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The antagonistic basic helix-loop-helix partners BEE and IBH1 contribute to control plant tolerance to abiotic stress

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ABSTRACT

The bHLH family is composed by canonical and non-canonical transcription factors (TFs) that differ in the presence or absence of their DNA-binding domain, respectively. Since both types of bHLH proteins are able to dimerize, their relative abundance impacts their biological activity. Among this TF family BEE and IBH are canonical and non-canonical bHLHs, respectively and previous reports indicated that BEE2 and IBH1 dimerize. Wondering whether BEE TFs participate in the abiotic stress response and how the dimerization with IBH1 could regulate their role in Arabidopsis, double *bee1/bee2* and triple *bee1/bee2/bee3* mutants were tested under salinity and drought stresses. The *bee1/bee2/bee3* mutant showed an enhanced tolerance whereas the double mutant behaved similar to wild type plants. These results indicated that *BEE* genes play a role in the stress response and also put in evidence the redundancy within the BEE family. Moreover, ectopic expression of *IBH1* on different mutant backgrounds improved plant tolerance to abiotic stress, independently of the background. However, the yield of these transgenic plants was penalized with abortive seeds. Our results suggest that *BEE* genes are negative regulators of physiological responses to abiotic stress whereas IBH1 is a positive modulator via different pathways, one of them involving BEE TFs.

1. Introduction

Regulation of gene expression in plants occurs at different stages involving different molecules which in a coordinated way balance plant needs modulating transcription, translation, post transcription and post translation by varied ways. In plants, transcription constitutes the most important regulatory step in which transcription factors (TFs) play key roles.

Transcription factors are especially important in the plant kingdom representing between 3–6% of total encoding genes. The Arabidopsis genome encodes 1500 TFs and among them, 45% belong to plant specific families [1]. Plant TFs have been classified in families and subfamilies according to structural features. There are twenty one plant-specific TF families and 14 of them are only present in land plants [2]. Using fully sequenced genomes, it was shown that most of these families were already incorporated before land plant colonization [3]. In addition, plant radiation was accompanied by an expansion in already existing plant TF families [3,4]. This expansion is not solely caused by diversification but also by the unique combination of conserved domains. Members of such divergent or expanded families have been shown to participate in biological processes unique to plants like development in response to environmental factors and particularly to stress [5].

Stress responsive TFs belong to MYB (MYeloBlastosis oncogene), bHLH (basic helix–loop–helix), AP2/ERF (APETALA 2/ethylene-responsive element-binding factor), basic leucine zipper (bZIP), NAC (NAM, ATAF, and CUC), HD, and WRKY families [1,2,5,6].

The bHLH family is the second largest in number of members in Arabidopsis and these proteins have two conserved regions, the HLH and the basic domain, responsible for DNA binding. In general, these domains are able to bind E- or G-boxes (CANNTG and CACGTG, respectively) [7]. To date, several bHLH have been associated to stress responses: drought, salinity, freezing, etc. In fact, the most characterized member of this family is ICE1 which controls CBF expression, a TF involved in freezing tolerance in Arabidopsis [8].

BEE1, BEE2, and BEE3 are bHLH redundant TFs that have been described as involved in the early response required for brassinosteroids (BRs) action [9]. The expression of these genes is regulated by BRs and also by abscisic acid (ABA), antagonist of BRs. At early developmental stages, the triple mutant *bee1/bee2/bee3* has shorter hypocotyls than those of wild type (WT) and at later stages, this mutant exhibits smaller floral organs. These phenotypic alterations were not observed in the

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single and double mutants suggesting an important degree of functional redundancy between these genes [9]. *BEE1* overexpressors exhibited a weaker ABA response and have larger flowers than controls. Based on these experimental data, it was suggested that BEE proteins may function as signaling molecules in multiple pathways [9].

IBH1 is part of a small clade known as atypical bHLH and it is able to dimerize with specific bHLH members through the HLH domain; however IBH1 lacks the basic binding domain required for DNA binding [10,11]. Hence, when such dimerization occurs, the partner is inactivated losing its capability to bind DNA. By this way, IBH1 represses cell elongation dimerizing with ACE and/or CIB5 [10,11]. IBH1 was also reported as able to dimerize with BEE2 [11,12].

