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TREHALOSE ACCUMULATION PROVIDES DROUGHT TOLERANCE TO GENETICALLY-MODIFIED MAIZE IN OPEN FIELD TRIALS

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Maize is a staple crop in many developing countries and its cultivation is challenged by both biotic and abiotic stresses. Trehalose is frequently used as a carbon source in many organisms but, its function is equally important as an osmoprotectant and signaling molecule. In this work, we explored the effect of the expression of a trehalase antisense RNA, hypothesizing the inhibition of trehalose hydrolysis would provide drought tolerance to maize. Down-regulation of trehalase mRNA in GM plants was achieved; furthermore, this resulted in higher levels of trehalose accumulation relative to wild type plants. Physiological evaluation was performed in both greenhouse and field conditions. We observed improved plant growth, flowering and grain production under abiotic stress. These results indicate that inhibition of trehalase results in a significant protective effect in maize against water stress and low temperatures, as well as a positive impact on plant growth and photosynthesis.
Keywords: Maize, drought stress, marker-assisted breeding, open field trial, trehalose, antisense RNA, MS medium
Abbreviations: AS-RNA, Antisense RNA; CaMV 35S-Promoter, Cauliflower Mosaic Virus 35S Promoter; CIEA9, Genetically-modified Maize Line; CR, Landrace; B73, Wild Type Control; CRxCIEA9, Hybrid F1; ddPCR, Droplet Digital PCR; GM, Genetically Modified; HMG13, High Mobility Group protein; mRNA, messenger RNA; ORF, Open Reading Frame; PEG, Polyethylenglycol; RT-ddPCR, Real Time Droplet Digital PCR; RWC, Relative Water Content; siRNA, small-interferent RNA; Ter-Nos, Nopaline Synthase Terminator; TRE, Trehalase.

INTRODUCTION

Drought stress has a profound negative effect on agriculture. It is one of the most important factors limiting plant growth, which in turn affects animal and human consumption (Ahuja *et al*., 2010; Gong *et al*., 2014). In agricultural areas, crop growth depends on water availability. It is neither necessarily a choice of the producer nor is controlled by consumer demand. Different experimental approaches have been developed to obtain drought-tolerant plants, such as traditional breeding, molecular marker-assisted breeding and genetic engineering (Xoconostle-Cázares *et al*., 2010).

Drought tolerance is a quantitative trait thus, several gene products are involved in determining such a trait. Several metabolites have been associated with the coordination of both physiological and biochemical mechanisms that underlie drought tolerance in certain plant species. These in principal could be employed to provide such properties to susceptible varieties. In the resurrection plant *Selaginella spp*, a drought tolerant plants, the accumulation of the disaccharide trehalose is correlated to their tolerance to abiotic stress (Adams *et al*., 1990; Müller *et al*., 1995; Van-Dijk *et al*., 2002). Trehalose is synthesized in two enzymatic steps by trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) (Cabib and Leloir, 1958; Avonce *et al*., 2004).

Trehalose levels are negatively regulated by the enzyme trehalase, which hydrolyzes this disaccharide, yielding two glucose molecules (Gibson *et al.*, 2007). Extant evidence has shown that trehalose and trehalose-6-phosphate (T6P) can also modulate other processes in plants, such as regulation of carbon metabolism and flower induction (Schluepmann *et al.*, 2003; Van Dijken *et al.*, 2004). Indeed, *Arabidopsis tps1* mutants have a delay in flowering (Wahl *et al.*, 2013), while its overexpression associated to the vasculature induces early flowering (Ruiz-Salas *et al.*, 2016). Trehalose has also been described as a modulator of photosynthetic rate by inducing the expression of photosystem II core complex proteins (psbY), Rubisco small subunit (RBCS 3B), Phosphoglycerate kinase (PGK1) and Fructose-bisphosphate aldolase (ALDP1) (Paul *et al*., 2001; Kondrák *et al*., 2011). Indeed, a truncated trehalose-6-phosphate synthase from *Arabidopsis* and

Selaginella improves its catalytic activity in yeasts (Van-Dijck *et al*, 2002).

Antisense RNA (AS-RNA) mediated gene silencing is a powerful tool to decrease the expression of desired genes in plants, fungi and animals. AS-RNA targets the endogenous mRNA, forming a double strand hybrid, thus hindering its translation, and reducing its expression (Iki, 2016). Additionally, AS-RNA can trigger post-transcriptional gene silencing, via DICER-LIKE (DCL) RNase III by recognition of double stranded RNA and cleavage into 21-24 nt fragments; one strand is then incorporated into the RNA-Induced Silencing Complex (RISC), which degrades the complementary mRNA target and amplifies the silencing signal (Fukudome and Fukuhara, 2016).

In the present work, trehalase AS-RNA was constitutively expressed in genetically-modified B73 maize (CIEA9) with the aim of decreasing trehalase levels and therefore allowing the accumulation of trehalose. Two independent lines (CIEA9.1 and CIEA9.2) were characterized and evaluated in greenhouse and subjected to drought stress. Genetically Modified (GM) maize CIEA9.1 and its isogenic B73 line were then selected for evaluation in an open field trial in Northern Mexico, under drought and cold stress. In the trial, performed in the winter of 2013, a landrace x GM (CR x CIEA9) hybrid was also assayed, showing drought tolerance under the aforementioned conditions. Maize decreasing trehalase expression by antisense RNA could accumulate trehalose, providing tolerance to drought and cold.

MATERIALS AND METHODS

***Plant materials*:** Transgenic plants of maize inbred line B73 were grown in the green house and were self-pollinated. Maize seeds were germinated in a moist chamber (Conviron S10H) at 26°C for 5 days. Later the seedlings were shifted to cylindrical pots (60 cm) containing 40% peat moss (Kekkila - Rosenlewripv), 40% soil and 20% Agrolite. The pots were placed in greenhouse at a temperature ranging from 26-30°C, relative humidity ranging from 40 to 50%, and a photoperiod of 16 h light and 8 h dark cycle. The plants were irrigated with Miracle-Grow nutrient solution during vegetative growth whereas TR Nutrigarden fertilizer (nitrogen-phosphorus-potassium (17-17-17). The immature ears were isolated 10 to 12 days after pollination as described by Frame *et al.* (2002) and Chen *et al.* (2014), and sterilized with 1.8% sodium hypochlorite, 0.1% Tween-80 for 20 min. Ears were then submerged in 70% ethanol for 10 min and rinsed three times with sterile, distilled water. Ninety immature embryos (1-1.5 mm) were excised and cultured as previously described (Armstrong and Green 1985; Chen *et al.* 2014), cultured on MS medium (Murashige and Skoog, 1962) augmented with 2 mg/L Dicamba, 3% Sucrose, 100 mg/L Inositol and 3 g/L phytagel (Sigma-Aldrich, Mexico). The embryos were maintained for one month in darkness at 26°C until their use in transformation procedures.

