Plant Omics Journal POJ

POJ 7(2):94-101 (2014) ISSN:1836-3644

Stress-inducible expression of a gene encoding C-repeat binding factor 4 (CBF4) from Arabidopsis improved performance of transgenic maize under drought condition

Huawen Zou[1,3†], Xiaohan Wang[1, 2†], Conglin Huang[1], Jinsong Chen[1,2], Xiuhai Zhang[1], Chang Luo[1], RongYu[2], Zhongyi Wu[1,*]

[1]Beijing Agro-Biotechnology Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China
[2]College of Life Sciences, Capital Normal University, Beijing 10048, China
[3]College of Agriculture, Yangtze University, Jingzhou 434023, China

***Corresponding author: wuzhongyi@yahoo.com**
†These authors contributed equally to this work.

Abstract

Drought is one of the significant limiting factors affecting crops yields in the world. AtCBF4 (C-repeat binding factor 4) is a homolog of the CBF/DREB1 (dehydration-responsive element-binding protein 1) transcription factors that plays important roles during drought tolerance. Here, we report the functional characterization of transgenic maize (inbred line Mo17) expressing *AtCBF4* under the control of RD29A (responsive to dehydration 29A) promoter from Arabidopsis. Some morphological and physiological traits related to drought tolerance such as relative water content (RWC), proline and MDA (malondialdehyde) content, etc. were measured. The results show that expression of *AtCBF4* in maize significantly enhanced drought tolerance. Under dehydration stress, *AtCBF4* transgenic plants L3, L4 and L8 showed lower cell membrane damage (decreased 21-24%), higher relative water content (increased 7-12%), higher total soluble sugars (increased 7-29%), higher proline content (increased 23-37%), better growth and development, greater biomass and higher grain yield (increased 20-25%), compared to wild type. Furthermore, the transgenic lines kept significantly higher germination index and drought tolerance index under PEG condition. These results strongly indicate that expression of *AtCBF4* could increase drought tolerances in maize.

Keywords: Transgenic maize, *AtCBF4*, drought tolerance, grain yields.
Abbreviations: CBF_C-repeat Binding Factor; DREB_dehydration responsive element binding; RD29A_responsive to dehydration 29A; PCR_reverse transcription-polymerase chain reaction; ABA_abscisic acid; MDA_malondialdehyde; RWC_relative water content; CRT_C-repeat; EREBP_ethylene-responsive element binding protein; CaMv_cauliflower mosaic virus; PEG_polyethylene glycol; FW_fresh weight.

Received: 29 Sep 2013; Revised: 8 Jan 2013; Accepted: 16 Jan 2014.

Introduction

During growth and development, plants often encounter various environmental stresses such as high or low temperature, drought and salinity, which are important factors limiting agricultural production. Many countries in the world are suffering from water shortage and the increasingly serious drought problems. However, transgenic plants can produce new breeding resources with resistance to drought, cold and other stresses.

The responses to stresses in plants are regulated by multiple signaling pathways (Knight et al., 2001), and the responses to different stress factors can induce an overlap in the patterns of genes expression such as the transcription factors. Transcription factors are a group of important regulator genes, which can build a complicated regulating network in plants and regulate gene expression at different time and environment (Chen et al., 2002). Dehydration Responsive Element Binding Protein/C-repeat Binding Factor (DREB/CBF) can specifically bind cis-acting element Dehydration-Responsive Element/C-repeat (DRE/CRT). The DRE/CRT cis-acting elements exist in the promoter of some genes and are usually activated by low temperature, water deficit or high salinity. The core sequence of DRE/CRT cis-acting elements is CCGAC (Hsieh et al., 2002). The CBF/DREBs family belongs to the AP2 super family, and all of them contain a typical AP2/EREBP (ethylene-responsive element binding protein) DNA-binding domain (Oh et al., 2007). To date, 4 members are found in *CBF* family. *CBF1*, *CBF2* and *CBF3* are essential for low temperature responses (Gilmour et al., 1998). They are proved to be induced within 15 minutes when exposed to low temperature. Furthermore, studies show that plants overexpression of Arabidopsis *CBF* genes leads to the increased freezing tolerance in transgenic plants (Gilmour et al., 2000; Pino et al., 2007).

CBF4, another homolog of CBF/DREB1 genes from Arabidopsis, is different from the above 3 *CBF* genes. It is proved to be induced by ABA and drought but not by low temperature. Moreover, *CBF1*, *CBF2* and *CBF3* are proved to be involved in ABA-independent signaling pathway while *CBF4* is involved in ABA-dependent signaling pathway (Haake et al., 2002). Besides ABA, drought and low temperature, transcription factor *DREB/CBF* is proved to be induced by adverse stimulation; thus, can activate a series of genes which depend on the function of DRE/CRT cis-acting

element. Consequently it can enhance low temperature, water deficit and high salinity tolerance (Agarwal et al., 2006). The transgenic Arabidopsis over-expressing *CBF4* under the CaMv (cauliflower mosaic virus) 35S promoter were more tolerant to low temperature and water deficit, indicating that it plays important roles in the drought signal transduction pathway (Haake et al., 2002). The CaMv 35S, as a constitutive promoter element, drives the gene over-expression all the time, but many reports described that expression of CBF/DREB often produces smaller transgenic plants (Haake et al., 2002; Hsieh et al., 2002). It seems to be a good choice to substitute RD29A for CaMv 35S promoter (Kazuko et al., 1993). Stress inducible RD29A promoter is expressed at a low level under normal conditions which has minor effects on plant growth, but it is strongly induced by each of the stress treatments. The induced RD29A could provide an even greater tolerance than that of CaMV 35S promoter (Kasuga et al., 1999).