The phytohormone ABA plays a central role in abiotic stress response of the plant and modulates multiple aspects of different acclimation responses. The core perception mechanism of ABA was recently elucidated [13] but downstream molecular players remain unclear. *BEE* genes are part of early response genes that control trade-off responses of the plant, between growth and defense or growth versus stress response. The expression of *BEE* genes is strongly induced by BRs, growth promoting hormones, and strongly repressed by ABA and pathogen signals [9,14]. The putative contribution of *BEE* genes to ABA-induced responses *in planta* remains poorly understood so far. With the hypothesis that IBH1 could be capturing BEE TFs and as a consequence, inactivating them, we decided to investigate the role of BEE1, 2 and 3 and their putative interaction with IBH1 in the abiotic stress response.

In the present work, we show that *BEE* genes are redundant negative regulators of plant responses related to abiotic stress. In addition, we found that ectopic expression of *IBH1* in Arabidopsis plants also improved plant tolerance to abiotic stress through various ways. One of these pathways involves *BEE* genes, since the ectopic expression of *IBH1* in the triple *bee1/bee2/bee3* mutant improved to a higher extent some physiological responses of this mutant.

2. Materials and methods

2.1. Plant material, growth conditions and plant treatments

Arabidopsis thaliana plants were grown on Klasmann Substrate No. 1 compost (Klasmann-Deilmann GmbH, Germany). Growth chamber conditions were set in 22–24 °C under long-day conditions (16/8 h light/dark cycles) with a light intensity of approximately 120 µmol $m^{-2} s^{-1}$ in 8 × 7 cm pots. The Col-0 ecotype was used as the WT control in all experiments. All plant experiments were done with four plants per pot. The insertional mutants used in this study were described before: the *ibh1* single mutant (SALK 049177) [15], the double *bee1/bee2* and the triple *bee1/bee2/bee3* mutants [9].

2.2. Transgenic plants carrying the IBH1 gene

The *IBH1* cDNA was obtained from an ABRC clone U16375 (Ohio State University, Columbus). The cDNA was provided into a pEntr/SD-DTopo vector. The fully re-sequenced clone was used as a substrate for an LR-Clonase reaction using the pFK247 vector as a destination vector. The pFK427 vector is derived from the pGreen vector series: genetic fragments were recombined by Gateway cloning into a modified pGreen vector (pFK210) conferring resistance to BASTA [16]. A *35S CaMV* promoter drives the expression of an N-terminal fusion protein with GFP, here named $35S_{pro}$:*GFP-IBH1* (*IBH1* OE). A sequence-verified clone was used for transforming *Agrobacterium tumefaciens* strain LBA4404 and then generating transgenic Arabidopsis plants with the floral dip method [17]. In order to screen for low-expressing lines, we followed a two-step selection process. First, T1 seedlings were screened on soil trays for resistance to BASTA (50 mg ml^{-1}) and second, we used a Leica TCS SP8 Compact confocal microscope to check for nuclear localization of GFP-IBH1 in BASTA resistance plants with no visible effect on rosette expansion. All experiments were done with transgenic lines containing a single T-DNA insertion based on T2 segregation test following a 3:1 ratio of the herbicide resistance marker. Homozygous T3 lines were further used to analyze transgene expression levels and plant phenotypes.

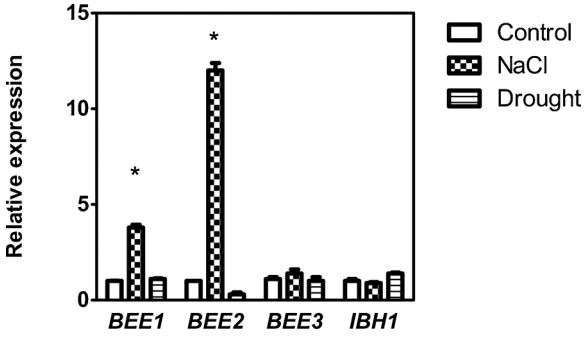

Fig. 1. *BEE1* and *BEE2* expression is induced by salinity in Arabidopsis plants. The relative transcript levels of *BEE1*, *BEE2* and *BEE3* were measured in rosette leaves of 25 day-old WT (Col-0) plants after 48 h of salinity and drought stress treatments. *BEEs* transcript abundance was measured and expressed relative to the level detected in control plants of Col-0 genotype. Error bars represent the standard error of three independent biological replicates. Statistical significance was computed by Student's *t*-test (Asterisks indicate $P < 0.05$).