***Genetic transformation by biolistic procedures and plant regeneration*:** The 684 bp sequence of the trehalase-encoding ORF was obtained from the maize genome database Phytozome, accession number: GRMZM2G162690. The antisense ORF is driven by the Cauliflower Mosaic Virus 35S promoter (CaMV 35S-promoter, 835 bp) and employs the nopaline synthase terminator (Ter-NOS, 253 bp), flanked by EcoRI sites. The cassette expression unit was cloned in the T-vector pTOPO 4 (Invitrogene). B73 maize embryos were then transformed with a biolistic procedure, utilizing the high-pressure Helium PDS 1000-He® system (Biorad). EcoRI fragments (1772 bp) were bombarded on the embryogenic callus growing in axenic culture. The preparation of the tungsten particles, DNA coating and bombardment parameters were performed according to protocols described by Sanford *et al*. (1993) and Cabrera-Ponce *et al*. (1997). Transformants were selected in the presence of 4% polyethylene glycol (PEG) 8000 (Sigma-Aldrich); after the transformed embryogenic callus grew in the selection medium, they were regenerated and transferred to greenhouse for further development as described by Cabrera-Ponce *et al*. (1997).

The genetically-modified maize was named CIEA9; two independent lines (CIEA9.1 and CIEA9.2) were selected for further characterization at greenhouse., For the open field trial, the hybrid CR x CIEA9.1 was obtained and its T1 offspring was simultaneously grown with T6 CIEA9.1, their parental lines CR and CIEA9.1.

MOLECULAR ANALYSIS

***DNA extraction and detection of construct by PCR*:** Total DNA was extracted from leaves of four-weeks old, CIEA9 lines and B73 wild type control plants. The tissues were pulverized by freeze fracture with liquid nitrogen and DNA extraction was performed by employing DNeasy Plant mini Kit (QIAGEN, Hilden, Germany) according to the supplier's recommendations. To assess the presence of the construct, the detection of the CaMV 35S-promoter was performed by PCR employing the primers 35SP-F 5'-GTGGATTGATGTGATATCTC-3'' 35SP-R 5'-TGTCCTCTCCAAATGAAA-3' in the following mixture: 1x Ex Taq buffer, 2.5 µM dNTPs mixture, 0.25 µM of each primer, 100 ng of genomic DNA and 1U Takara Ex Taq HS enzyme (Takara, Japan). Conditions for thermocycling were as follows: 94°C, 1 min and 30 cycles of 94°C, 30 sec; 60°C, 30 sec, 72°C, 30 sec. The amplified product was resolved by electrophoresis on a 1% (w/v) agarose gel and visualized by ethidium bromide staining. The images were digitized with Kodak Digital Science EDAS 120.

***Determination of transgen copies inserted in transgenic maize CIEA9*:** Determination of transgenes copies in

CIEA9was performed by droplet digital PCR ddPCR), employing the QX200 droplet-reader (Bio-Rad) in 20 µl ddPCR reaction mixture (containing 100 ng total DNA, 900 mM primers and 250 nM probe). The primers used for amplifying CaMV 35S-promoter were 35Sdd-F 5´-CCTCTGCCGACAGTTGTCCCAA-3´, 35Sdd-R 5´-TGCGAAGGATAGTGGGAT TGTGCG-3´ and 35Sdd-Probe 5´-(FAM) TGGACCCCCACCCACGAGGAGCA (BHQ1)-3´. High Mobility Gene HMG13 was employed as a maize endogenous gene, employing the primers HMG1-F 5´-TTGGACTAGAAATCTCGTGCTGA-3´, HMG1-R 5´-GCTACATAGGAGCCTT GTCCT-3´ and HMG1-Probe 5´-(HEX)-CAATCCACACAAACGCACGCGTA-(BHQ1)-3´. Thermocycling was performed in a Biometra PCR device with the following settings: 94°C, 10 min (1 cycle); 94°C, 30 sec and 60°C, 1 min (45 cycles); 94°C, 10 min (1 cycle).

Digital quantification of maize TRE transcript was assayed by RT-ddPCR, using RNA extracted with the Direct-zol TM RNA Kit (Zymo Research) and QX200 droplet-reader (Bio-Rad). Total RNA 10 ng were assayed in 20µl of the One-Step RT-ddPCR reaction mix. The final concentrations were 900 mM for the primer and 250 mM for the probe. The primers used for target gene TRE detection were TRE-F 5´-GCATGAAGTAATCCAGATG-3´, TRE-R 5´-GAAGCCTAACTCACAGAA-3´ and probe TreAS-probe 5´-(FAM)-ACTTCGTTCCGCTGTGGCTA-(BHQ1)-3'. The thermocycler settings were 60°C, 30 min (1 cycle); 94°C, 5 min (1 cycle); 94°C, 30 sec and 60°C; 1 min (40 cycles); 94°C, 10 min (1 cycle).

***Quantification of Trehalose in plant tissues*:** Leaves were sampled from fully-irrigated and drought-stressed CIEA9 and B73 wild type plants. 1 g of plant tissue was ground in 1 ml of 10 mM Tris-Cl pH 7. Homogenate was centrifuged to obtain the aqueous phase. Trehalose was extracted with a chloroform-methanol mixture using the protocol described by Lunn *et al*. (2006). The trehalose was quantified by using a modified fluorometric assay (Carillo *et al*., 2013) with the following modifications: the trehalase reaction was incubated at 37°C for 30 min with trehalase concentration at 0.1 nkat trehalase and standard solutions containing 1.0-4.0 µM trehalose. The trehalose reaction was measured using a Qubit® 3.0 Fluorometer (Life-Technologies).

***Field trial and crop phenology*:** A Field trial was performed in Concordia City, State of Sinaloa, Mexico (Latitude 23.332621 and Longitude -106.078915), and started on October 26, 2012 according to the federal permit 0027/2011, issued by the Mexican federal authorities. The classification system used to evaluate the phenology development was the Iowa State University system based on the plant vegetative and reproductive development (Fassio *et al*., 1998).

***Meteorological conditions*:** Weather conditions during the field crop were based on the report by CONAGUA (www.conagua.gob.mx). In addition, a datalogger system (Thermotracker^{TM}) to monitor temperature and humidity was installed in the field trial, set to register every hour 24h/7d during all the trial.

***Plantation design in the open field trial*:** Cultivation area was divided in 8 m^2 blocks, and 1 kg of soil was sampled from every block to determine its composition. Planting frame was designed to employ 8m^2, containing 60 plants, considering each plant as an experimental unit. Four blocks were employed as replicates for each treatment.