To gain comprehensive insight into the biological function of AtCBF4 gene in transgenic maize, here, we report a detailed analysis of gene transformation and some morphological and physiological traits related to drought tolerance. The primary mechanism of *AtCBF4* gene tolerance to drought is discussed.

Results

Molecular characterization of transgenic maize

The expression vector (Fig.1) was transferred into maize wild-type Mo17. After herbicide selection, 20 individual herbicide resistant lines were obtained from T_1 seeds. PCR (reverse transcription-polymerase chain reaction) analysis from T_1 seedling suggested that most herbicide resistant plants possessed the *AtCBF4* gene (date not show). The T_2 plants of three independent lines L3, L4 and L8 were confirmed to be homozygous lines by herbicide screening. DNA samples from the above three homozygous lines and L9 (segregation ratio is 3:1) were proceeded to the PCR amplification. The result showed that all these lines contain the *AtCBF4* gene (Fig. 2A). The Southern blot (Fig. 2B) and Northern blot analysis (Fig. 2C) provided further molecular evidence of *AtCBF4* in transgenic plant. As shown in Fig. 2B, all the four lines had a specific band in the southern blot analysis, which showed the exogenous gene *AtCBF4* had been integrated into transgenic plants genome with single copy. Meanwhile, Northern blot analysis showed that *AtCBF4* was expressed in transgenic maize but not in wild-type under drought stress and the L9 expression level was lower than the homozygous lines.

Higher drought tolerance index and higher survival rate of AtCBF4 transgenic maize plants under drought treatment

The drought tolerance index was used for the evaluation of seed germination index under drought stress condition. As shown in Table 1, under normal water condition, the seed germination rate of wild-type and three transgenic lines were 0.74 and 0.74, 0.64, 0.65, respectively. There is no obvious difference between the wild-type and three transgenic lines. But when treated with PEG (polyethylene glycol), the seed germination rate of wild-type is much lower than that of three transgenic lines. The drought tolerance indices of three transgenic lines are significantly higher than that of wild-type, and the L3 showed highest drought tolerances. From the result, we can see that different transgenic lines have different tolerances level. This may be due to the position effect of transgene in different transgenic lines.

The healthy and uniform plants of the wild-type and transgenic lines in three-leaf-stage (Fig. 3A) were selected for drought experiment. Seedlings were held without watering for 12 days, then re-watered and grown under normal conditions for another 3 days. After treatment, the wild-type withered, but the three transgenic lines showed a better growth recovery phenotype (Fig. 3B). This result showed that *AtCBF4* expression in maize can increase drought tolerance of transgenic maize.

Lower cell membrane damage of AtCBF4 transgenic seedlings under drought stress

The electrolyte leakage and MDA content of leaf cells from maize seedlings were determined. The electrolyte leakage and MDA content in leaf cells increased gradually with increasing drought treatment, but the damage to *AtCBF4* transgenic plants was much less than that to wild type plants (Fig. 4A). As shown in Fig. 4A, under normal condition, the leaf electrolyte leakage of transgenic lines L3, L4 and L8 was 16.5%, 16.4% and 16.6%, respectively. The leaf electrolyte leakage wild-type was 16.9% which slightly higher than that of the transgenic lines. After 3 days of drought treatment, the leaf cell electrolyte leakage in wild-type was 23.1%, and only 18.3%, 18.0% and 17.5%, in the transgenic lines L3, L4 and L8, respectively, which are significantly higher than that in transgenic lines. The data of the sixth day and the ninth day treatment exhibited the similar results.

As shown in Fig. 4B, under normal condition, the MDA content of transgenic lines L3, L4 and L8 was 12.3, 12.4, and 12.4 nmol/g FW, respectively, while it was 12.7 nmol/g FW (fresh weight) in the wild-type, close to the transgenic lines. After 3 days of drought treatment, the MDA content in the transgenic lines was significantly lower than that in the wild-type. These results indicated that expression of *At*CBF4 gene could enhance drought tolerances in maize.

Higher RWC of AtCBF4 transgenic plants under drought stress

RWC is considered as a parameter to assess the water status in plants and is used to be an ability to hold moisture and estimating water requirement degree of index in plant. As shown in Fig. 4C, under normal condition, the RWC in transgenic lines L3, L4 and L8 was 95.0%, 94.1% and 94.8%, respectively, while the value was 93.9% in wild-type. After 3 days of drought treatment, the RWC in wild-type decreased to 84.6%; however, the RWC in transgenic lines L3, L4 and L8 was 94.7%, 90.1% and 92.5%, respectively, which was very close to the data before stress treatment. A higher water loss rate was observed in wild-type, and the difference between the transgenic lines and wild-type was significant. The higher RWC in transgenic maize seedlings enabled them to maintain normal cell turgor under drought stress, which is beneficial for growth.

