controls were planted in a greenhouse. Four-week-old seedlings were exposed to 15 days of no-water treatment (drought) followed by 7 days of rewatering. Data were collected for leaf relative water content (RWC) after 10 days and 15 days of drought stress (before rewatering for recovery), and percentage of plant survival was determined 7 days after rewatering.

2.6. Salinity Tolerance Tests. The T3 *HVA1, mtlD, and HVA1+mtlD* plants were initially obtained from seeds of a single T0 plants. The single seed was germinated *in vitro* in MS medium containing 15 mg/L of glufosinate ammonium selection medium, and this single plantlet was transferred into soil, grown for molecular testing followed by seed collections. After T0–T3 plants were tested via molecular methods, a total of 40 seeds of T3 of *HVA1* transgenic lines were also allowed to germinate *in vitro* in MS medium containing 15 mg/L of glufosinate ammonium selection medium. The surviving seedlings and the same age wild-type control seedlings were transferred to pots and treated daily with one liter of each of four different concentrations (0, 100, 200, and 300 mM) of NaCl by gradually increasing 50 mM per day to reach each of the final concentrations in 10 days. Commercial 20-20-20 fertilizer was also equally added to the salt solutions as a supplement for nutritional needs. Plastic trays were placed under each pot to avoid unequal run-offs. Shoot and root length and dry weights data collections took place on plants after 10 days of salt treatments. Dry matters were obtained in an 80°C oven for two days and weighed to

determine shoot and root dry weights. Also, 40 seeds of *mtlD* T3 plants were allowed to germinate *in vitro* under 15 mg/L glufosinate ammonium selection. Thirty seedlings of selected T3 plants and nontransgenic control seeds were transferred to pots and watered daily with normal tap water for two weeks before being salt treated. Seedlings at their four-leaf stage of growth were treated with salt for 10 days. For the plant height, the distance from ground level to the tip of the longest leaves was measured for the absolute growth rate (AGR) using the formula: AGR $= (h_2 - h_1)/(t_2 - t_1)$, where h_2 and h_1 were final and initial height of the plant while t_2 and t_1 were the final and initial days, respectively [49].

2.7. Leaf Relative Water Content (RWC) Tests. Leaf samples from *HVA1* transgenic and nontransgenic 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 8 a.m., their mid-ribs were cut and discarded, and the rest of the leaves were immediately placed in plastic bags to limit water evaporation. Leaf sections were removed from the plastic bags and weighed and then bathed in distilled water for four hours to achieve full turgidity at room temperature. After hydration, the samples were placed between two tissue papers to remove any water on the leaf surface and then immediately weighed to obtain their weight at full turgidity (TW). Samples were then dried at 80°C overnight and then weighed to determine their dry weight (DW). Leaf relative water content (RWC) was calculated by the following formula, as reported [50]:

$$\text{RWC } (\%) = \left[\frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}\right] \times 100, \quad (1)$$

where RWC is relative water content; FW, fresh weight; DW, dry weight; TW, total weight.

2.8. Mannitol Accumulation Test of T3 mtlD Plants. Carbohydrate extraction, derivatization, and analysis procedures in this section followed modification of Keller and Loescher [51]. Briefly, fresh leaves of T3 transgenic and nontransgenic control plants were collected 7 days after they were exposed to different salt concentrations. One gram of each fresh leaf sample was ground in liquid nitrogen. The tissue powder was extracted with 95% ethanol, partitioned against chloroform, and the samples were dried with a SC 200 Speedvac (Thermo Fisher Scientific, Asheville, NC, USA). Samples were then oximated and the oximes were then converted to trimethylsilyl derivatives. Gas chromatography was performed on a 6890 N GC system (Agilent Technologies, Santa Clara, CA, USA). Peak identities were confirmed by GC-mass spectroscopy.

2.9. Photosynthesis Analysis of T3 mtlD Plants. Leaf net photosynthetic rate (μmol CO_2 m^{-2} leaf area s^{-1}) and stomatal conductance (mmol H_2O m^{-2} leaf area s^{-1}) were determined on the second-uppermost expanded leaf of plants using the LI-6400XT portable photosynthesis system (LICOR, Lincoln NB) as described by Wang et al. [52].

