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The sunflower transcription factor HaHB11 confers tolerance to water deficit and salinity to transgenic Arabidopsis and alfalfa plants

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ABSTRACT

Homeodomain-leucine zipper (HD-Zip) transcription factors are unique to the plant kingdom; members of subfamily I are known to be involved in abiotic stress responses. HaHB11 belongs to this subfamily and it was previously shown that it is able to confer improved yield and tolerance to flooding via a quiescent strategy. Here we show that *HaHB11* expression is induced by ABA, NaCl and water deficit in sunflower seedlings and leaves. Arabidopsis transgenic plants expressing *HaHB11*, controlled either by its own promoter or by the constitutive *35S CaMV*, presented rolled leaves and longer roots than WT when grown under standard conditions. In addition, these plants showed wider stems and more vascular bundles. To deal with drought, *HaHB11* transgenic plants closed their stomata faster and lost less water than controls, triggering an enhanced tolerance to such stress condition and also to salinity stress. Concomitantly, ABA-synthesis and sensing related genes were differentially regulated in *HaHB11* transgenic plants. Either under long-term salinity stress or mild drought stress, *HaHB11* transgenic plants did not exhibit yield penalties. Moreover, alfalfa transgenic plants were generated which also showed enhanced drought tolerance. Altogether, the results indicated that HaHB11 was able to confer drought and salinity tolerance via a complex mechanism which involves morphological, physiological and molecular changes.

1. Introduction

During their life cycle, plants must deal with diverse environmental stress factors which affect their growth and production (Shinozaki and Yamaguchi-Shinozaki, 2000; Qiu and Yu, 2009). Abiotic stress factors include water deficit and excess, soil salinity, high and low temperatures, high and low light intensities. Such stressing situations impair plant growth and alter cellular processes such as photosynthesis, carbon partitioning, carbohydrate and lipid metabolism, protein synthesis, gene expression and osmotic homeostasis. Depending on the severity of the stressing situation, plants would have reduced biomass, shorter stems and less yield production (Singh and Laxmi, 2015).

Among stressing factors, drought is the most serious one limiting the productivity of agricultural crops worldwide, with devastating economical and sociological impact. To adapt themselves to such stressful condition, plants have evolved different physiological and molecular strategies either to escape stress or display tolerance (Shinozaki and Yamaguchi-Shinozaki, 2000). One of such mechanisms is stomata closure, an instant response in order to minimize water loss; however, at the same time this strategy reduces photosynthesis and concomitantly, growth. Some plants combine high growth rate with short life cycle during wet season; regrettably, this strategy is combined with yield penalty (Skirycz et al., 2011).

Among the molecular mechanisms displayed by plants to deal with stress, most involve the activation of certain specialized transcription factors (TFs), able to regulate entire signal transduction pathways. As a natural response, these special TFs are capable to convert stress-induced signals into protective cellular responses (Century et al., 2008).

One of the more important challenges of plant scientists is to obtain crops with enhanced drought tolerance. To enhance the natural adaptive response seems a good strategy and, for this reason, various TFs from different families were expressed as transgenes, mostly in Arabidopsis (Qiu and Yu, 2009; Raineri et al., 2015; Yao et al., 2016). These TFs belong to varied families such as APETELA2 (AP2), bHLH, bZIP, HD-Zip, NAC, ZF, MYB and WRKY and,

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as expected, transgenic plants expressing some of these TFs were more tolerant than controls to varied abiotic stress factors, including drought (Ribichich et al., 2014). However, most of the transgenic genotypes expressing TFs also exhibited pleiotropic effects such as growth detriment and yield loss, especially, when the plants were grown in standard or moderate stress conditions (Skirycz et al., 2011). The most common scenario observed was high stress tolerant plants which in standard growth conditions yielded significantly less than controls. Such characteristics are not suitable for crop improvement because climate and rain are difficult to predict and it is probable that for this reason, drought tolerant crops are not available yet in the market. So, the next challenge is to obtain drought tolerant plants combined with increased yield or at least, without yield penalties.

Homeodomain-leucine zipper (HD-Zip) family TFs are only present in plant kingdom. They were classified in four subfamilies (I to IV) and HD-Zip I members are intimately related to abiotic stress tolerance (Ariel et al., 2007; Ribone et al., 2015).

HaHB11 is a member of the sunflower HD-Zip I subfamily previously described as conferring improved yield in standard growth conditions and flooding tolerance via a quiescent strategy (Cabello et al., 2016). Like the sunflower HaHB4 which was described as conferring drought tolerance by repressing ethylene signaling (Manavella et al., 2006), HaHB11 is a divergent member possessing an atypical carboxy terminus (Arce et al., 2011). Its closest Arabidopsis homologs are AtHB12 and AtHB7. However, even when the expression of these genes is induced by water deficit, they did not confer flooding or drought tolerance neither improved biomass nor seed yield when they were overexpressed in Arabidopsis (Olsson et al., 2004; Ré et al., 2014).

