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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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Abbreviated running headline: *HaHB11* confers drought and salinity stress tolerance to Arabidopsis and alfalfa plants

Highlights

- The expression of the sunflower gene *HaHB11* is induced by drought, ABA and salinity.
- Transgenic *HaHB11* Arabidopsis plants have enhanced drought and salinity tolerance.
- Transgenic plants have longer roots, rolled leaves and more vascular bundles than WT.
- *HaHB11* alfalfa transgenic plants exhibit drought tolerance.

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.

Keywords

HaHB11; Homeodomain-leucine zipper I; transcription factor; drought tolerance; seed yield***;*** vascular bundles

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 et al., 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, 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; Re 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.

Experimental Procedures

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).

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 ºC 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 x 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 ºC 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 x 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.

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. (1993) 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, 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.

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 minutes 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 the pot weight and maintained by weighting the pots and adding the necessary quantity of water every two days.

Arabidopsis salinity stress tolerance evaluation: 25-day-old plants (16 plants per genotype, 4 per pot) were irrigated with 50 mM NaCl (1 l). After 7 days, an additional litter of 150 mM NaCl was added and fourteen days after the first NaCl treatment, 200 mM NaCl (1 l) was further added. Photographs were taken a week after the last addition.

Mild salinity stress was applied for yield assessment. For this treatment, 100 mM NaCl (1 litter to the tray with 16 pots) was added to 21-day-old plants (N=16), each one in an individual pot. Two additions of 100 mM NaCl (1 l each) were done 7 and 14 days after the first one. Then, the plants were normally watered with H_2O until seed harvesting.

Arabidopsis water loss treatments during water deficit treatments: five leaves for four different plants from each genotype were removed at the times indicated in the figure and weighted (W1). After that, the same leaves were incubated in demineralized water for 3 h, and weighed again (W2). The difference in weight (W2-W1) was considered as water loss (Jakab et al., 2005).

Alfalfa drought stress treatment: one-month-old alfalfa plants (4 per pot) grown under standard conditions and well watered in a growth chamber at 24°C under long photoperiod, were subjected to drought stress assays. Watering was stopped during 10 days and then rewatered until recovery. Photographs were taken one week after and water loss was evaluated as described above for Arabidopsis plants.

RNA isolation and expression analyses by real time RT-PCR

RNA for real-time RT–PCR was prepared with Trizol® reagent (InvitrogenTM) according to the manufacturer´s instructions. RNA (2 µg) was used for the RT reactions using M-MLV reverse transcriptase (Promega). Quantitative PCRs were carried out using a MJ-Cromos 4 apparatus in a 20 µl final volume containing 1 µl SyBr green (10 x), 8 pmol of each primer, 2 mM $MgCl_2$, 10 µl of a 1/25 dilution of the RT reaction and 0,12 µl Platinum Taq (Invitrogen Inc.). Fluorescence was measured at 78-80 ºC during

40 cycles. Sunflower RNA was also prepared with the Trizol (Invitrogen Inc.) technique, but with a different dilution of the RT reaction, 1/50.

Specific oligonucleotides for each gene were designed using publicly available sequences (Arabidopsis.org web page and ncbi.nlm.nih.gov). For sunflower HaHB11, specific oligonucleotides were previously designed (Cabello et al., 2016). The designed sequences are described in Supplementary Table 1.

Histology and microscopy

Stem vasculature: Arabidopsis inflorescence sections were harvested from the base of the first internode of 30 cm-height stems, 0.5-1 cm length sections were fixed at 24°C for 1 h in a solution containing 3.7 % formaldehyde, 5 % acetic acid, 47.5 % ethanol and then dehydrated through a graded series of ethanol (70 %, 80 %, 90 %, 96 % and 100 %; 30 min each one) followed by 1 h in 100 % xylene. The samples were placed into plastic molds finally embedded with 100 % Histoplast (Biopack™). Each block was incubated overnight at room temperature to ensure solidification. Transverse stem sections (10 µm thick) were obtained using a Leica Microtome (Microtome RM2125, Leica). Cross sections were mounted on slides coated with 50 mg/ml poly-d-Lys (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris-HCl pH 8.0 and dried during 16 h at 37°C. After removing the paraffin with 100 % xylene for 15 min at room temperature, sections were rehydrated using a graded series of ethanol (100%, 96%, 90%, 80%, 70% and 50%; 1 min each one) to finish in distilled water. Samples were then stained with 0.1% Toluidine blue, rinsed and mounted on Canadian balsam (Biopack™) for microscopic visualization in an Eclipse E200 Microscope (Nikon) equipped with a Nikon Coolpix L810 camera.

Results

HaHB11 expression is up-regulated by ABA and drought

To investigate the expression pattern of *HaHB11*, total RNA was isolated from sunflower 7-day-old seedlings and V3 plants. Before the extraction, seedlings were fractioned in cotyledons, hypocotyls, first pair of leaves and roots whereas V3 plants in leaves, petioles, stems, cotyledons, hypocotyls and roots. Transcript levels were assessed by RT-qPCR and the results are shown in Figure 1. In 7 day-old seedlings (Figure 1A) expression was evident in cotyledons and hypocotyls whereas in V3 plants transcripts were increased in petioles and leaves (Figure 1D).