2.3. RNA isolation and expression analyses by real time RT-PCR

Total RNA for real-time RT-PCR was isolated from Arabidopsis leaves using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (500 ng) was reverse-transcribed using oligo(dT)18 and M-MLV *RevertAid Reverse* Transcriptase (Thermo Scientific). Quantitative real-time PCR (qPCR) was performed using a Mx3000P Multiplex qPCR system (Stratagene, La Jolla, CA) and the following primers: BEE1_FP (TAAggCTATgggAATggCTACg); BEE1_RP (TTgCTgCAgTgAgTTTCATCg); BEE2_FP (ACCACATCTCTCggCgCT); BEE2_RP (CAgAATCggTTCTCAAgCTgTTg); BEE3_FP (CAgAACgggTTCgACgAgg) and BEE3_RP (TCAAgCATAgTAgCCATTCCCAT); IBH1-FP (AgAggCTgAggAATCTTgTTCCg); IBH1-RP (ATgAgCCgTCTCTTCCATCAgC). Quantification of mRNA levels was achieved by normalization against actin transcripts levels (ACTIN2 and ACTIN8) according to the ΔΔCt method using the following primers: Actin-FP (ggTAACATTgTgCTCAgTggTgg) and Actin-RP (AACgACCTTAATCTTCATgCTgC). Three biological replicates, tested by duplicates, were used to calculate the standard deviation. Each replicate was obtained by pooling tissue from 3 to 4 individual plants. The samples were obtained from plants treated for 48 h with the corresponding stress condition.

2.4. Drought and salinity stress treatments

Treatments in soil started when plants were 25-day-old. Each plant genotype had 16 pots (8 × 7 cm) with 4 plants per pot. The genotypes were distributed on different trays following a completely randomized design. Both drought stress and salinity treatments extended up to the end of the plant life cycle. After two weeks of treatment, we measured different plant traits including: 1) total leaf chlorophyll, 2) water loss in whole plant and isolated leaves, 3) leaf densitometry. The plant architecture attributes including silique number and seed yield were scored at the time of the harvest. Plant pictures were taken at different stages of the experiments. The drought stress treatment consisted in applying a mild stress defined as 60% of field capacity. To determine the field capacity, pots were watered, left to drain out the water in excess and weighted. This initial weight was considered 100% field capacity. The salt stress was applied at the same plant age watering the plant with NaCl solution every 5 days. The irrigation treatments were as follows: day 1, 1000 mL of 50 mM NaCl; day 5, 1000 mL of 100 mM