In this work, we show that *HaHB11* expression is up-regulated by ABA, NaCl and drought. The ectopic expression of this sunflower gene in Arabidopsis and alfalfa conferred to both species an increased tolerance to drought and salinity stresses without yield penalties. To display such phenotype, this sunflower TF regulates several abiotic stress related genes, promotes main root growth and leaves rolling which reduces transpiration surface.

2. Experimental procedures

2.1. Constructs and transgenic plants

35S:HaHB11, *PrHaHB11:HaHB11* constructs and the corresponding Arabidopsis transgenic plants bearing these constructs were previously described (Cabello et al., 2016).

2.2. Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). WT and transgenic plants were grown directly on a mix of vermiculite, perlite, peat moss and soil (3:2:2:1) in a growth chamber at 22–24 °C under long-day photoperiod (16 h of illumination with a mixture of cool-white and GroLux fluorescent lamps) at an intensity of approximately 150 μE $m^{-2} s^{-1}$, in 8 cm diameter × 7 cm height pots, during the periods of time indicated in the figures. For several experiments (indicated in the corresponding Figure Legends), seedlings or young plants were used. In these cases, seeds were germinated and grown in Petri dishes containing Murashige and Skoog medium, 1% agar. The dishes were kept at 4 °C for 2 days and then transferred to the growth chamber in the conditions described above for variable periods of time.

Helianthus annuus (cv. HA89) seeds were germinated on wet filter paper for 7 days and then transferred to 8 × 7 cm pots containing a vermiculite-perlite mix, one plant per pot and well-watered. Then, the plants were placed in a 45-cm plastic square tray until treatments.

2.3. Alfalfa transformation

The regenerative clone C2-3, kindly provided by Drs. B. McKersie and S. Bowley (Plant Biotechnology Division, Department of Plant Agriculture, University of Guelph, Canada), was used for the transformation of alfalfa plants (*Medicago sativa*, L.). Petioles of alfalfa were infected with previously transformed *Agrobacterium tumefaciens* and cultured *in vitro* as described by D'Halluin et al. (1990) with modifications. Axenic explants, previously injured with a scalpel, were inoculated for 2 min with a bacterial culture ($OD_{600\,nm}$ 0.5–0.8) previously grown at 28 °C. After 3 days of co-cultivation in darkness at 25 °C, on a solid callus inducing medium supplemented with 100 μM acetosyringone, the explants were washed to eliminate bacteria and then, placed on callus induction medium SHK (Schenk and Hildebrandt, 1972; modified by McKersie et al., (1993)), with kanamycin (25 mg/l) and cefotaxime (400 mg/l). The explants were maintained in a culture chamber at 25 °C under long photoperiod conditions until they formed and matured somatic embryos. These mature embryos were placed in a rooting medium composed of Murashige and Skoog Basal Medium (Cat. # MS 519, Sigma) diluted 1:2 with water. After rooting, the seedlings were taken to the greenhouse for rustification under controlled moisture conditions.

2.4. Plant treatments

Arabidopsis treatments with ABA: 3-week-old Arabidopsis plants grown in MS Petri dishes were transferred to a fresh MS medium dish supplemented with 100 μM ABA for 1 h. After that, seedlings were harvested and frozen in liquid nitrogen until RNA extraction.

Sunflower seedlings treatments: *Helianthus annuus* (cv. HA89) seeds were germinated on wet paper for 7 days and then transferred to a fresh MS medium dish supplied with different hormones (100 μM ABA, 20 μM ACC, 100 μM SA, 100 μM BAP) or NaCl as indicated in the corresponding Figure Legend. For darkness treatment, 7-day-old seedlings grown as described above were transferred to a fresh Petri dish with MS medium and kept in completely darkness during 2 h. For water stress treatments, 7-day-old seedlings were transferred to a dry paper during 15 min and harvested for RNA extraction.

Plants grown on soil as described above were subjected to drought stress by stopping watering when they arrived to V3 stage, approximately 14 days after the transfer to pots. At different times, as indicated in the Figure legends, leaves were harvested for RNA extraction. Salinity stress to V3 plants was applied by watering the plants each week with 50, 150 and 200 mM NaCl. For RNA extraction, leaf samples were harvested 1 and 3 days after each NaCl addition.

Arabidopsis plants severe drought stress: four plants per pot germinated and grown as described above, were water-saturated. The water saturated pots were weighted and this initial weight was considered 100% field capacity; all the pots had equal quantities of soil and water. Four pots per genotype (16 individual plants) were used for each experiment repetition. Twenty-five days after sowing, watering was completely stopped until plant damage was clearly observed and then rewatered. Photographs were taken during the treatment whereas survival% was calculated two days after recovery.

Arabidopsis plants mild water-stress treatments: starting the treatment all the pots were water saturated to achieve 100% field capacity (FC) and maintained in this FC until day 25. Twenty five-day-old plants were subjected to stress by stopping watering until the desired FC was reached. Field capacity was evaluated as the% of