***Drought stress assay*:** Drought stress was applied in 8 m^2 sections by quadruplicate, applying 40, 50, 60 or 70% of the normal irrigation recommended for the region. Relative water content (RWC) was measured in the soil of each treatment by triplicate. After irrigation, the actual RWC was calculated with the following equation:

$$RWC = \frac{(FW - DW)}{(SW - DW)} * 100$$

where FW is the fresh weight of soil samples, SW the saturated water weight of samples and DW stands for the dry soil weight after drying for 12 h at 105ºC.

***Photosynthesis measurements*:** The photosynthesis rate was measured in fully expanded flag leaves using the LI-6400 XT Portable Photosynthesis System XT (LICOR, Lincoln, NE). For this non-destructive technique, each leaf was introduced in the chamber and measured twice after one minute of stabilization. Data collected from the plants were averaged in each block. These measurements were performed under a constant light intensity of 1500 mol/m^2/s, like the detected light intensity in the field. The temperature registered during the measurements was 26°C within the equipment chamber.

***Harvest of maize kernels*:** Maize cobs containing 16-18 % RH were manually harvested; then their husks were removed and weighed. Cobs were also photographed to characterize their color and seed content.

***Bromatological and toxicological analyses*:** Bromatological analyses of maize flour of previously ground seeds were referred to an authorized laboratory (*Laboratorio de Constatación Agroindustrial*, Mexico City). The toxicological acute analysis was evaluated and approved by the Biosafety and Ethical Committee of our Research Center and held during 30 days in our experimental animal facility. For this purpose, fifty, 6-8 week old female BALB/c mice were evaluated. Body weight of each mouse was initially registered, and a blood sample was withdrawn from the vein tail by puncturing with a syringe for hematic biometry evaluation. Mice were fed with diet supplemented with GM and conventional maize flour at free demand. After 30 days, mice were weighed, and blood samples were also obtained for further analyses. Animals were euthanized with CO_2 according to the authorization of the Biosafety and Ethical Committee.

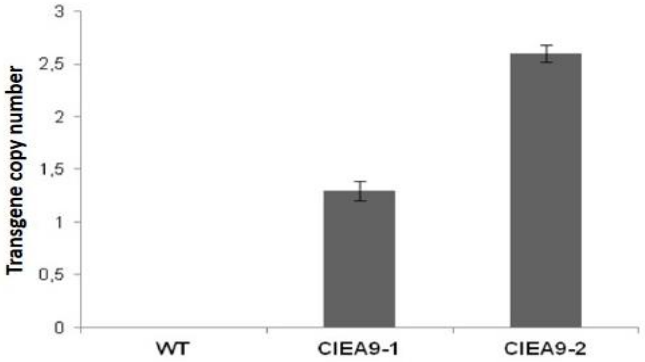
***Statistical model and analysis*:** The data were analyzed by means of two-way ANOVA using Excel 2010 software. Averaged data, with similar standard deviation were

compared using a Tukey test (HSD) with a confidence interval of 95% (alpha < 0.05).

RESULTS

***Selection of maize calli transformed with a trehalase AS construct*:** Introduction of an antisense trehalase DNA construct into maize, which corresponds to the antisense DNA sequence from positions 54 to 737 bp of the maize trehalase (TreAS) open reading frame (Genbank accession no. NM_001153335.1) fused to the CaMV 35S promoter and the nos terminator (Ter-NOS), was performed in order to reduce trehalase mRNA and protein levels (Fig. 1a). Of note, this construct is devoid of marker genes, and that harbored by the construct lacks the potential for protein coding, namely TreAS. This construct was delivered via biolistics to B73 embryogenic calli, shown in (Fig. 1b); these were then selected using 4% PEG-8000 (Sigma-Aldrich) in MS medium, where clear calli were observed; in contrast smaller, phenolized calli, presumably not transformed, are also present (Fig. 1c). Maize embryos capable of growing in selective medium were regenerated and the resulting plants were grown in the greenhouse to obtain transgenic seeds for further characterization (Fig. 1d). After flowering and self-fertilization, cobs were obtained, with a yellowish color, similar to that of wild type B73 line. (Fig. 1e).

***Determination of transgene copy number in transformed plants*:** Wild type and transgenic maize were analyzed to determine the number of inserted transgenes in their genome. The use of droplet digital PCR (ddPCR) to quantify the CaMV 35S promoter (221bp) and endogenous maize gene HMG13 (101 bp) was assayed to determine the presence of a single insertion in CIEA9.1. In the second line CIEA9.2, two copies per genome were detected (Suppl. Fig. 1). The selection of CIEA9 for further studies was based on the presence of a transgene single copy, considering that a second copy could provoke complete gene silencing.



Suppl. Figure 1. Transgene copy number. Quantification of transgene copy number was assayed by droplet digital PCR. CaMV 35S promoter and HMG endogenous maize gene were quantified in B73 wild type maize, and the independent GM lines CIEA9-1 and CIEA9-2. Ratio of CaMV 35S promoter and HMG are graphed. Standard Deviation is indicated from three independent replicates.

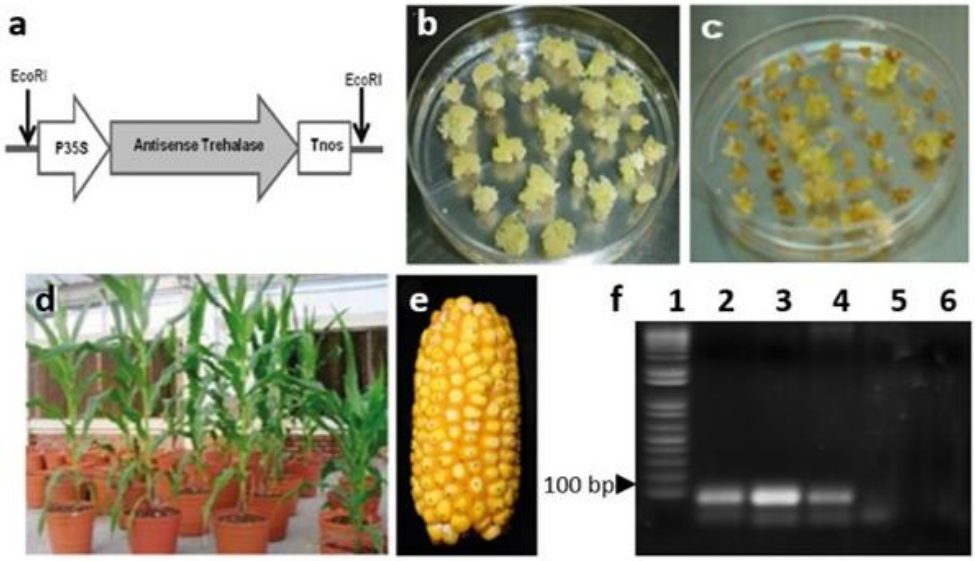


Figure 1. Development of transgenic maize by biolistic transformation. (a) Representation of the casette encoding the CaMV 35S-Promoter, the antisense sequence of trehalase gene, and Ter-Nos, flanked by *Eco*RI sites. (b) Embryogenic B73calli bombarded with the aforementioned construct. (c) Embryogenic calli selection with 4% PEG after particle bombardment. (d) Regenerated plants in the greenhouse. (e) Cob with seeds obtained from transgenic plants in greenhouse. (f) Agarose gel showing the 100 bp PCR fragment of CaMV 35S promoter; 1, Molecular weight marker ladder 1 kb; 2, Positive control; 3, Total DNA CIEA9.1; 4, CIEA9.2; 5, Wild Type B73; 6, Non-template control.