Higher total soluble sugar and proline content in transgenic seedlings under drought stress

As shown in Fig. 4D, under normal condition, the content of total soluble sugars was at similar low levels in lines. The total soluble sugars content of wild-type and transgenic lines L3, L4 and L8 were 3.47 and 3.64, 3.42 and 3.49 mg/g FW, respectively. There was no significant difference among them.

Table 1. Germination index and drought tolerance indexes under normal or PEG condition.

Line	Germination index under H_2O condition	Germination index under PEG condition	Drought tolerance index
Wild-type (Mo17)	0.74 ± 0.1255	0.02 ± 0.0000	0.027
L3	0.74 ± 0.1091	0.38 ± 0.0522**	0.514
L4	0.64 ± 0.1358	0.25 ± 0.1024**	0.391
L8	0.65 ± 0.0845	0.31 ± 0.0427**	0.477

Error bars indicate ±SE (n=3). * and ** indicate significant differences at the 0.05 level and 0.01 level using the student's *t*-test, respectively. Average of three independent experiments.

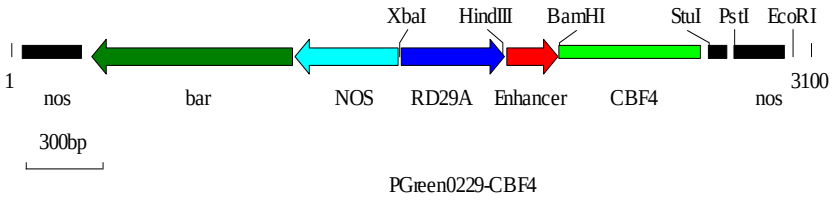

Fig 1. Partial diagram of expression vector pGreen0229-*AtCBF4*.

When treated with drought stress, the significant difference of sugar content was observed between transgenic and wild-type seedlings (Fig. 4D). After 3 days of drought treatment, the total soluble sugars content in transgenic lines L3, L4 and L8 were 5.82, 4.87 and 5.23 mg/g FW, respectively, higher than that in the wild-type, which was 4.52 mg/g FW.

As shown in Fig. 4E, under normal watering condition, the proline content in transgenic lines L3, L4 and L8 was 37.84, 35.32 and 36.32 μg/g FW, respectively, a slightly higher than that in wild-type plants, which was 33.76 μg/g FW. After 3 days of drought treatment, the proline content in transgenic lines L3, L4 and L8 was increased to 88.80, 79.84 and 82.56 μg/g FW, respectively, significantly higher than that in the wild type plants, which was 65.04 μg/g FW. These results also suggested that inducible expression of *At*CBF4 gene could enhance drought stress tolerance in maize.

Grain yields of AtCBF4 transgenic plants under drought treatment

Field experiment was conducted to investigate the production. Under drought treatment, the transgenic plants grew healthier than the wild-type (date not shown). As shown in Fig. 5, under drought treatment, the ear of transgenic lines were significantly larger than the wild-type (Fig.5).

Seed number per ear and 100 seed weight of maize are important factors which directly influence the grain yields. The seed number per ear and 100 seed weight of transgenic lines were slightly lower than that of wild-type under well watering conditions. However, under drought conditions, the seed number per ear of transgenic lines L3, L4 and L8 was 299.4, 234.7 and 293.3 respectively; significantly higher than that of the wild-type, which was 144.6 (Table 2). Ultimately, the 100 seed weight of transgenic line increased 20-55% than that of wild-type under drought treatment condition.

Discussion

Abiotic stresses lead to more than 50% yield losses in major crops, and drought was the primary factor which limits crop productivity globally (Hussain et al., 2011).

There were 1500 transcription factors in Arabidopsis (Ratcliffe et al., 2002), in which most of such genes had been identified and analyzed. CBF/DREB were important transcription factors in many plants, expression of *CBF* genes could improve drought or freeze tolerance in Arabidopsis (Haake et al., 2002; Gilmour et al., 1998; Medina et al., 1999; Liu et al., 1998). The CBF/DREB transcription factors exist widely in Arabidopsis (Gilmour et al., 1998), maize (Qin et al., 2007), tomato (Jaglo et al., 2001), tobacco (Park et al., 2001), rice (Dubouzet et al., 2003) and et al, which have the typical AP2 / EREBP DNA-binding domain.

In this study, *AtCBF4* transgenic maize lines were generated by pollen-tube pathway mediated transformation. The results showed that *AtCBF4* gene had been integrated into maize genome and expressed in transgenic plants under drought condition. There were no apparent phenotypic differences between *AtCBF4* transgenic lines and wild-type under normal growth condition. This might be due to that the *AtCBF4* gene was driven by RD29A promoter and was expressed at low levels under normal conditions. However, there were significant differences between *AtCBF4* transgenic lines and wild-type under drought condition. Compared to the wild-type, the *AtCBF4* transgenic lines displayed improved drought tolerance. The physiological parameters of seedlings were examined at 0, 3, 6 and 9 days after the drought treatment, respectively, to determine drought tolerance of the *AtCBF4* transgenic lines. Like other abiotic stresses, drought leads to damages the cell membrane. Our results showed that although drought stress influences the growth of both wild-type and transgenic lines, the two parameters in the transgenic lines, especially the L3, were significant lower than that in wild-type. After 3 days of drought treatment, the RWC in wild-type decreased from 93.9% to 84.6%, but it only decreased 0.3% in the transgenic line L3, Furthermore, after 9 days of treatment, the RWC in L3 was 76.2%, which was much higher than that in wild-type (60.5%). The detection of proline and total sugar content showed the similar results. All the transgenic lines, especially, the L3 showed to accumulate higher content of proline and total sugar than the wild-type. These indicated that expression of *AtCBF4* in maize could increase the capacity to absorb and retain water by lower solute potential, and thus maintain normal cell turgor.