Two DNA plasmids, pBY520 containing the barley *HVA1* gene (Figure 1) and JS101 containing the bacterial *mtlD* gene (Figure 2), were cobombarded with the ratio of 1 : 1 into immature maize embryogenic calli as described above. Transgenic plants were selfed and *HVA1-mtlD* T3 transgenic plants (line no. 51-1) were tested for drought and salt tolerance in comparison to single transgene *HVA1* transgenic plants (line no. 161) and *mtlD* transgenic plants (line no. 27-1).

2.10. 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.

3. Results and Discussions

3.1. Confirmation of the Stability of the Transgenes Integration and Transcription. The *HVA1*, the *mtlD*, and the *HVA1-mtlD* transgenic plants were tested for the presence and stability of the transgenes. Figure 3 confirms the cointegration and coexpression of the *HVA1* and the *mtlD* in plants.

All T3 surviving plants initially originated from one T0 plant. T3 seeds were given numbers as 27, 28, and 51 (Figure 3) which were grown into mature plants, and plants were tested via PCR to confirm which plants carried both transgenes, and northern blotting confirmed the transgenes transcriptions. Some of the PCR positive plants did not show the transgene transcription either due to gene silencing or because the PCR results were false positive. We only kept plants that showed transcription of both transgenes. Data were not collected on plants that did not transcribe both transgenes.

Molecular analyses (Figure 3) confirmed the cointegration and co-transcription and the stability of transgenes in the *HVA1+mtlD* plants. These results agree with Agrawal et al. [14] who obtained cointegration of up to two mixed (1 : 1 ratio) bombarded other transgenes in plants. When both transgenes cassettes are linked in the same construct, both genes are usually expected to be integrated in the same chromosomal position and thus may be inherited together in all their progenies [53]. It is rare in cobombardment, but chemical selection of both transgenes can assist in transmissions of both transgenes into their plant progenies. In this case, occasionally, multiple transgenes from various plasmid cotransformations can cointegrate and coinherit stably in subsequent progenies [54]. In the cotransformation research presented here, we assume that the *HVA1* and the *mtlD* transgenes were not integrated in the same chromosomes because the two transgenes were not linked in one plasmid. Therefore, not all *HVA1+mtlD* progenies showed the integration of both transgenes.

3.2. Drought Stress Tolerance Test. Figure 4 represents the relative water content (RWC) after 10 and 15 days of water withholding of T3 transgenic plants versus their wild-type nontransgenic control plants.

Figure 4 results show that after 10 or 15 days of normal watering, there were no significant differences between the