***Expression level of trehalase and trehalose accumulation*:** Absolute levels of trehalase mRNA in control and transgenic plants were determined by RT-PCR using ddPCR (Fig. 2a). Trehalase mRNA levels in transgenic plants were lower than in control plants. However, the accumulation of trehalose to higher levels did not cause aberrant phenotypes, as those observed in Arabidopsis and other plants when trehalose was obtained by trehalose synthase activities (O'Hara *et al*., 2013).

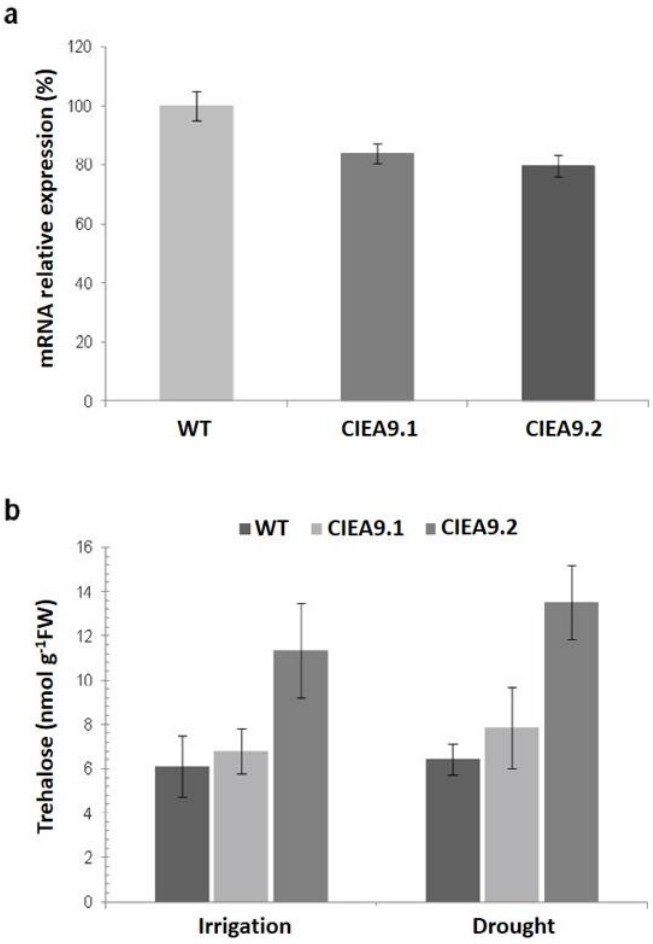
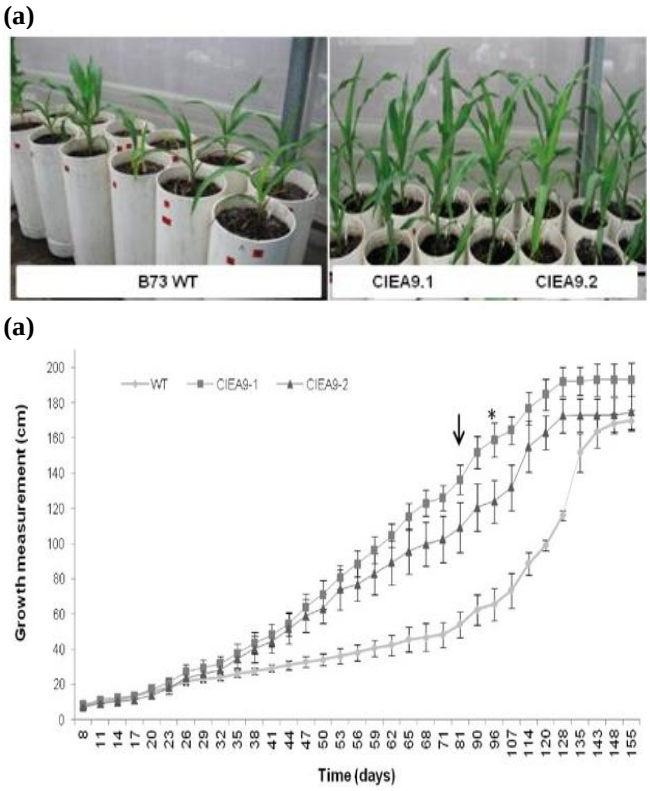


Figure 2. Expression levels of trehalase encoding transcript and trehalose accumulation. (a) Accumulation levels of trehalase mRNA in Wild Type andtransgenic CIEA9.1 and CIEA9.2 plants, by RT-ddPCR(b) Trehalose content in maize leaves grown under irrigation and drought stress. SD bars are indicated in each treatment.

Trehalose accumulation was determined using a fluorometric assay on samples of irrigated transgenic and control plants, as well as those under drought conditions (Fig. 2b). In fully irrigated conditions, the control and CIEA9.1 transgenic plants did not show significant differences in trehalose content. Conversely, in CIEA9.1 and CIEA9.2 lines under drought treatment, a higher trehalose content was detected. CIEA9.2 accumulated higher levels of trehalose in both full irrigation and drought regimes.



Suppl. Figure 2. Genetically-modified maize performance under drought stress in the greenhouse. (a) Left panel, B73 Wild Type displaying 4 leaves (Stage V4); right panel, CIEA9.1 and CIEA9.2 displaying 6-7 leaves (Stage V4-5). (b) Maize phenology under drought stress in the greenhouse. Arrow indicates start of drought, during tassel and cob initiation (Stage VT). Asterisk indicates kernel development in both lines of GM maize.