Table 2. Seed number per ear and 100 seed weight of maize with and without drought treatment.

Line and treatment	Seed number per ear	100 seed weight (g)
Wild-type without drought	332 ± 30.45	35.44 ± 1.08
L3 without drought	308 ± 25.41	34.57 ± 2.45
L4 without drought	316 ± 37.18	33.96 ± 1.71
L8 without drought	307 ± 29.52	34.14 ± 2.38
Wild-type with drought	144.6 ± 19.59	20.76 ± 0.50
L3 with drought	299.4 ± 40.64**	31.94 ± 4.42**
L4 with drought	234.7 ± 22.27**	27.62 ± 1.29**
L8 with drought	293.3 ± 82.34**	29.80 ± 3.79**

Error bars indicate ±SE (n=20). * and ** indicate significant differences at the 0.05 level and 0.01 level using the student's *t*-test, respectively. All values represent the average of two independent experiments.

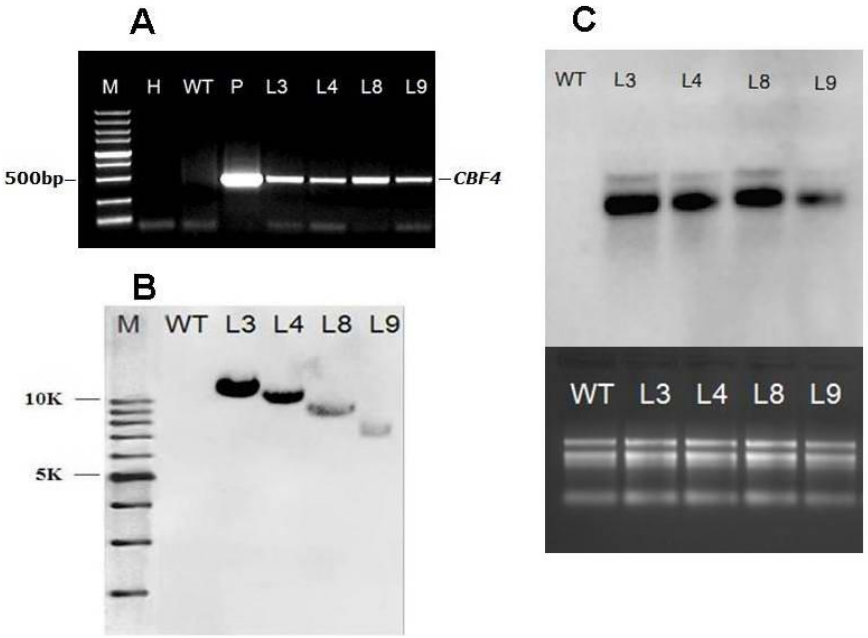

Fig 2. Molecular identification of transgenic lines. (A) PCR analysis of T_2 transgenic lines and the expected fragment of *AtCBF4* (523bp) was amplified from the plasmid DNA and the total DNA of all transgenic lines, but not from the wild type plant or water control. M, DNA ladder; H, H_2O; WT, wild-type (Mo17); P, plasmid; L3, L4, L8, L9, transgenic lines. (B) Southern blot analysis of partial transgenic lines and the hybridization signal band of *AtCBF4* was observed from the *Bam*HI digested genomic DNA of four transgenic lines, but not from the wild type plant. M, DNA ladder; WT, wild-type (Mo17); L3, L4, L8 and L9, transgenic lines. (C) Northern blot analysis of *AtCBF4* expression in different transgenic lines. The hybridization signal band of *AtCBF4* was observed from the total RNA of four transgenic lines, but not from the wild type plant. WT, wild-type (Mo17); L3, L4, L8 and L9, transgenic lines.

As a result, the transgenic lines had a higher survival rate and better growth condition than wild-type under drought treatment. Further experiment proved that the *AtCBF4* transgenic maize not only enhanced drought tolerances but also improved the harvest yield than Mo17 wild-type under continuous drought stress in field. The results (Table 2) showed that the transgenic lines had much greater seed mass than wild-type under continuous drought environment in field. Compared to the wild-type, the seed number per ear of L3, L4 and L8 increased 107.1%, 32.6% and 102.8%, respectively. The 100 seed weight of L3, L4 and L8 increased 53.6%, 22.9% and 25.2%, respectively, indicating that the *AtCBF4* transgenic lines could obtain more harvest yield than wild-type growing under drought conditions. However, comparing to well watering conditions, there still existed some yield loss in transgenic lines under drought conditions. This yield in field was consistent with the expression level of *AtCBF4* in different lines, more expression level of *AtCBF4*, more harvest yield under drought stress.