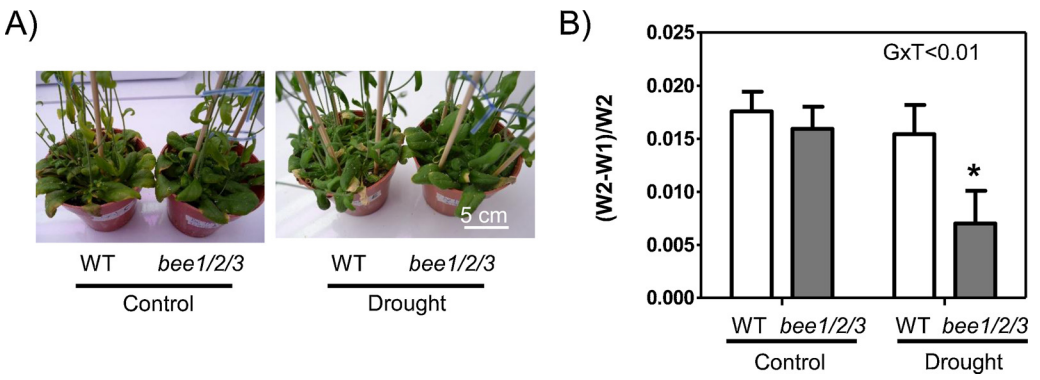

Fig. 2. The triple *bee1/bee2/bee3* mutant shows enhanced tolerance to mild drought stress. (A) Illustrative pictures showing Col-0 and *bee1/bee2/bee3* plants grown with a mild drought treatment. Scale bars are shown in the picture. (B) Leaf rehydration assay after 14 days of mild drought stress. This assay was done using 5 replicates per genotype. In all cases, thin bars represent standard error. The significance of the relevant terms of the analysis of variance (G, genotype; T, Treatment; GxT, G by T interaction term) is indicated in each panel. Asterisk indicates significant differences between means in cases in which the interaction term was significant ($P < 0.05$, Bonferroni posttests).

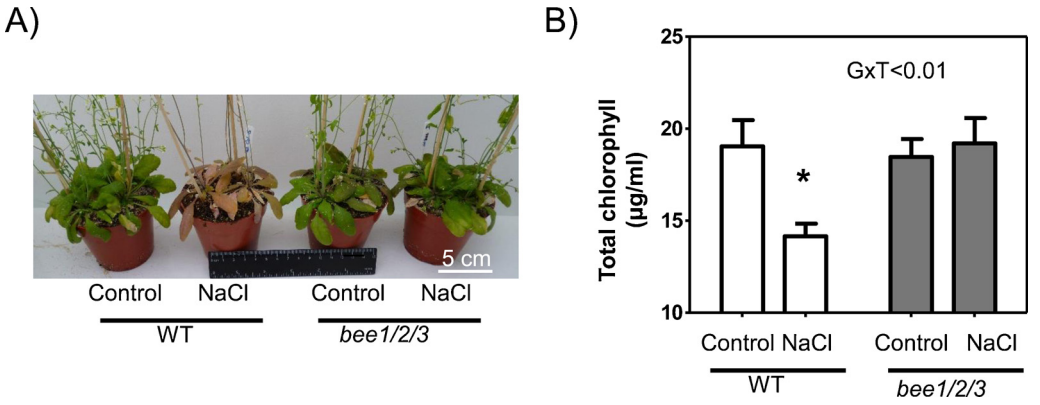

Fig. 3. The triple *bee1/bee2/bee3* mutant has increased tolerance to salinity. (A) Illustrative picture showing Col-0 and *bee1/bee2/bee3* plants after salinity treatment. Scale bars are shown in the picture. (B) Total chlorophyll content of leaf discs of NaCl treated plants. Extracts were prepared in triplicates from a pool of 4 leaf-discs each, harvested 7 days after treatment initiation. In all cases, thin bars represent standard error. Asterisks indicate significant differences between means within the treatment ($P < 0.05$, Bonferroni posttests). G states for Genotype and T for treatment, whereas GxT is the interaction of the factors.