***Genetically-modified maize under drought stress*:** GM maize CIEA9 and its isogenic line B73 wild type maize (B73 WT) were grown in a greenhouse to evaluate the effect of limited watering on their phenology. (Suppl. Fig. 2a). Plant height was monitored, registering CIEA9 plants were taller, relative to control plants. Maize is more susceptible to drought stress during germination and in late vegetative development (before pollination and kernel development). Drought stress was applied in late vegetative development, interestingly, even during drought treatment (81 to 98 days post germination) plants were still capable of sustaining growth (Suppl. Fig. 2b). After drought treatment, transgenic plants were starting the development of flowering (phenological stage VT), while control plants were still in vegetative growth stage, developing leaves (phenological stage V12). Drought treatment was suspended after thirteen days, in which wild

type plants shown drought-associated symptoms, consisting in chlorosis and loss of turgor. In contrast, these symptoms were milder in CIEA9.1 plants, and barely visible in CIEA9.2 GM maize (Data not shown).

Drought, photosynthetic parameters and productivity in open field trial: GM maize CIEA9 showed a higher capacity to tolerate drought in the greenhouse as described above. Furthermore, they were able to continue their growth, presumably through higher levels of trehalose accumulation. Mexico is a signatory of the Cartagena protocol, and a Federal law regulates the planting of Genetically Modified organisms (GMO) in open field trials. GM maize was characterized, and a risk assessment was performed, selecting an ecorregion located in Concordia, Sinaloa, a northern state where no maize landraces have been identified. To test whether a similar phenomenon occurred in the field a replicate were performed in randomized blocks (Fig. 3a,b), using the CIEA9, hybrid F1 (CR x CIEA9), landrace (CR) and parental B73 wild type (B73) plants (Fig. 3c,d,e,f). A biological barrier was employed by planting the maize one month after the planting of conventional maize, located at least at 5 km. A physical barrier consisted of maize of the region, known as CR (Criollo Rendidor), a high yield landrace. Soil analysis shown a composition of 17% clay, 32% silt and 51% sand, classified as loamy sand soil, with low water retention, according to

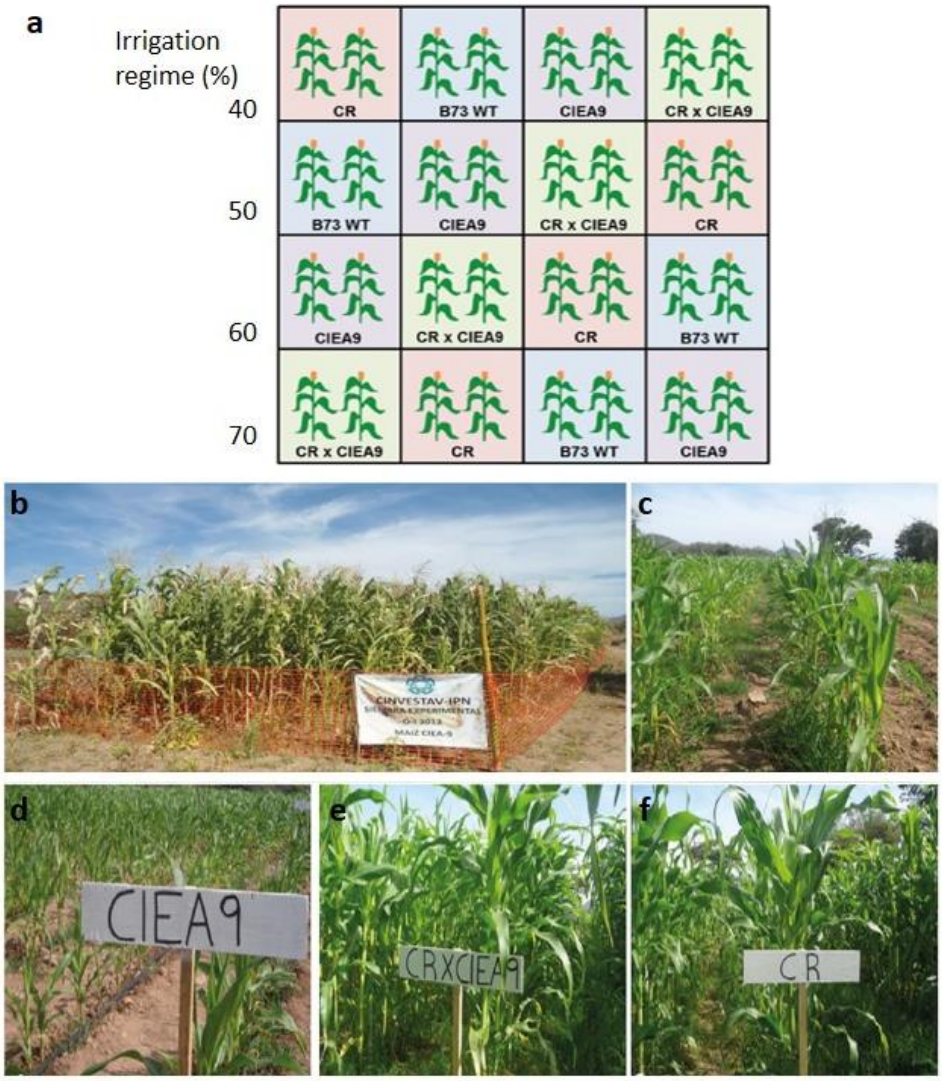
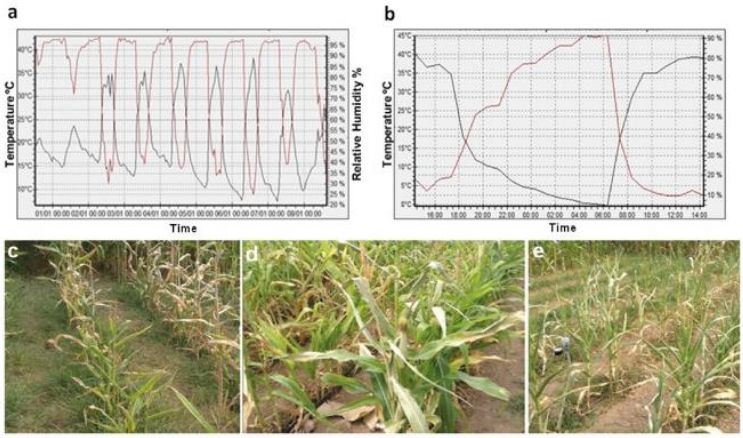


Figure 3. Open field trial of transgenic and control maize plants. (a) Experimental planting area where different irrigation treatments were applied (70, 60, 50 and 40%, respect to the normal irrigation in the area) and location of the following genotypes: CR, regional landrace; B73 WT, Conventional B73; CIEA9, GM CIEA9.1 maize; CR x CIEA9, hybrid. (b) Overview of the plantation. (c) B73 control plants in field (Stage V7, cob and tassel initiation). (d) CIEA9 transgenic plants in field (Stage V8, Cob development). (e) Hybrid CR x CIEA9 in stage V8. (f) Regional Landrace CR in V7 stage.