To know which hormones or stressing factors regulate *HaHB11* expression, seedlings and plants from the same age were subjected to different treatments. Seven-day-old seedlings presented *HaHB11* induction after treatments with BAP, GA, ABA (Figure 1C) and water deficit (Figure 1B) whereas in V3 plants, only ABA (Figure 1F), mannitol and, to a lesser extent, NaCl (Figure 1E) were able to induce this gene expression.

To further characterize the response to drought and NaCl, kinetics of induction was performed subjecting V3 plants to drought during 10 days (Figure 1G) or gradually adding NaCl during the same period. *HaHB11* response to drought was evidenced late, at the 10th day when the plants were close to death whereas the response to NaCl picked at the 3rd day (Figures 1 G and H).

Transgenic Arabidopsis plants expressing HaHB11 exhibit tolerance to drought and salinity

Considering the up regulation of *HaHB11* by drought, Arabidopsis plants transformed with the construct *35S:HaHB11* (Cabello et al., 2016) were subjected to severe drought stress using WT plants as controls. Individuals from three independent lines showing different expression levels (H11-A, H11-B and H11-C: high, medium and low, respectively) were grown in standard conditions during 4 weeks and then, watering was stopped during 14 days. This treatment slowly resulted in a severe drought condition. At day 15, the plants were watered to recover themselves; survival rate and health were surveyed and showed clear differences between transgenics and WT (Figure 2C). Figure 2A shows an illustrative image of *HaHB11* and WT plants after the drought treatment. Water loss during the treatment was also evaluated, indicating that the three independent *HaHB11* lines lost less water than controls, independently of the transgene

expression level (Figure 2B). The reduced water loss is undoubtedly a key for drought tolerance. Considering other drought tolerance related traits, transgenic plants closed their stomata faster than WT; *HaHB11* lines closed 50% of their stomata at 3rd day, whereas WT at 8th day (data not shown). Water consumption was also surveyed by weighting the pots during the drought treatment and adding water to conserve the same weight. The results shown in Figures 2 D and 2E indicated that high expression level lines were able to better keep water than the WT. As it can be appreciated in Figure 2E, even with the same available quantity of water, all the transgenics looked healthier than controls. The experiments were repeated several times, both in the vegetative and in the reproductive stage with similar results.

Longer roots and rolled leaves could explain the increased drought tolerance exhibited by HaHB11 transgenic plants

Aiming at understanding the physiological and molecular mechanisms by which *HaHB11* transgenic plants presented increased drought tolerance compared to controls, further analyses were carried out. A detailed observation of the plants lead to detect leaf rolling, a trait closely related with drought tolerance because such rolling allows the plant to expose less transpiration surface (Figure 3A). Cross sections of leaves were done to further analyse this trait (Figure 3B). These sections evidenced wider leaves generated by larger palisade mesophyll cells and larger spongy mesophyll cells (Figure 3B).

Other differential traits that could be related with improved drought tolerance were the longer roots and shorter hypocotyls exhibited by *HaHB11* plants compared to their controls (Figure 3C).

Stem anatomy changes between transgenic HaHB11 and WT plants are closely related with drought tolerance

Severe drought stress seriously affects plant growth and development. However, moderate water deficit is a more frequent situation in the field. To test how transgenic and WT plants respond to such situation, mild stress treatments were carried out. Plants were irrigated during all the life cycle to grow at 40 % or 65 % field capacity by daily limited watering. These treatments provoked mild stress but both type of plants survived and could be harvested. Seeds were collected for each individual plant and, surprisingly, high expression level *HaHB11* plants yielded less than controls at 40 % field capacity

whereas the low expression level line behaved more similar to WT. At 65 % field capacity, all the genotypes presented similar yields. Wondering if high expression levels play somehow a negative role considering yield in drought conditions, transgenic plants in which the transgene expression is driven by the own promoter (Cabello et al., 2016) were assessed in two stressing growth conditions: 30 and 40 % field capacity (Figure 4A, lower panel). In both tests, these plants yielded more seeds under stress conditions than the WT or the transgenic lines bearing the constitutive promoter. Moreover, yield increase was related to the transgene expression level. These results were particularly surprising because the same plants (bearing the inducible promoter) were tested in severe drought conditions and did not show increased tolerance compared to controls (not shown).

To understand the latter results, morphological studies were carried out comparing all the studied genotypes under different growth conditions. In normal irrigation conditions, *HaHB11* plants have wider stems and more vascular bundles than their controls, probably explaining the significant increased yield presented in such conditions (Cabello et al., 2016). Moreover, histological sections evidenced that transgenic stems were more lignified than WT ones (Figure 4 B). Interestingly, at 40 % field capacity, stems of *HaHB11* and WT plants were indistinguishable considering the stem width, number of vascular bundles and lignification (Figure 4B). At 60 % field capacity the stems of *HaHB11* plants recovered the morphology of those grown in normal conditions (Figure 4B and 4C). These results suggested a strong relationship between the drought condition, the stem width, the number of vascular bundles and yield; indicating that plants are more capable of producing seeds if they achieve to enlarge the stem width and vasculature.