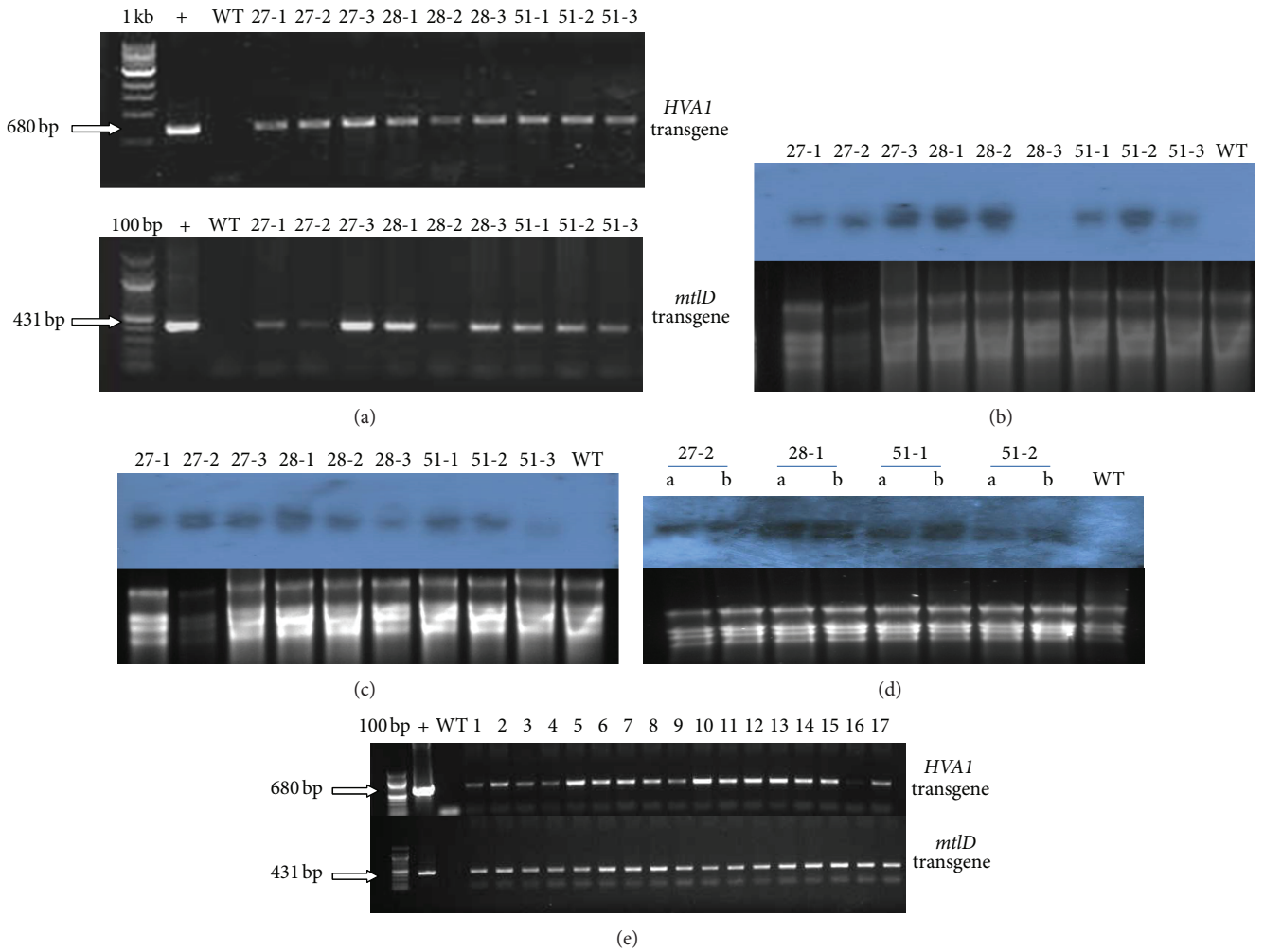

Figure 3: (a) PCR analysis of the combination of *HVA1-mtlD* in T1 maize lines. (b) Northern blot hybridization of T1 *HVA1* in *HVA1-mtlD* transgenic lines using the *HVA1* probe. (c) Northern blot hybridization of T1 *mtlD* in the *HVA1-mtlD* transgenic lines using the *mtlD* probe. (d) Northern blot hybridization of T2 *HVA1* in *HVA1-mtlD* transgenic lines using the *HVA1* probe. (e) RCR analysis of the T3 *HVA1-mtlD* confirming the stability of both transgenes in the fourth (T3) progeny obtained from northern blot positive HVA1 and mtlD T2 line number 51-1.

leaf RWC of the wild-type control nontransgenic and all three sets of transgenic plants (*HVA1, mtlD,* and *HVA1+mtlD*). After 10 days of water withholding, the leaf RWC of the wild-type control plant was lower (81.9%) as compared to all three sets of transgenic plants (HVA1 RWC was 94.5, mtlD RWC was 92.7, and the *HVA1+mtlD* RWC was 94.3). After 15 days of water withholding, the leaf RWC of the wild-type control plant was much lower (57.1%) as compared to all three sets of transgenic plants (HVA1 RWC was 81%, mtlD RWC was 77.6%, and the *HVA1+mtlD* RWC was 85.0%).

Table 1 represents the effect of drought tolerance on survival of T3 single versus double transgene transgenic plants as compared with their wild-type nontransgenic control plants.

All three sets of transgenic lines showed quick recovery after watering and a higher percentage of these plants survived compared to their wild-type nontransgenic controls that were grown under 15 days of water deficit followed by 7 days of watering. Table 1 shows that the *HVA1+mtlD* plants had highest percentage of survival (67%), followed by *HVA1* single transgene transgenic plants (52%) and the *mtlD* transgenic plants (45%), while only 35% of the wild-type nontransgenic control plants survived. In this experiment, a high number of T3 seeds did not germinate probably due to the fact that seeds were not stored under refrigerated conditions.

In the research presented here, transgenic maize plants expressed both *HVA1* and the *mtlD* gene resulted in improved RWC, plant survivals, and in increased drought tolerance as compared with their wild-type nontransgenic plant counterparts and also as compared with plants expressing only one of the two single-transgenes.