Our result revealed that expression of *AtCBF4* in transgenic maize could enhance drought tolerances and improve the harvest yield under drought conditions. But as shown in Fig. 5 and Table 2, the harvest yield of transgenic lines was slightly decreased than that of wild-type under well watering condition in the field. It was interesting and unexpected because RD29A promoter was expressed at low levels under normal conditions. Moreover, some reports described that substituting RD29A promoter for CaMv 35S in CBF/DREB transgenic plants could avoid the retardation growth of transgenic plants (Kasuga et al., 1999). There were no obvious growth phenotype differences between the transgenic plants and Mo17 wild-type in the whole growth process, but the size of the ear and the harvest yield of the transgenic plants were slightly smaller than that of wild-type, and this phenomenon was not found in the empty vector control lines (the date not shown). This suggests that low level

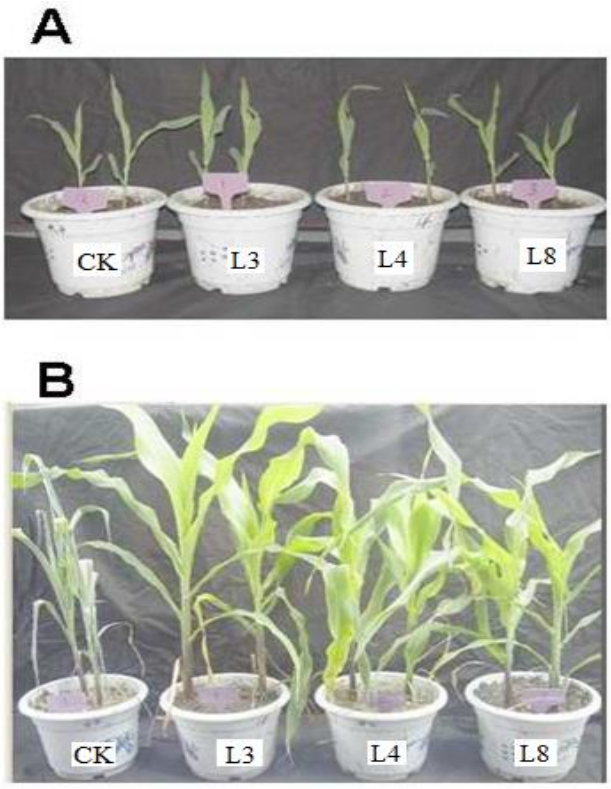

Fig 3. *AtCBF4* expression conferring the obvious effect of drought tolerance in transgenic maize. (A) The wild-type and transgenic lines before drought stress treatment in three-leaf stage. (B) The wild-type and transgenic lines were held without watering for 12.d, then re-watered and recovered for 3d.

expression of *AtCBF4* (CBF/DREB) had no effect on the growth of transgenic plants but may cause the slightly decrease of the harvest yield of transgenic lines under well watering conditions in the field.

Materials and Methods

Plant materials

The maize cultivar used in this study was inbred line 'Mo17' (wild-type), originated from the United State of America and grown as a parent line in northern China.

Construction of plasmids

The plant expression vector pGreen0229 with the *PAT* gene conferring resistance to the glufosinate herbicide was provided by John Innes Center, UK. The full cDNA of *AtCBF4* (GenBank accession no. AB015478: coding sequence 18601-19275) from Arabidopsis (Columbia) was sub-cloned into the *Bam*HI/*Stu*I site under the control of RD29A promoter from Arabidopsis to generate the vector pGreen0229- *AtCBF4* as shown in Fig 1. The construction was confirmed by restriction enzyme digestion and sequencing.

Plant transformation

The basic procedures for the transformation method were conducted as described by Wang et al. (2001) with minor modification. The transformation experiment was performed in Yacheng, Hainan Province. The well grown wild-type maize was chosen for artificial pollination and covered by parchment paper bags. After artificial pollination (22 h), the parchment paper bags were taken off, the top of the spike-stalk was cut off and dropped on 200 µl of the vector solution (500 ng/µl purification by Plasmid Maxi prep System from New Industry Company), and then covered by parchment paper bags again until harvest. At maturity stage, the T_1 seeds that developed from the treated spike were harvested.

Screening of transgenic maize

The T_1 seeds were grown and screened for transgenic lines in the green house in Beijing, China. The seedlings of four-leaf stage from T_1 seeds were sprayed with 200 mg/L glufosinate 3 times every 5 days. Two weeks later, the possible transformed seedlings were still survived but the untransformed seedlings were dead. After PCR analysis, the transgenic seeds were grown and continued to be screened for herbicide resistance in order to obtain homozygous transgenic lines. Among them, 3 independent lines of T_3 homozygote seeds were used for further analysis.

PCR analysis

Total genomic DNA was extracted from the fresh leaves of transgenic maize seedlings using the Wizard Genomic DNA Purification Kit (Promega), and DNA from wild-type plant and double distilled water were used as negative controls. The gene-specific primers sequences designed corresponding to *AtCBF4* gene were as follows: *AtCBF4*-forward, 5'- TTACTCTACAT-TCCCAGACTCGT-3', and *AtCBF4*-reverse, 5'- CCATATA-AAACACACCACCATTC-3'. The predicted PCR product is 523 bp.