Genes involved in ABA signalling are regulated in HaHB11 transgenic plants

To elucidate if ABA signalling was involved in the drought tolerance phenotype exhibited by *HaHB11* plants, ABA related genes were assessed for transcript levels in 14-day-old transgenic and WT plants, grown in MS-agar and supplemented with or without 100 μM ABA. Genes involved in ABA biosynthesis (*ABA1* and *ABA2*), in ABA dependent signalling (*ABI1*, *ABI2* and *ABI5*), in response to ABA (*RD29A*, *RD29B*, *EM6* and *RAB18)* as well as in ABA independent signaling (*COR15A and COR47*) were selected for this assessment. *ABA1* and *ABA2* were down-regulated in *HaHB11* compared to WT plants, independently of the presence of ABA (Supplementary Figure

1). *ABI1*, *ABI2*, *ABI5* and *RAB18* were also repressed in *HaHB11* compared to WT in standard conditions or after ABA treatment, although they were induced by ABA in both genotypes. *COR15a*, *COR47* and *RD29a* were down-regulated in *HaHB11* compared to WT untreated plants and ABA repressed their expression, independently of the genotype. *EM6* and *RD29b* were induced in *HaHB11* plants compared to WT in both conditions (Supplementary Figure 1). These results indicated that *HaHB11* differentially regulates genes involved in ABA biosynthesis and ABA independent signaling pathway and genes involved in cellular protection. This differential regulation would contribute somehow to the generation of the complex phenotype observed in *HaHB11* transgenic plants.

HaHB11 confers drought tolerance to transgenic alfalfa

Arabidopsis is a model plant which exhibits multiple advantages for the experimental work including routinely transformation methods, genetic tools and a short life cycle. This species allows acquiring knowledge in a relative fast form; however it is not evident that an observed differential phenotype caused by a transgene would be repeated in other plant species or crops. With this question in mind, a second species was chosen to test *HaHB11* as a biotechnological tool. Alfalfa (*Medicago sativa*) plants were transformed with the constructs *35S:HaHB11* and *ProH11:HaHB11*. Several independent transgenic lines were obtained and *HaHB11* transcripts levels evaluated by RT-qPCR (Supplementary Figure S2). Six independent lines transformed with *35S:HaHB11* (H1, H2, H7, H10, H16 and H17) and two of those transformed with *ProH11:HaHB11* (Pr1 and Pr7) were chosen for further analysis. One of the lines (H21), in which it was not possible to detect *HaHB11* transcripts was chosen as negative control. Morphological and developmental traits were assessed and no significant differences were detected between transgenic and WT plants in F1 plants. A severe drought treatment was applied by stopping watering during ten consecutive days until plants looked truly damaged. At day 11^{th} plants were watered and observed (Figure 5A). Lines H1, H2, H10 and H16, which showed higher expression levels, recovered themselves whereas other plants died. Water loss during the treatment was also evaluated and, in general, no big differences between lines were detected (Figure 5B). However, at 7 and 8 days after treatment, *35S:HaHB11* plants lost less water compared to controls (Figures 5C and 5D).

HaHB11 transgenic Arabidopsis plants are tolerant to salinity stress

Drought and salinity stresses are frequently but not always related. On the other hand *HaHB11* expression was induced by a NaCl treatment which indicated a possible role in such condition. To investigate if *HaHB11* is able to confer a differential trait in front of salinity stress, transgenic Arabidopsis plants and controls in the vegetative stage were subjected to salinity stress by gradually adding NaCl to the pots as described in Methods. High expression level lines tolerated the treatment and remained green whereas controls became yellow and lost chlorophyll (Figure 6A).

To evaluate yield after salinity stress, plants were irrigated three times separated by 7 days each with NaCl 100 mM which generated a mild stress. At the end of the life cycle, seeds were harvested and yield evaluated. The results indicated that all the plants tolerated such moderate stress without significant losses and a slight improved yield was observed in the transgenic genotypes compared to WT (Figure 6B).

Discussion

HaHB11, a sunflower divergent transcription factor belonging to the HD-Zip I family, was previously described as a biotechnological tool. It conferred improved yield and tolerance to flooding, both waterlogging and submergence, to transgenic Arabidopsis plants by a quiescent mechanism. Moreover, after flooding treatments death of controls occurred by dehydration, called desubmergence effect (Cabello et al., 2016). This latter result indicated a possible tolerance to water deficit displayed by these plants. A role in flooding was firstly suspected by the differential regulation of several genes related to this stress condition (Bailey-Serres and Voesenek, 2008; Bailey-Serres et al., 2012). Here, we investigated the differential behavior of *HaHB11* plants in front of drought and salinity stresses.