When plants are exposed to severe drought stress, the results include reduced leaf RWC and closed stomata. Such conditions also tend to be associated with increases in ABA and/or sugar contents in plants [55]. We measured the leaf RWC because it is considered an appropriate indicator of

Table 1: 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	Seeds not germinated	Total number of plants	Number of plants surviving	Percentage (%)
HVA1	9	21	11	52
mtlD	10	20	9	45
HVA1-mtlD	9	21	14	67
WT	10	20	7	35

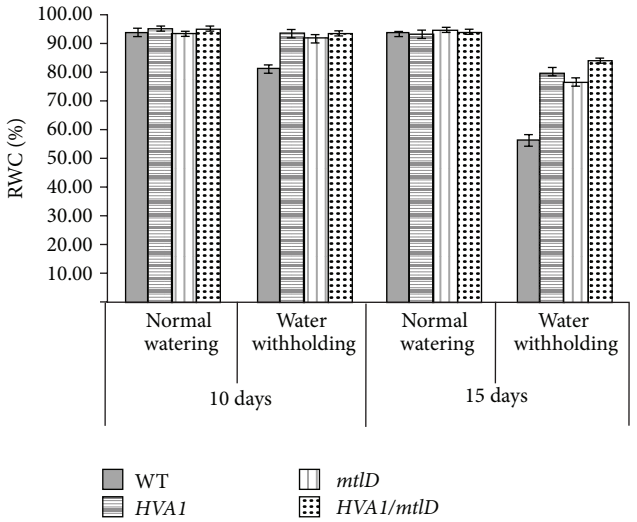

Figure 4: Percentage of leaf relative water content (% RWC) of the T3 transgenic lines after 10 and 15 days of water withholding. Bars are mean ± SE of three samples. The percentages of least square means (LSMeans) are shown on the top of each column.

tissue water retention capacity. Also, when the leaf RWC is reduced below some critical threshold (e.g., below 0.3 g $H_2O\,g^{-1}$ DW), there is insufficient water for preferential hydrations [56]. As such, the wild-type control nontransgenic plants in the present study were more affected by water stress than those that were transgenic.

The present work agrees with Su et al. [57] work in transgenic poplar when five effector genes (vgb, SacB, JERF36, BtCry3A, and OC-I) stacked in a transgenic plant resulted in improved total biomass and chlorophyll concentrations and showed greater water use efficiency under drought conditions. The present work also agrees with Wei et al. [5] work where two transgenes (*betA*, encoding choline dehydrogenase and *TsVP*, encoding V-H^+-PPase) stacked in maize plants could further improve drought tolerance as compared to their wild-type nontransgenic control plants and as compared with transgenic plants expressing only *betA* or *TsVP* transgenes.

3.3. Salinity Tolerance Test of HVA1, mtlD, and HVA-mtlD T3 Plants. Table 2 shows that, as salinity concentrations increased from 100 mM NaCl to 300 mM NaCl, the results included reduced shoot length, shoot fresh weight, and shoot and root dry weights of all three sets of transgenic plants (*HVA1*, *mtlD*, and *HVA1+mtlD*) and the wild-type control plants. However, the wild-type controls were far more affected by salt treatments than any of the three sets of transgenic plants.

At 100 mM NaCl, the *HVA1+mtlD* transgenic plants were less damaged by salt stress treatment as compared to single transgene *HVA1* or *mtlD* plants and as compared with the wild-type controls under the same conditions. The percentage of reductions of shoot fresh weight and shoot and root dry weights of the *HVA1+mtlD* plants were 13%, 18.4%, and 21.4%, respectively. These reductions were 32.1%, 14.8%, and 25.0% for *HVA1* transgenic plants and 25.9%, 27.0%, and 21.9% for the *mtlD* transgenic plants, respectively.

At high salinity concentrations, there were no significant differences in the reductions of shoot fresh and dry weights between the *HVA1+mtlD* versus single transgene *HVA1* or *mtlD* transgenic plants. Roots of the *HVA1+mtlD* and *mtlD* transgenic plants had accumulated more mannitol and therefore were less affected by salt stress, that is, displaying a lower percentage reduction in root dry weight relative to *HVA1* transgenic and wild-type plants. With increasing salt concentrations, the *HVA1+mtlD* plants showed more shoot length reductions as compared with the *HVA1* or *mtlD* plants.