Suppl. Figure 3. Effect of low temperatures in maize growing in the open field trial. (a) The temperature oscillated between 12 and 31°C. (b) The lowest temperature registered was minus 2°C. (c, d and e) An overall damage on leaf tissue was recorded, however, cob initiation and kernel development continued in GM maize (CIEA9 and CR x CIEA9).

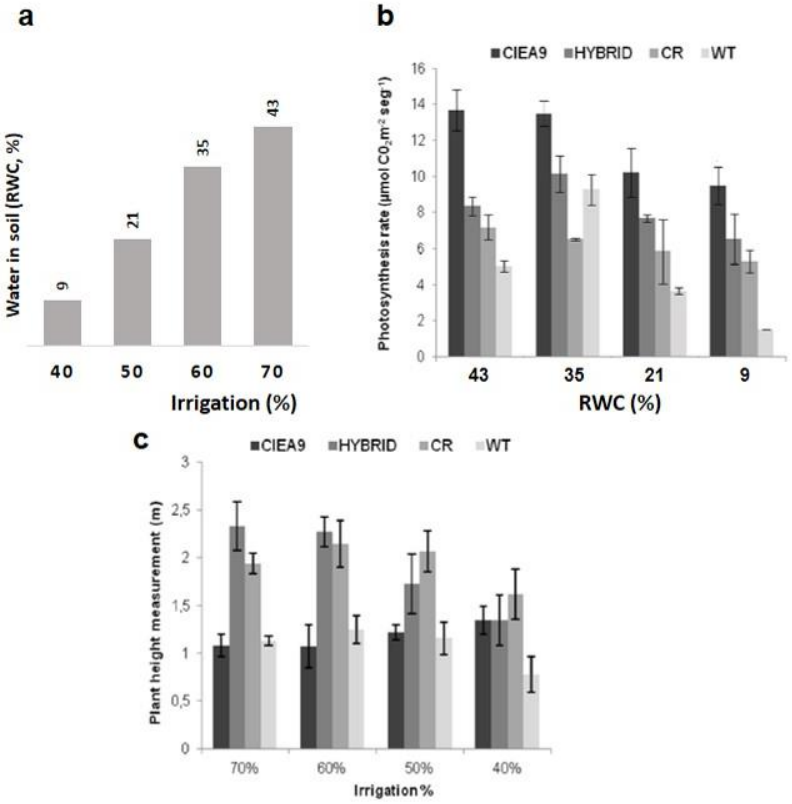


Figure 4. Evaluation of GM maize. In the open field trial. (a) Soil water content. Irrigation regimes assayed respect to the water usage in the region (70%, 60%, 50% and 40%), related to actual relative water content (RWC) in soil. (b) Photosynthesis in genotypes CIEA9; Hybrid (CR x CIEA9); CR, Landrace of the region; WT, Wild Type B73tested under decreasing water regimes. of. (c) Plant height recorded under different water regimes. SD is indicated for each bar. Data are average of 10 biological replicates.

Kaufmann and Cleveland (2008). Temperature and relative humidity measurement in the field was registered, recording in November and December 2012temperature range of 14.2°C to 30.4°C. From January to May, temperatures oscillated between 12.4°C and 31.5°C, and no rainfall was registered during the trial. In January 2013, a mass of cold air hit the northern states of Mexico, which caused average minimum temperatures between -5 and 0°C. On February 1st,

2013 Southern Sinaloa suffered 5 to 10 days of frost, with a recorded temperature below 0°C, registered in the field.

Interestingly, while B73 wild-type (WT) plants displaying seven leaves, with tassel initiation phenology stage V7, 70 cm), GM CIEA9 maize had already developed tassels and started cob development (phenology state V8, height 66 cm). Furthermore, the hybrid CIEA9.1 x CR developed cobs (phenology V8 stage, height 165 cm) inheriting the size of CR. Wild type CR developed to V7 stage, with height 120 cm) Randomized blocks were treated with different irrigation regimes (70%, 60%, 50% and 40%) representing several degrees of RWC in soil (Fig. 4a). The application of 40% irrigation represented an actual RWC in soil of 9%, which corresponded to severe drought. In addition, an irrigation of 70% and 60% represented an actual RWC of 43% and 35% respectively. Photosynthetic capacity decreased with low watering regimes; however, despite an overall photosynthetic decrease, the transgenic and hybrid plants exhibited higher photosynthetic rates compared to control and CR plants, in all irrigation conditions tested (Fig. 4b). Plant height was also registered in the field trial, in which the transgenic line maintained its growth at 70% to 40% tested irrigation; however, control wild type B73 and the landrace CR developed a smaller height at irrigation of 50 and 40% of the normal watering in the region. The hybrid CIEA9.1 x CR also displayed an impairment in its growth at lower irrigation status (Fig. 4c).

Analysis of the maize phenology indicated that tassels in GM plants appeared twenty-one days earlier than in WT plants; meanwhile tassels emerged on average 11 days after flower emergence in GM maize. Accelerated development is an important agronomic trait because it could allow shorter agricultural cycles, which translate into saving culturing time and its associated field labor, to reach similar crop productivity. During the field test described here, freezing temperatures were recorded once (-2°C) (Suppl. Fig. 3a,b) although during the rest of the trial the temperature oscillated between 12°C and 31°C (Suppl. Fig. 3a,b). Temperature and humidity were monitored at the center of the field. Although the air current affected the actual temperature, which was heterogeneous due to the topography of the terrain, some parts were more damaged by frost, which were in most cases located at the southeast end of the field. However, genetically modified plants and hybrids showed less damaged stems, in contrast to unmodified materials, which began to dry out due to the frost (Suppl. Fig. 3c,d,e).

Transgenic and hybrid plants showed normal grain filling at 70% irrigation (Fig. 5a), compared to CR and control plants, which had less grain filling and were more affected by the frost. Indeed, transgenic plants exhibited more grain development, even in low irrigation (40%). In the cultivation area there was a higher productivity in terms of seed filling in transgenic and hybrid plants compared to the wild type plants, especially at 70% and 60% of irrigation (Fig. 5a). Landrace CR and control wild type B73 yielded very few cobs with seeds at irrigation of 40%. The calculation of productivity consisted in weighing the grains detached from the cobs, adjusting their humidity to 17% and calculating the productivity in one hectare (Fig. 5b).