Firstly, an expression analysis of *HaHB11* in two developmental stages indicated a positive regulation by ABA, NaCl and drought. This result was not surprising because several members of this TF family, and especially those more close in the phylogenetic tree to HaHB11, like AtHB7, AtHB12, MtHB1 were also described as regulated by these factors (Ariel et al., 2010; Henriksson et al., 2005; Ribone et al., 2015; Ré et al., 2014). It was reported that *ATHB7* and *ATHB12*, both strongly induced by water-deficit and ABA, act as positive transcriptional regulators of phosphatases type 2C genes and as negative ones of ABA signaling (Valdes et al., 2012). These conclusions derived from chromatin immunoprecipitation and gene expression analyses. It was also demonstrated that those Arabidopsis HD-Zip I TFs repress the transcription of ABA receptors genes (*PYL5* and *PYL8*) in response to ABA stimulus (Valdes et al., 2012). However, the overexpression of both closest Arabidopsis members did not trigger a drought-tolerant phenotype (Olsson et al., 2004; Romani et al., 2016). *HaHB11* induction occurred late, at day 10th of the treatment and the induction of *HaHB1*, another member of the sunflower HD-Zip I family which conferred drought tolerance via a membrane stabilization mechanism, picked at the 4th day (Cabello and Chan, 2012). At the 10th day of the drought treatment, plants were seriously dehydrated, almost died, and this was a scenario very similar to that observed when *HaHB4* transgenic plants were studied. These latter plants exhibited a high drought tolerance (Dezar et al., 2005). These results might suggest that drought late-response genes would have an active role in front of severe stress.

Considering salinity stress, both *HaHB1* and *HaHB11* transcript levels were induced after three days of 50 mM addition showing a similar behavior (this work and Cabello and Chan, 2012).

Drought and salinity tolerances are precious traits but they would be truly appreciated when they were accompanied by improved yield, or at least by no yield penalties. *HaHB11* transgenic plants exhibit an enhanced tolerance to several abiotic stress factors (flooding, drought, salinity) without yield penalties when plants are grown in standard or mild stress conditions compared to controls. Moreover, these plants had a considerable yield increase in normal growth conditions (Cabello et al., 2016). Although the transgene is heterologous in Arabidopsis and alfalfa, the analyses showed in this manuscript allowed us to know how a combination of physiological and molecular changes triggered in the plants by the expression of this foreign TF can generate a multiple tolerance phenotype. This combination of responses is schematized in Figure 7.

Leaf rolling, stomata closure and reduced leaf area are different physiological mechanisms conducting to drought avoidance. Such mechanisms are useful for the plant when it suffers terminal drought; however, they are usually associated with a reduced biomass and yield in milder drought scenarios (Tardieu, 2012). *HaHB11* plants displayed at least two out of these three mechanisms because they presented leaf rolling and stomata closure under stress. However, these plants have not exhibited yield penalties when grown in mild stress conditions (drought or salinity). On the contrary, they exhibited increased yield and this is probably because they elongate primary roots and have wider stems and more vascular bundles than their controls. Other authors described plants with similar characteristics; such is the case of transgenic rice plants transformed with *OsHI1* which had increased yield, more vascular bundles and branches (Terao et al., 2010). Notably, *HaHB11* plants also had wider stems and more vascular bundles than controls, strongly indicating that these traits can equilibrate the loss produced by leaf rolling and stomata closure occurring under drought conditions.

Interestingly, *HaHB11* plants stems wide and vascular bundles significantly varied with the hydration condition and this variation was concomitant with the obtained yield. After a severe drought, the stems of *HaHB11* and WT plants were indistinguishable considering wide and number of vascular bundles. Accordingly, seed production decreased in both genotypes. It is already known that seed production is mainly determined at flowering and slightly after it (Taiz and Zeiger, 2006). In most species,

the number of ovules largely exceeds the number of seeds, and water deficit reduces even more the seed/ovule ratio via abortion (Dosio et al., 2010). This adaptive mechanism allows the remaining seeds to be appropriately filled in spite of reduced photosynthate supply, sometimes with effects (positive or negative) on seed quality (Tardieu, 2012). Notably, *HaHB11* plants subjected to mild stress conserved stem morphology and yield production, which were similar to those evaluated in normal growth conditions.

Another anatomic change observed in *HaHB11* plants was the lignin content. When the plants were subjected to drought, stem lignification increased. It was described that different types of abiotic stresses, including drought, caused changes in the lignin contents (Moura et al., 2010). Moreover, the key enzyme in lignin biosynthesis, CAD (CINNAMYL ALCOHOL DEHYDROGENASE) from *Ginkgo biloba* and from sweet potato increased its expression in stems under abiotic stress (Kim et al., 2010; Cheng et al., 2013).

It is clear that plant survival and plant performance under water deficit are complex processes depending on different physiological and molecular mechanisms (Tardieu, 1996; Skirycz et al., 2011). *HaHB11* displayed several of them concomitantly generating a complex but beneficial phenotype.

When induced by drought and salt stresses, it acts as a positive regulator of ABA responsive genes, leading to enhanced drought and salt tolerance. On the other hand, the down-regulation of ABA biosynthesis genes indicated that under drought or salt stress, *HaHB11* needs to decrease ABA levels in plants. ABA signaling may be stronger in *HaHB11* transgenic plants, inducing genes that have cellular protection function, like *EM6* and *RD29b* (Ding et al., 2009; Msanne et al., 2011).