Southern bolt analysis

Southern bolt analysis was performed to further confirm the transgene in the genome of the transgenic plants. Genomic DNA was extracted from the fresh leaves of the *AtCBF4* transgenic and wild-type plants using CTAB protocol and quantified after RNAse treatment.

30 µg of genomic DNA samples from the maize plants were digested with the restriction enzyme *Bam*HI (Promega) at 37 °C for 16 h, then separated by gel electrophoresis in 0.8% agarose gel, and transferred into Hybond nylon membranes (Amersham Bioscience) by vacuum blotting using standard protocols (Bio-rad 785). The 523 bp of *AtCBF4* gene fragment was amplified from plasmid pGreen0229- *AtCBF4* with the gene-specific primers by DIG DNA Labeling Kit (Roche), which was used as the probe. The membrane was hybridized with a DIG-labeled *AtCBF4*-specific probe at 42 °C for 16 h. The hybridized membrane was washed and detected according to the protocol of DIG Nucleic Acid Detection Kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche). The signals of hybridization were captured using the phosphor image analyzers (FLA 4000, Fuji).

Northern bolt analysis

For northern blot analysis, the maize seedlings were water withheld for a week. Total RNA from five-leaf stage seedlings of transgenic maize and wild-type plants were extracted using TRIzol Reagent (Invitrogen). 20 µg of total RNA were electrophoresed in 1.2% agarose gel containing 5% formaldehyde, and then transferred onto Hybond nylon membranes (Amersham Bioscience) using standard protocols. The probe for northern blot was same as the probe for southern

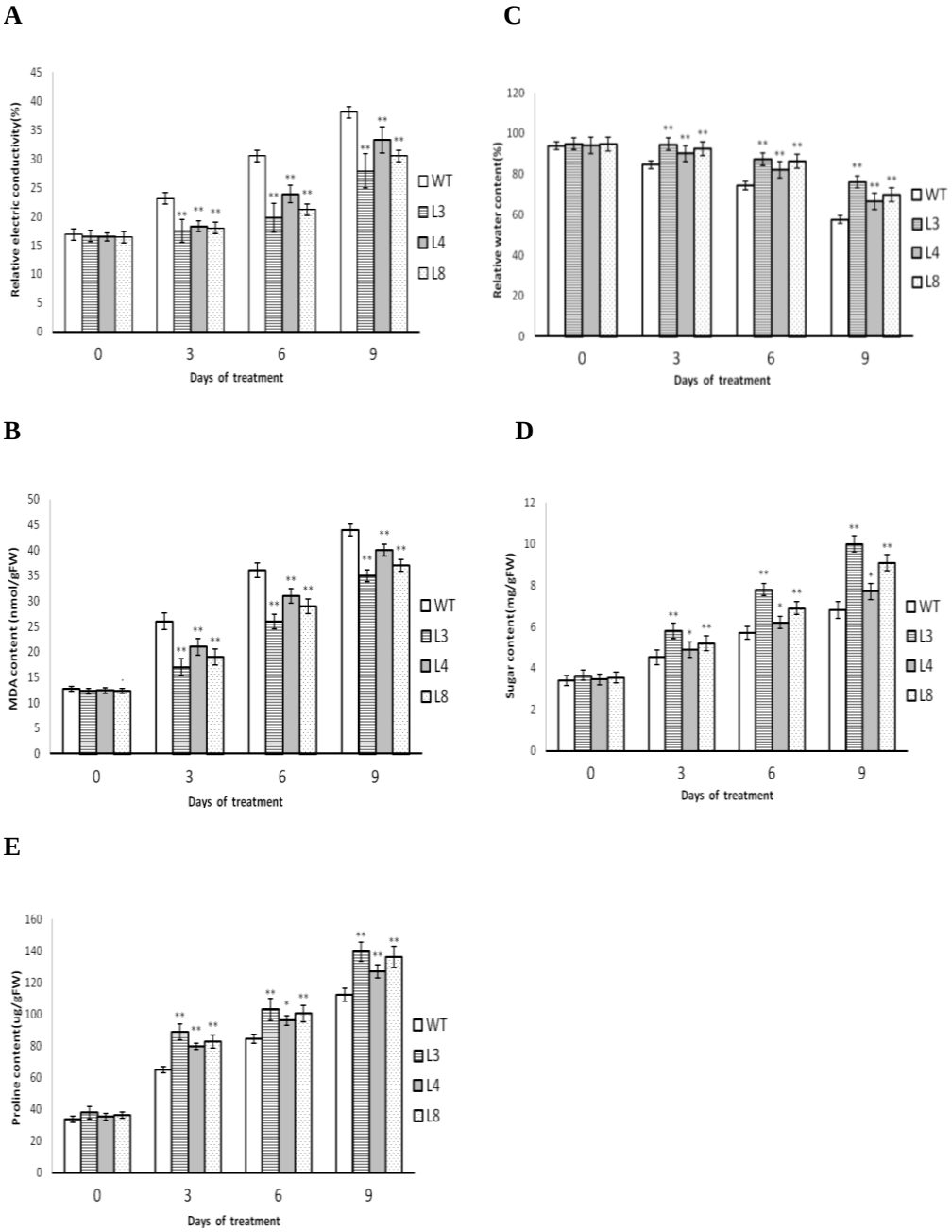

Fig 4. Physiological parameters of seedlings at 0, 3, 6 and 9 days after the drought treatment, respectively. WT, wild-type (Mo17); L3, L4, L8: transgenic lines. Error bars indicate ±SE (n=3). * and ** indicate significant differences at the 0.05 level and 0.01 level using the student's *t*-test, respectively. (A) Electrolyte leakage of leaf cell under drought treatment. (B) MDA content of the leaves under drought treatment. (C) RWC under drought treatment. (D) Changes in total soluble sugar under drought treatment. (E) proline content under drought treatment.