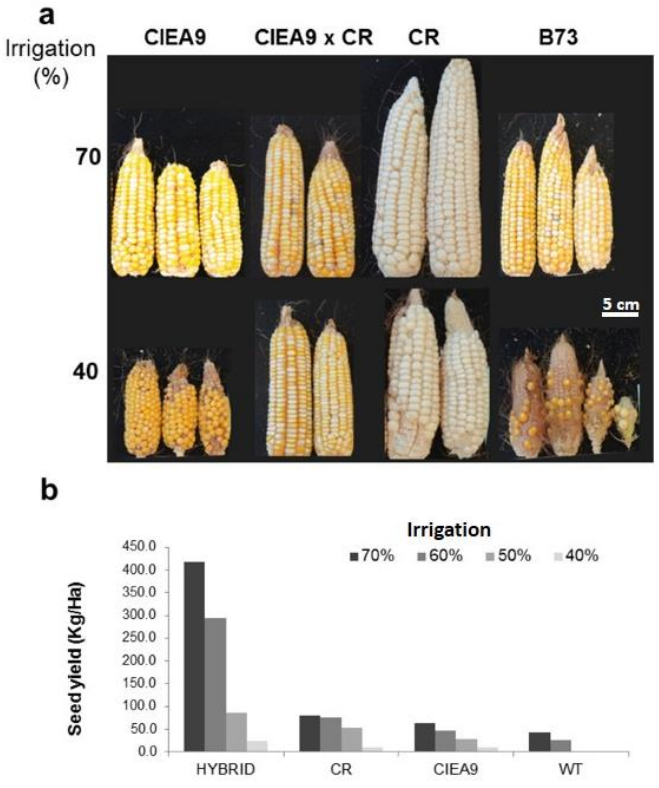


Figure 5. Cobs developed in different levels of irrigation. (a) GM CI9EA Hybrid CIEA9 x CR; Regional landrace CR; Wild Type B73. (b) Theoretical productivity of genotypes tested in the field trial under drought stress (70, 60, 50 and 40% of normal irrigation).

***Bromatological and toxicological analysis*:** Bromatological analyses were performed on corn grains of transgenic and control plants, showing similar levels of nutritional content (Fig. 6a) without a significant difference between the grains ($p<0.05$). Toxicological analyses were performed using 6-8 week old female BALB/c mice; in a 30 day subchronic assay, where no significant statistical differences were found in neither the weight records nor the hematic biometry analysis ($p<0.05$). The livers of mice were macroscopically analyzed, and no differences were identified when compared to livers of mice fed with conventional maize.

Bromatology assays included quantifying protein, fiber, ash, humidity, fat, and digestible protein content, in which no statistical differences were registered (Fig. 6b,c,d). Hematic biometry quantified erythrocytes, hemoglobin content,

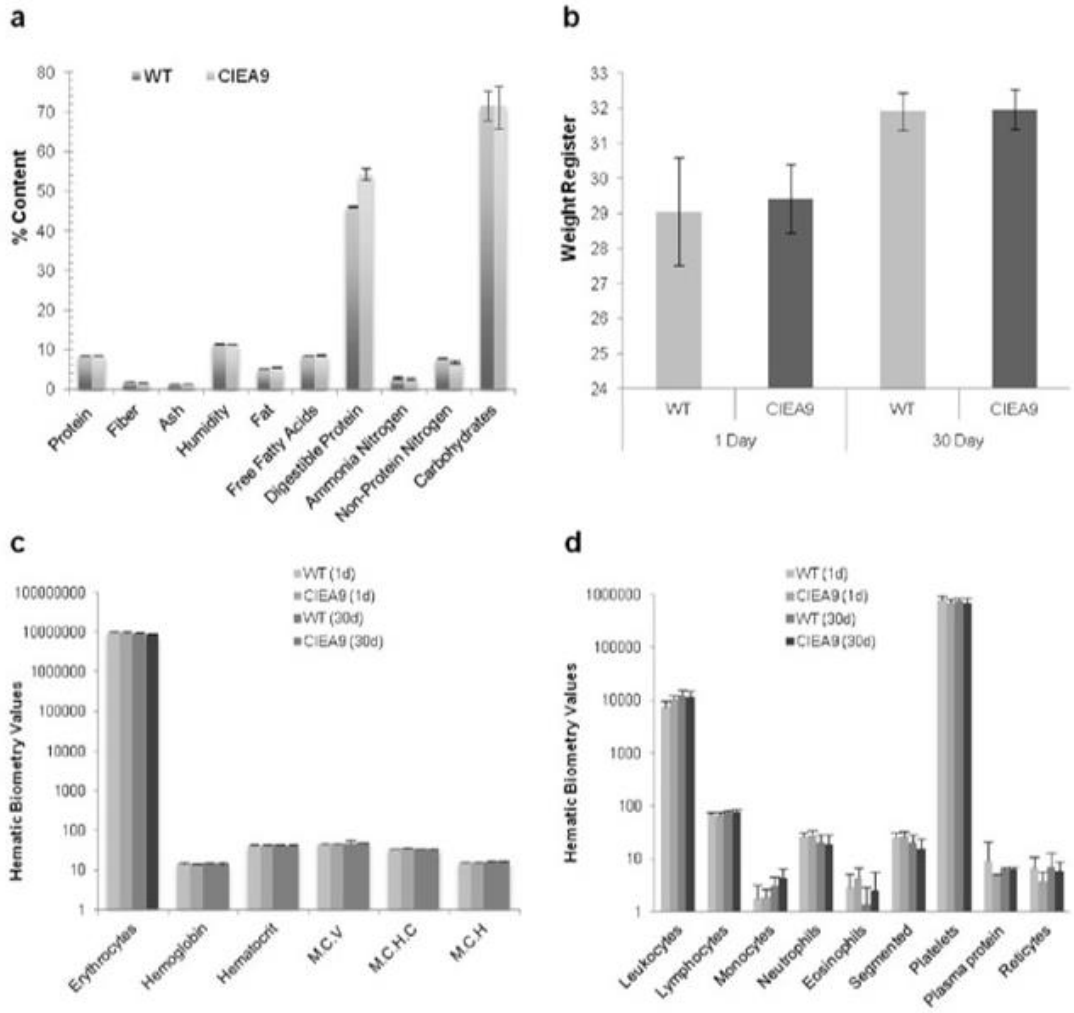


Figure 6. Bromatological and toxicological analysis of GM seeds. (a) Bromatology of GM CIEA9 and its isogenic line B73 Wild Type. Evaluated traits: Protein, Fiber, Ashes, Humidity, Fat, Free fatty acids, Digestible protein, Ammonia nitrogen, Non-protein nitrogen, Carbohydrates. (b) Semi-chronic toxicological test in BALB/c mice, fed with WT and GM CIEA9 maize, comparing body weight at day 1 and 30 days. (c) Biometry of blood red line cells. Erytrocytes, Hemoglobin, Hematocrit, M.C.V. (Mean Corpuscular Volume); M.C.H.C. (Mean Corpuscular Hemoglobin Concentration); M.C.H. (Mean Corpuscular Hemoglobin). (d) Biometry of blood white line cells. Leukocytes, Lymphocytes, Monocytes, neutrophils, Eosinophils, Segmented cells, Platelets, Plasma protein and Reticulocytes. Standard Deviation is indicated. Experimental values were compared with Tukey (HSD) Test employing a confidence interval of 95% ($p < 0.05$).

hematocrit, M.C.V, M.C.H.C, M.C.H., leukocytes, lymphocytes, monocytes, neutrophils, eosinophil, segmented cells, platelets, plasma protein content and reticulocytes. Basophils, Myelocytes and juvenile Neutrophils were not included in the graphics of the white formula, since the values in these cases were negligible (close to zero), with no statistical difference.