Conclusions

The transcription factor HaHB11 confers drought and salinity tolerances by the combination of different changes provoked to transgenic plants. Among the physiological variations, leaf rolling, root elongation and stomata closure seem to be the more important to generate the tolerant phenotype. Transgenic plants, both Arabidopsis and alfalfa, consume less water which conducts to a more efficient use of water compared to controls.

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Figure legends

Figure 1. ***HaHB11 expression is induced by drought, NaCl and ABA in sunflower seedlings and V3 plants***

HaHB11 transcripts were measured by RT-qPCR in different organs and two developmental stages. (**A**), (**B**) and (**C**) 7-day old seedlings in different organs (A), after abiotic stress (**B**) or hormone (**C**) treatments. (**D**), (**E**), (**F**), (**G**) and (**H**) 21-day-old plants organs (**D**), after abiotic stress (**F**), kinetics of water deficit stress (**G**) and salinity stress (**H**) treatments. All the values were normalized with the lowest expression value (roots, untreated plants or time 0, respectively) using the ΔΔCt method. *Actin* transcripts (*ACTIN2* and *ACTIN8*) were used as a reference. Error bars represent the standard deviation of three independent biological replicates.

Figure 1

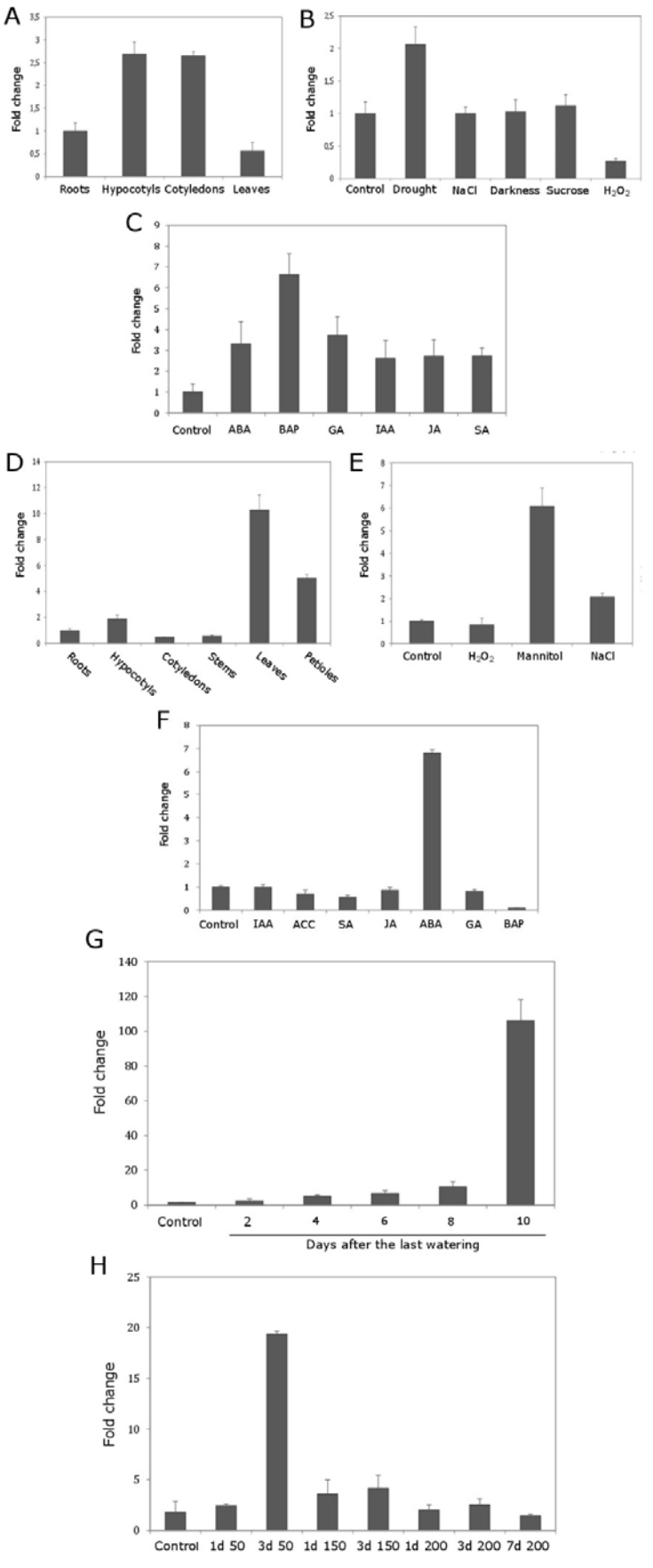
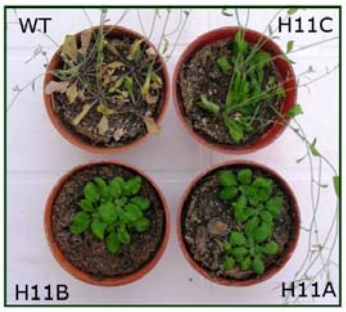


Figure 2. ***HaHB11 Arabidopsis transgenic plants use water more efficiently than WT***

A severe drought treatment was applied to 30 day-old WT and *HaHB11* transgenic plants. (**A**) Illustrative photograph was taken during the experiment. (**B**) Water loss evaluation during 13 days after stopping watering. (**C**) Survival percentage after recovery. (**D**) Water consumption was evaluated by weighting the pots during the drought treatment. (**E**) Illustrative photograph of plants during a water deficit treatment in which pots weight was equalized in all the pots by adding water to WT and H11-C plants. Statistical significance was determined by T-test. Asterisks depict $P \leq 0.05$.