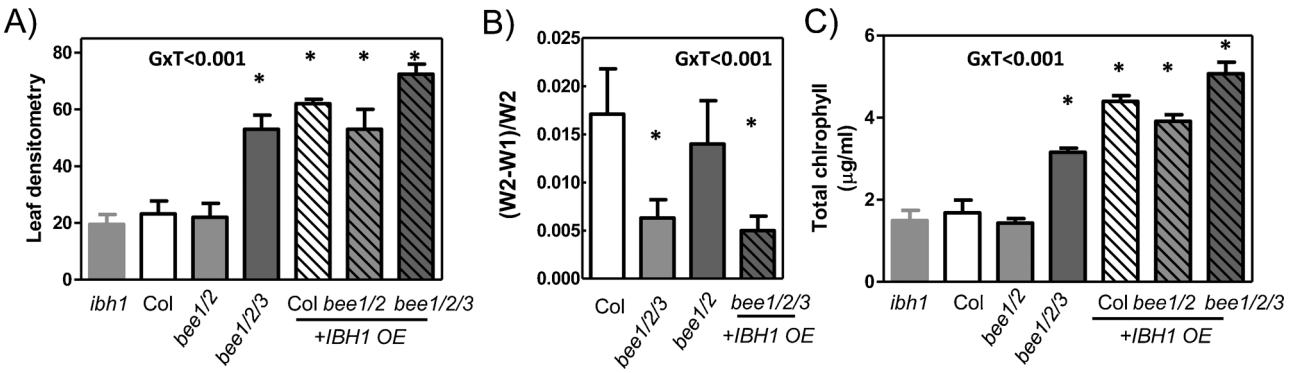

Fig. 4. Plant tolerance to drought and salinity is improved by *IBH1* overexpression. (A) Effect of drought stress on leaf densitometry. (B) Water loss evaluation in the indicated genotypes after a drought stress treatment (C) Impact of salinity on total chlorophyll content of leaf-discs. Extracts were prepared from a pool of 4 leaf-discs in triplicates, harvested 15 days after treatment initiation. In all cases, thin bars represent standard errors. The significance of the relevant terms of the analysis of variance (G, genotype; T, Treatment; GxT, G by T interaction term) is indicated in each panel. Asterisks indicate significant interaction differences between means. Scale bars are shown in the picture.

NaCl; day 10, 1000 mL of 150 mM NaCl and day 15, 1000 mL of 200 mM NaCl solution.

2.5. Plant survival assays in response to severe drought and prolonged salinity stress

Arabidopsis 25-day-old plants were subjected to severe drought treatment and prolonged salinity stress. In all cases, each plant genotype had 4–6 pots (8 × 7 cm) with 4 plants per pot. The genotypes were distributed on different trays following a completely randomized design. Severe drought consisted in stopping watering at day 25 for two weeks until WT plants looked severely affected (Supplementary Fig. S2A). At this time, all genotypes were rehydrated and watered daily to score plant survival after 7 days (Fig. 5 and Supplementary Fig. S2B). In parallel, the salinity treatment consisted on watering the plants with increasing concentrations of NaCl solutions using the following pipeline: day 1, 1000 mL of 50 mM NaCl; day 5, 1000 mL of 100 mM NaCl; day 10, 1000 mL of 150 mM NaCl solution and irrigated with water until the end of the experiment. The NaCl-treated plants were monitored along the treatment (Fig. 5 and Supplementary Fig. S2C). Plant pictures shown in Supplementary Fig. S2 were taken after two weeks of treatment and after one week following plants rehydration with water.

2.6. Rehydration assay of detached leaves

The water loss in detached leaves of Arabidopsis plants was measured on 6 leaves from 5 different plants. The initial leaf weight was defined as the starting point of the experiment (W1). Subsequently,

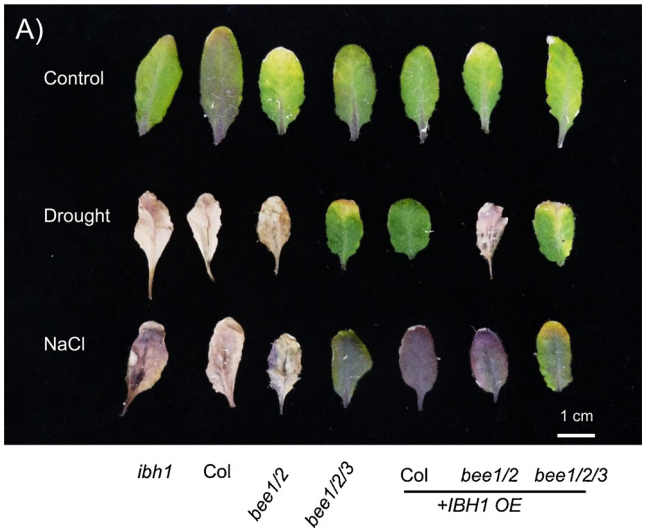

Fig. 5. *IBH1* OE plants exhibit improved performance upon drought and salinity stress. (A) Illustrative pictures showing the general aspect of detached leaves from plants growing in drought stress and NaCl treatment. The phenotype was scored in the following genotypes: Col-0, *ibh1*, *bee1/bee2* and *bee1/bee2/bee3* and those overexpressing *IBH1* in Col-0, *bee1/bee2* and *bee1/bee2/bee3*.