Our results are in agreement with the previous reports on transgenic celery [58] and loblolly pine (*Pinus taeda* L.) [9], where the expression of multiple transgenes showed higher accumulation of osmoprotectants as compared with their single transgene transgenic plants.

3.4. Accumulation of Mannitol and Other Soluble Sugars in mtlD and HVA1-mtlD Plants. Table 3 represents data collected on the effect of NaCl concentrations on mannitol, glucose, sucrose, fructose and inositol contents of transgenic versus wild-type nontransgenic control plants.

3.4.1. Mannitol Accumulation. The results from Table 3 indicate that there was an increase in detectable mannitol in all of the transgenic plants. Various other reports have also confirmed that the expression of *mtlD* in transgenic plants results in accumulation of mannitol, along with an improved drought and salt tolerance in different plant species including potato [41], sorghum [40], wheat [36], and canola [42].

3.4.2. Glucose Accumulation. Glucose levels increased in salt treated wild-type plants, especially when treated with 200 nm NaCl. However, no significant differences were found in glucose accumulation between the wild-type controls and the transgenic plants among the various NaCl treatments.

Table 2: Effects of salt treatments on shoot length, shoot fresh weight, and shoot and root dry weight of *HVA1*, *mtlD*, and *HVA1-mtlD* T3 plants.

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

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

Table 3: Effects of different NaCl concentrations on accumulation of different soluble sugars in T3 versus the wild-type control plants.

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

3.4.3. Sucrose Accumulation. Similar to glucose, sucrose accumulated more in wild-type plants that were stressed by 100–300 mM NaCl, especially those treated with 200 mM NaCl. Transgenic plants exposed to 200 mM of NaCl showed less sucrose accumulation as compared to their wild-type control but higher sucrose accumulation under 100 mM and 300 mM NaCl.

3.4.4. Fructose Accumulation. Fructose accumulation increased in the wild-type controls with NaCl treatments, especially with 200 mM of NaCl. Transgenic plants expressing a combination of *HVA1-mtlD* showed significantly higher fructose accumulation under NaCl concentrations as compared with their wild-type control plants, especially at 100 mM NaCl.

4. Conclusions

Drought can be a devastating and often recurring problem in agriculture. Although salinity is not unexpected, it is also as damaging as drought stress to crops, leaving farmers with low crop yields and less farm incomes. Global warming is

now a widely accepted phenomenon which can negatively affect crops such as maize yield because warmer temperatures contribute to higher plant transpiration and soil evaporation, enhancing the impact of drought and facilitating bringing salt to the soil surface via evaporation. Therefore, it is possible that most crops will face gradual increase of drought and salinity problems.

In the research presented here, the benefits of stacking the *HVA1* and the *mtlD* transgenes in maize genome on drought and salinity tolerance were studied.

With collaboration of BASF, Monsanto recently developed a patented transgenic maize genotype called "Drought-Gard Hybrids Corn" which expresses the gene for *Bacillus subtilis* cold shock protein *(CSPB)* [58]. When the Drought-Gard Hybrids Corn was tested in multiple field stations, it produced 92 BU per acre (5774.5 kg/hectare) of yield while its control nontransgenic crop only produced 76 BU per acre (4770.25 kg/hectare) under drought conditions [59]. The research presented here confirms that maize drought and salinity tolerance can be improved via transgene pyramiding. However, multiyear multilocation field tests are needed to further confirm the drought and salt tolerance of the HVA1+mtLD transgenic plants and the application of this research at commercial scales. Development of drought and/or salinity tolerant maize can improve farmers profits in regions of the world in which the unexpected drought and/or the salinity are factors.

Abbreviations

B: Billions
ABA: Abscisic acid
BAP: Benzylaminopurine
HVA1: *Hordeum vulgaris* abundant protein gene
IBA: Indole-3-butyric acid
LB: Luria-Bertani media
MS: Murashige and Skoog *in vitro* culture medium
pBY520: Plasmid containing *bar* and *HVA1* genes
PCR: Polymerase chain reaction
RT-PCR: Reverse transcription-PCR
CspB: Bacillus subtilis old shock protein.

Conflict of Interests

As per the journal guidelines, "a competing interest exists when professional judgment concerning the validity of research is influenced by a secondary interest, such as financial gain." Hereby, the corresponding author of this paper, Mariam Sticklen, reveals no conflict of financial and/or any other interests existing in this paper and the companies that the authors have purchased chemicals and supplies from for the purpose of this research.

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