Figure 2

A



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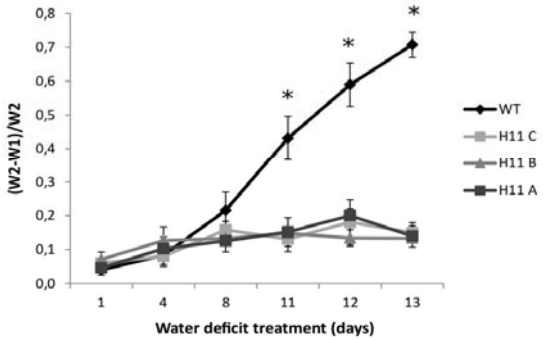
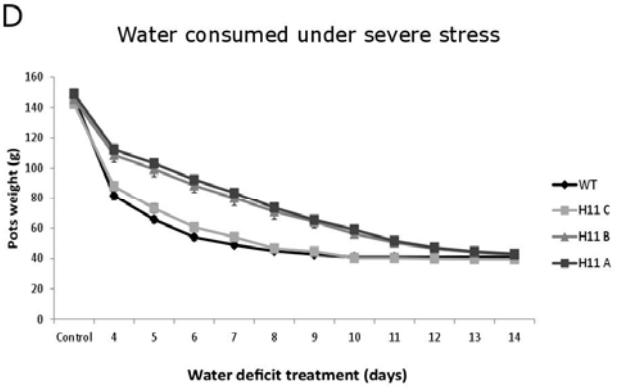


C

Survivors after drought stress				
	WT	H11A	H11B	H11C
Average	13	95	88	67
Deviation	6	5	6	8

D



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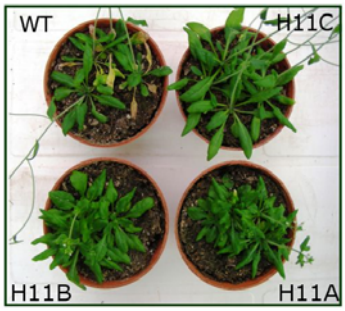


Figure 3. ***HaHB11 Arabidopsis transgenic plants have rolled leaves, longer roots and shorter hypocotyls compared to WT*** (**A**) Illustrative photograph of transgenic HaHB11 and WT plants grown in standard conditions. (**B**) 25-day-old leaves sections stained with toluidine blue. (**C**) 10-day-old seedlings from WT and H11-B lines grown in MS-agar 0,1 %. (**D**) Hypocotyls and roots length of 10-day–old seedlings from WT and H11-B lines (N= 25/genotype) in MS-agar 0,1%. Statistical significance was determined by T-test. Asterisks depict $P \leq 0.05$.