blot analysis. The nylon membrane was hybridized with a DIG-labeled *AtCBF4* specific probe at 50 ℃ for 16 h. The hybridized membrane was washed and detected according to the protocol of DIG Nucleic Acid Detection Kit (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche). Images were acquired by scanning the membranes with the LAS-4000 Image Reader (FUJIFILM)

Germination experiment

20 healthy seeds with same size from wild-type and homozygous transgenic lines were chosen for germination test. Seeds were surface sterilized with 70% ethanol for 2 min and washed 3 times with distilled water. Then the seeds were placed on double filter paper in culture dishes, and 20 ml 18% of PEG6000 was added to the filter paper for drought stress treatment. There was distilled water instead of PEG6000 for control group. The seeds were sprouted at 25 ℃ in a dark incubator. The number of sprouted seeds was calculated every 2 days until the eighth days (the 2 mm long radicle seeds were counted for germination). For the above measurement, three replicates were used. Germination index = $(1.00)\times nd_2$ + $(0.75)\times nd_4$+ $(0.50)\times nd_6$ + $(0.25)\times nd_8$ (nd_X = Germination rate of the experiment on the X day). The Drought tolerance index

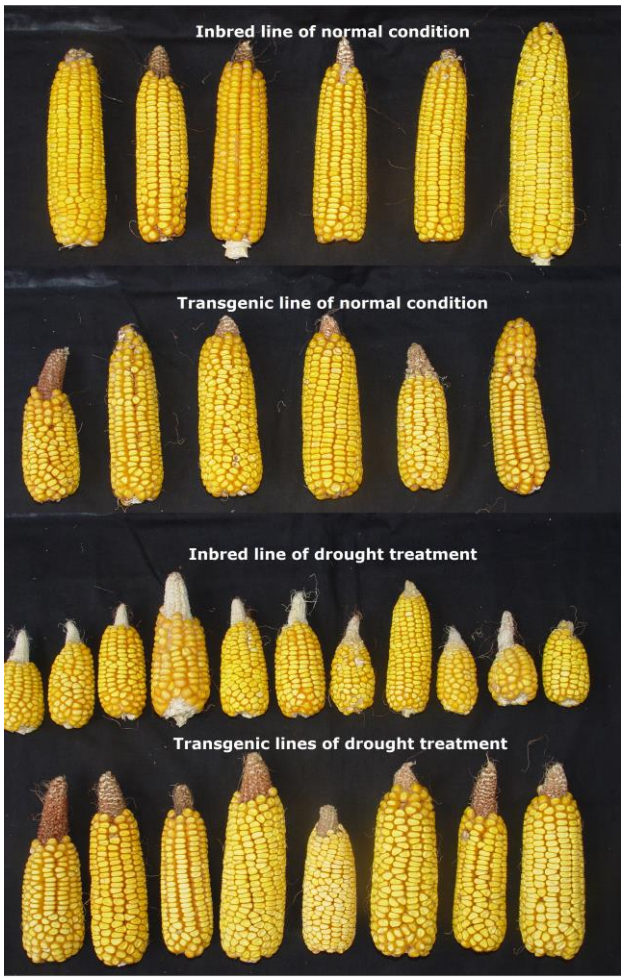

Fig 5. Grain yields of maize plants with and without drought treatment.

was measured based on the Germination index above. Drought tolerance index = Germination index of PEG treatment group / Germination index of distilled water treatment group.

Drought treatment

After surface sterilized, the seeds were grown in 23-cm plastic pots filled with 1:1 of vermiculite: nutrient soil mixture in a greenhouse at 27/16 °C (day/night) and 12/12 h light and relative humidity of 60-70%. All the pots were put in a big plastic box to ensure all the plant with the same watering condition. When the seedling grew to the five-leaf stage, health and uniform maize seedlings were selected and imposed to drought stress for 12 days. After that, all the pots were re-watered simultaneously. Three days later, the photographs were taken. The physiological parameters of seedlings were determined at 0, 3, 6 and 9 days after drought treatment, respectively. All the experiments were repeated three times independently.

Measurement of electrolyte leakage and MDA content

Membrane damage was assayed by measuring ion leakage and MDA from leaf discs. Measurement of electrolyte leakage and MDA levels was conducted at 0, 3, 6 and 9 days after the treatment, respectively.