DISCUSSION

***Decrease in trehalase mRNA can modulate phenological development*:** Biolistic transformation of embryogenic calli was carried out to obtain complete plants that express a trehalase AS-RNA, to inhibit the expression of the trehalase gene. At the same time, this construct was used as a selective marker, since putative transformants were able to grow in the presence of PEG, which simulates drought (Liu *et al*., 2014), thus eliminating the necessity to use selection markers based in antibiotic or herbicide resistance.

Genetically-modified plants continued to grow even when exposed to drought conditions. GM maize developed both male and female inflorescences earlier than control plants in greenhouse conditions. It is known that water deficit during pollination increases the frequency of kernel abortions in

maize and inhibition of ovary growth, which is associated with decreased levels of reducing sugars, depletion of starch and an increase in sucrose concentration (Zinselmeier *et al*., 1995). A water deficit during pollination could thus prevent the synthesis and movement of sugars to the seed and disturb grain filling. This same phenomenon could affect the production of seeds in the CR and wild type control plants in the field, which is compounded by the effects of low temperatures in the cultivated area.

***Higher trehalose content correlates with a decrease in trehalase expression levels*:** The detection of lower levels of trehalase mRNA levels suggested a direct relationship with the expression of the antisense mRNA. Trehalose content was measured by using a fluorometric assay in CIEA9 plants. In the independent transgenic line CIEA9.2, but not in 9.1, even though it had decreased mRNA levels like 9.2, trehalose content was higher than control maize under fully irrigated and drought conditions. Therefore, as demonstrated, the TreAS construct was able to inhibit the accumulation of endogenous trehalase mRNA and the synthesis of the trehalase enzyme; thus, allowing the accumulation of trehalose. Other strategies have approached the accumulation of trehalose by overexpressing trehalose synthase (Nuccio et al., 2015) identifying drought-tolerance phenotypes consistent with the results present in this research.

***Drought effects on development, photosynthetic parameters and field productivity*:** An outstanding feature of the transgenic material was the observed early flowering, both in greenhouse and field conditions, as well as the higher rates of seed production (mostly in the transgenic hybrid), compared to the controls. This trait is promising from an agronomic standpoint, as it could shorten the agricultural cycle of maize and, in principle, other crops, and can simultaneously reduce production costs, considering less culture maintenance will be employed. A decrease in the percentage of irrigation generated low levels of RWC in soil, leading to several stress symptoms such as turgor loss and necrotic leaves, which can be considered as effects of drought. The most affected plants by water deficit and frost were the controls and CR. These plants exhibited a decrease in the relative humidity, which has a direct effect on photosynthesis. Conversely, CIEA9 and CR x CIEA9 plants showed higher rates of photosynthesis, even under drought; this finding agrees with the work of Guo *et al*. (2010). Other research groups (Rodríguez-Salazar *et al*., 2009; Garg *et al*., 2002) reported that the accumulation of trehalose in transgenic plants gave rise to increased drought resistance and mitigated its physiological deleterious effects. Given the low endogenous levels of trehalose in plants, it is likely that the observed effects could also be related to a signaling mechanism for drought adaptation and tolerance. Indeed, a body of evidence indicates that trehalose-6 phosphate is involved in modulating the response to biotic stress and during inflorescence development growth; while trehalose appears to be involved in response to abiotic stress, as water deficit. The maize plants expressing trehalose phosphate phosphatase (TPP) in ears showed increased yield in fully irrigated plants and under drought conditions (Sheen, 2014; Nuccio *et al*., 2015). However, plants expressing TPP in all tissues showed a deleterious phenotype, in contrast to the expression of trehalase mRNA, as shown in the present work.

One of the most common responses to drought stress in plants is stomatal closure (which leads to reduced transpiration rate) and decreased water conductance since it is the most efficient way to reduce water loss. This physiological adaptation could be related to the observed mechanism in CIEA9 transgenic and control plants. Indeed, the transgenic and hybrid plants did not reduce photosynthesis drastically, suggesting its ability to continue the process of photosynthesis and water transport under drought conditions. This may be due to increased accumulation of trehalose in transgenic plants, likely acting also as an osmoprotectant. Moreover, this suggests that GM plants required less water for their development, and that trehalose accumulation afforded tolerance to drought stress. Thus, trehalose could well have an important role in carbon assimilation and fixation, as well as in stomatal closure, either direct or indirect.

The observed cold tolerance was a rather unexpected response evidenced by cold fronts that occurred during sowing (on March 2nd and 3rd), where the ability of plants to survive at low temperatures was evident as they could produce female flowers. The control and CR plants were stunted, exhibited bigger necrotic areas in leaves, and high levels of anthocyanin accumulation, in contrast to hybrid and GM plants. Maize is very sensitive to low temperatures; indeed, germination, seedling growth, leaf development and productivity can be affected. Exposure to low temperatures (below 18°C) is known to promote an accumulation of anthocyanins 30 times higher than the levels in plants at 23°C (Pietrini and Massacci (1998). In fact, anthocyanin accumulation is linearly related to the performance of photosystem II and CO_2 fixation. Foyer *et al*. (2002) reported the damage caused by low temperature occurring mainly in maize chloroplasts, thus resulting in the inhibition of photosynthesis and, consequently, premature plant senescence.

***Bromatological and toxicological analyses*:** Seeds of transgenic plants were subjected to bromatological analysis and showed no statistically significant differences in the content of protein, fiber, ash, humidity, fat, digestible protein, among other parameters, between GM and wild type plants. The toxicological analysis performed in mice in the present research, demonstrated the absence of significant changes between weight or blood count; in addition, the visual inspection of mice liver did not show anatomical abnormalities. These findings suggest that plant modification resulting in the accumulation of trehalose in GM maize did not cause bromatological nor toxicological changes in the final product compared to the controls.

***Conclusions*:** The overall results indicate that the accumulation of trehalose in plants via down regulation of trehalase (in the present case, via expression of trehalase AS RNA) is a suitable strategy to cope with drought and cold conditions in maize, which could also be applied to other important crops.

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