Figure 3

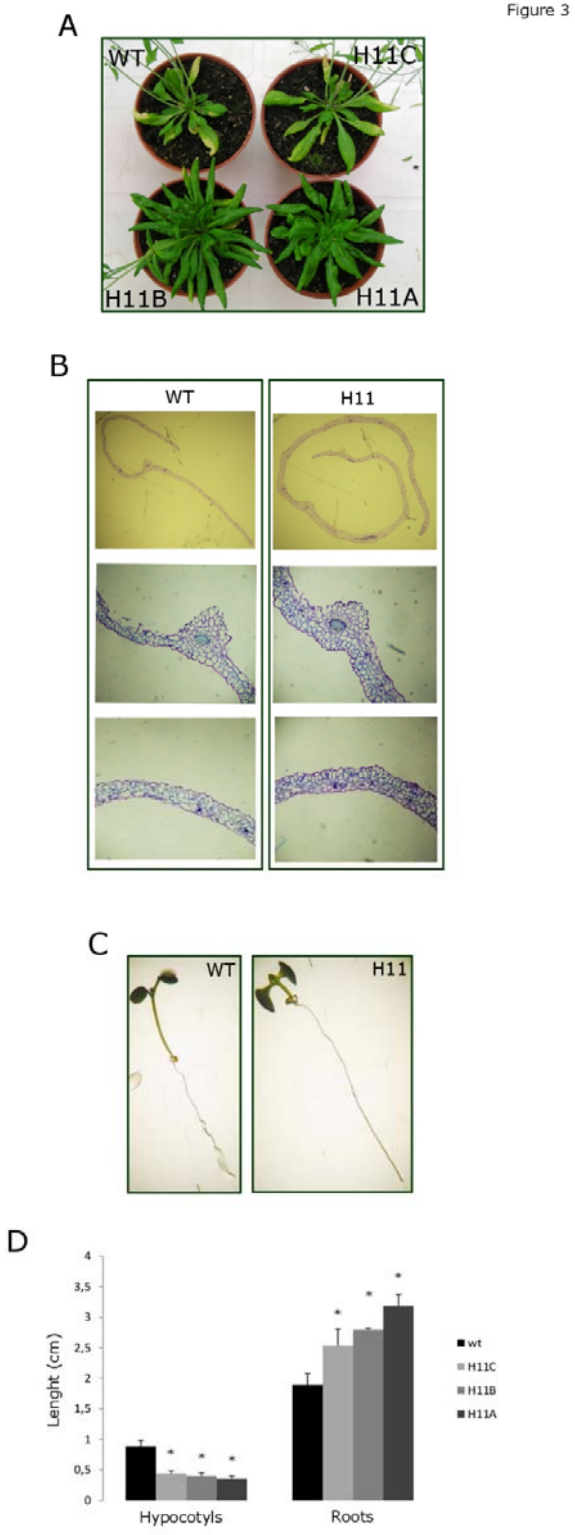
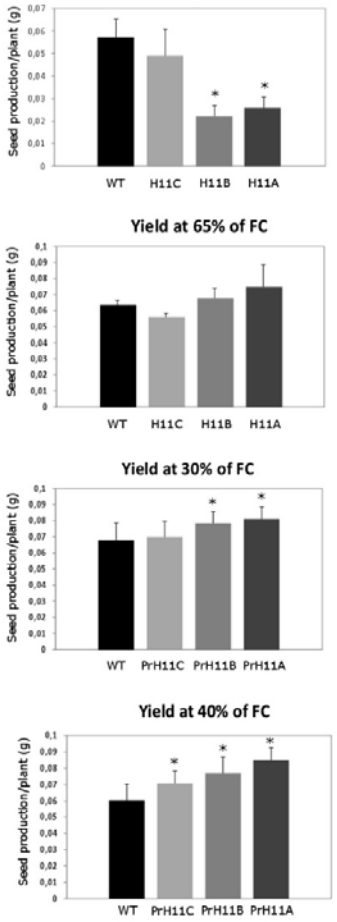


Figure 4. ***Plants yield is closely related with stem anatomy and the number of vascular bundles***

Seed production was evaluated in *HaHB11* transgenic and WT plants in different stress conditions. (**A**) Upper panels: seed production per plant in *35S:HaHB11* (H11-A, -B, -C) and WT genotypes in 40% and 65% field capacity conditions; lower panel: seed production of *ProH11: HaHB11* and WT plants at 30 and 40% field capacity. Four plants per genotype were considered for each evaluation; the experiments were repeated at least five times. (**B**) Stem sections (first internode) from 35 day-old WT and 50 day-old *35S:HaH11* plants grown in normal conditions, (C) under 40% and (D) 65% field capacity. Sections were stained with toluidine blue (left panel) or observed under UV in a fluorescence microscope (right panel, B). Asterisks indicate a T-test ≤ 0,05.

Figure 4

A



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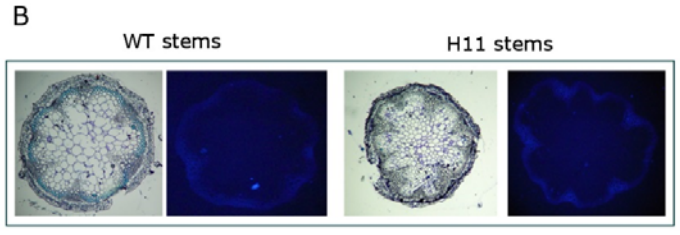

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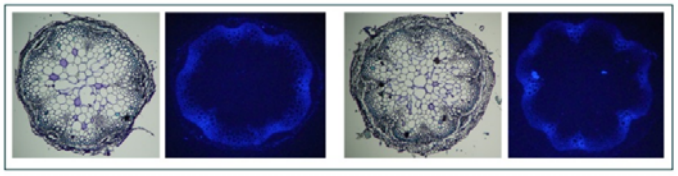

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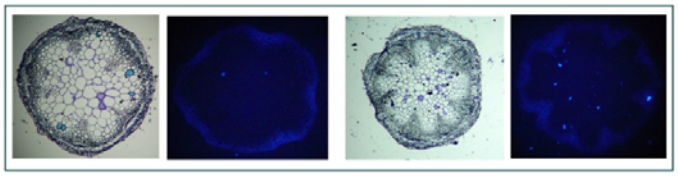

Figure 5. ***Alfalfa plants transformed with 35S:HaHB11 are tolerant to water deficit***

A severe drought treatment was applied to alfalfa plants transformed with *35S:HaHB11* or with *ProH11:HaHB11*. (A). Illustrative photograph of alfalfa plants taken after rehydration. H1, H2, H7, H10, H16 and H17 are independent lines transformed with *35S:HaHB11* whereas PR1 and PR7 are independent lines transformed with *ProH11:HaHB11*, playing as controls with *HaHB11* low-expression. (**B**) Water loss evaluation during a severe drought stress treatment applied to the same lines during 9 days. (**C**) and (**D**) Water loss evaluation at day 7 and 8, respectively, of the same lines as in **A** and **B**. In B, C and D, line H21 was used as a negative control