To measure ion leakage ratio, 0.1 g leaves in the same position were removed from different plants, rinsed briefly with deionized water, and immediately placed into a tube with 20 ml of deionized water. Conductivity EC1 was measured using an Electro conductivity Mater in room temperature (DDS-307, Shanghai, China) after the tubes were placed under 4 °C condition overnight. Then, the samples were heated at 100 °C for 20 min and conductivity EC2 was measured again. Ion leakage ratio was expressed as (EC1/EC2) ×100%. The MDA was determined by a color reaction with trichloroacetic acid according to the reported method (Heath et al., 1968; Zhao et al., 1994).

Quantification of RWC

The leaf discs were punched out from healthy and fully expanded leaves with similar ages, and the fresh weight (FW) of leaf discs was immediately measured. After soaking them in deionozed water at 4 °C overnight, their turgid weight (TW) was determined. Then, they were baked in an oven at 105 °C for 15 min and 75 °C for 72 h and their dry weight (DW) was determined. RWC was calculated from the equation (Gaxiola et al., 2001): RWC (%) = (FW-DW) / (TW-DW) × 100%.

Measurement of total soluble sugars and proline content

Measurement of total soluble sugars and proline content was conducted at 0, 3, 6 and 9 days after the treatment, respectively. Total soluble sugars of maize leaves were extracted in boiling water for 30 min and determined by anthrone reagent using glucose as the standard (Yemm et al., 1954). Proline in maize leaves was extracted with 3% sulfosalicylic acid and determined by the ninhydrin reaction method (Troll et al., 1953).

Drought stress of maize plants in field

All drought-stress experiments were conducted under the winter maize growing season in Sanya Hainan Province of China. Transgenic plants and wild-type plants were grown in field, when plants grew to five-leaf stage, 20 healthy, and uniform plants were exposed to drought stress without watering until harvest. There was almost no rain in Sanya during the whole growing season. The control group was watered every 12 days. After harvesting, the seeds were dried to constant weight naturally, and the seed dry weight (DW) of each plant and the grain number per ear from each line under drought stress were recorded.

Statistical analysis

Statistical differences between wild-type and transgenic plants on different treatments were analyzed following the Student's *t* test using SPSS (Chicago). Differences were considered significant at a probability level of $P \leq 0.05$, one asterisk indicated significant difference ($P \leq 0.05$), and double asterisks indicated significant difference ($P \leq 0.01$).

Acknowledgments

This study was financially supported by grants from National Special Program of Transgenic Research (No. 2009ZX08003-009B and 2014ZX08003-003B), and Foundation of Beijing Municipal Committee of Science and Technology (No. Z121100001512012, Z111100066111005 and KJCX20110 2003).

References

Agarwal PK, Agarwal P, Reddy MK (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in

plants. Plant Cell Rep. 25: 1263-1274.
Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang H, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002) Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell. 14: 559-574.
Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J. 33: 751-763.
Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc Natl Acad Sci USA. 98: 111-113.
Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 16: 433-442.
Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 124: 1854-1865.
Haake H, Cook D, Riechmann J, Pineda1 O, Thomashow MF, Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiol. 130: 639-648.
Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125: 189-198.
Hsieh TH, Lee JT, Charng YY, Chan M (2002) Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. Plant Physiol. 130: 618-626.
Hsieh TH, Lee JT, Yang PT, Chiu LH, Charng Y, Wang YC, Chan MT (2002) Heterology expression of the Arabidopsis C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol. 129: 1086-1094.
Hussain SS, Kayani MA, Amjad M (2011) Transcription factors as tools to engineer enhanced drought stress tolerance in plants. Biotechnol Prog. 27: 297-306.
Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in Brassica napus and other plant species. Plant Physiol. 127: 910-917.
Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol. 17: 287-291.
Kazuko YS, Kazuko S (1993) Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants. Mol Gen Genet. 236: 331-340.
Knight H, Knight MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci. 6: 262-267.
Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell. 10: 1391-1406.
Medina J, Bargues M, Terol J, Pérez-Alonso M, Salinas J (1999) The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. Plant Physiol. 119: 463-469.
Oh SJ, Kwon CW, Choi DW, Song SI, Kim JK (2007) Expression of barley HvCBF4 enhances tolerance to abiotic stress in transgenic rice. Plant Biotechnol J. 5: 646-656.
Park JM, Park CJ, Lee SB, Ham BK, Shin R, Paek KH (2001) Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell. 13: 1035-1046.
Pino MT, Skinner JS, Park EJ, Jeknić Z, Hayes PM, Thomashow MF, Hoisington D. (2007) Use of a stress inducible promoter to drive ectopic AtCBF expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnol J. 5: 591-604.
Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K (2007) Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in Zea mays L. Plant J. 50: 54-69.
Ratcliffe OJ, Riechmann JL (2002) Arabidopsis transcription factors and the regulation of flowering time: a genomic perspective. Curr Issues Mol Biol. 4: 77-91.
Troll W, Cannan RK (1953) A modified photometric ninhydrin method for the analysis of amino and imion acids. Journal Biol Chem. 200: 803-811.
Wang JX, Sun Y, Cui GM, Hu JJ (2001) Transgenic maize plants obtained by pollen-mediated transformation. Acta Bot Sin. 43: 275-279.
Yemm EW, Wills AJ (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochem J. 57: 508-514.
Zhao SJ, Xu CC, Zou Q (1994) Improvement of method for measurement of malondialdehyde in plant tissues. Plant Physiol Commun. 30: 207-210.