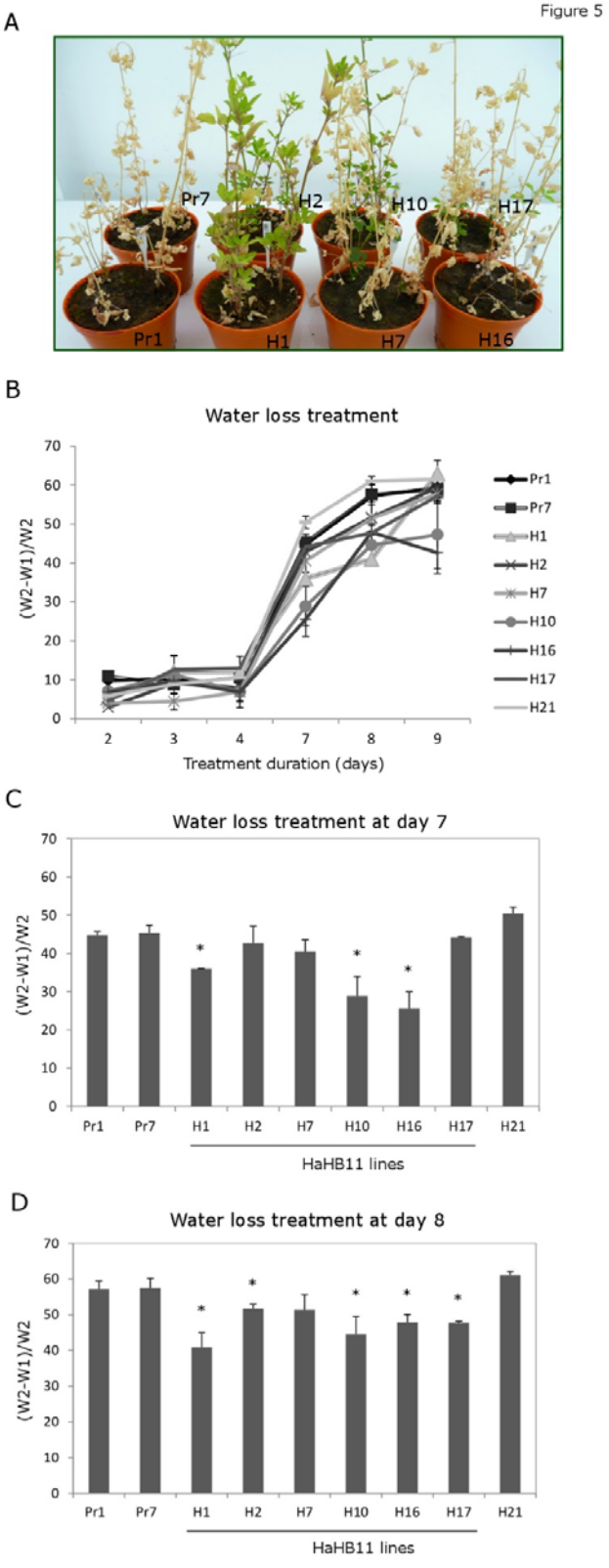

Figure 6. ***HaHB11 Arabidopsis transgenic plants are tolerant to salinity*** Thirty day-old WT and *HaHB11* transgenic plants were subjected to two different salinity stress

conditions. (**A**) Illustrative photograph of plants subjected to a severe salinity stress. (**B**) Seed production per plant (4 plants of each genotype repeated at least five times) after a moderate salinity treatment. Error bars represent the standard deviation of 20 independent biological replicates.

Figure 6

A

B

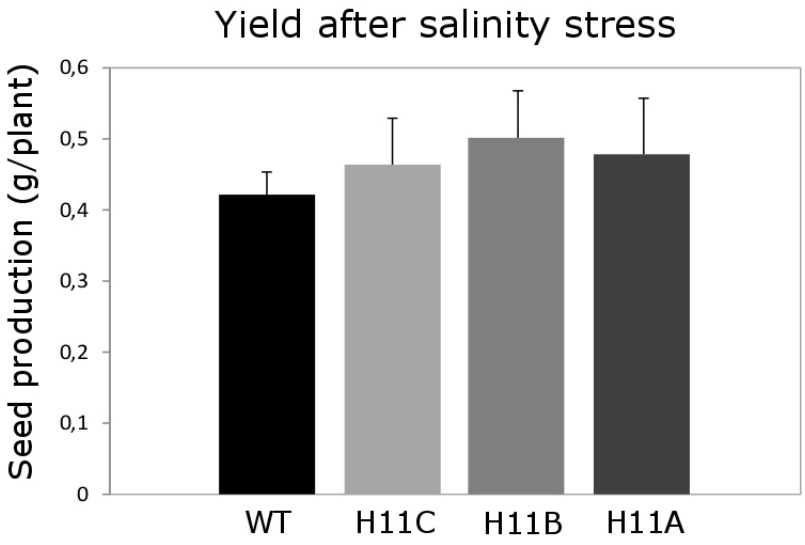

Figure 6. Proposed model for *HaHB11* regulation and effects on transgenic plants