Table 1
Survival rates of different genotypes after severe drought and salinity treatments.

Treatment	Genotype	No. of plants per experiment	No. of survivors	% survivors after rehydration
Drought	*ibh1*	16	0 ± 1	0
Drought	Col	16	0	0
Drought	*bee1/2*	16	0	0
Drought	bee1/2/3	16	14 ± 1	87.5
Drought	Col (+*IBH1 OE*)	16	16 ± 1	100
Drought	*bee1/2* (+*IBH1 OE*)	16	6 ± 2	37.5
Drought	*bee1/2/3* (+*IBH1 OE*)	16	15 ± 1	93.7
Salinity	*ibh1*	16	0 ± 1	0
Salinity	Col	16	0	0
Salinity	*bee1/2*	16	0	0
Salinity	bee1/2/3	16	14 ± 1	87.5
Salinity	Col (+*IBH1 OE*)	16	16 ± 1	100
Salinity	*bee1/2* (+*IBH1 OE*)	16	6 ± 2	37.5
Salinity	*bee1/2/3* (+*IBH1 OE*)	16	15 ± 1	93.7

Average numbers of plants surviving after exposure to stress. In the control treatment, all plants were alive.

detached leaves were incubated in demineralized water for 3 h, and weighed again (W2). The difference in weight (W2-W1)/W2 was considered as water loss [18].

2.7. Total leaf chlorophyll and leaf densitometry

Chlorophyll was extracted from four 0.5-cm diameter leaf discs in 1.0 mL of 80% acetone. Following a thorough vortexing and a 30 min incubation in total darkness, the absorbance of the solution was read at 645 and 663 nm. Total chlorophyll was determined according to the equation: $20.2\,A_{645} + 8.02\,A_{663}$ [19]. We used a non-destructive method to estimate the proportion of green tissues in the rosette called leaf densitometry. We used ImageJ software to quantify green and total rosette areas from pot pictures taken at the indicated times [20].

2.8. Plant architecture characterization

Architectural parameters were scored on 40-day-old plants including: rosette diameter, main stem height and width, number of secondary branches, number of secondary stems. Total silique number and seed yield were scored at the end of the plant life cycle. Measurements were performed manually or with the aid of a ruler or Vernier caliper. Plant architecture was assessed in 8 replicates (4 plants per pot) per genotype.

2.9. Statistics and analysis

In all cases, the statistical analyses were carried out using INFOSTAT software (professional version 1.1). Abiotic stress assays comparing WT and the triple *bee1/bee2/bee3* mutant were analyzed using a two-way ANOVA with genotype and treatment as factors. When interaction terms were significant, differences between means were analyzed using Bonferroni comparisons. Data comparing the effect of *IBH1* overexpression were analyzed using a one-way ANOVA with the genotype as main factor. In these cases, the genotypes were compared against WT plants within the same treatment. Appropriate transformations of the primary data were used when needed to meet the assumptions of the analysis. The gene expression data was analyzed by Student's *t*-test.

3. Results

3.1. BEE1 and BEE2 transcription factors are regulated by salinity

In view of previous reports in which BEE1, BEE2 and BEE3 TFs were described as repressed by ABA, we wondered if such regulation was related to abiotic stress responses. Transcript levels of *BEE1*, *BEE2* and *BEE3* as well as of *IBH1* were assessed in 25 day-old WT Arabidopsis plants after for 48 h to drought and salinity treatments (27 day-old plants). *BEE1* and *BEE2* expression was induced after salinity stress whereas neither *BEE3* nor *IBH1* presented differential transcript levels compared to those measured in control conditions. *BEE2* levels were significantly enhanced compared with *BEE1* and none of these genes seemed to be regulated by drought, at least at this developmental stage and by this treatment that were further used to test the functional contribution of these genes (Fig. 1).

3.2. The triple mutant bee1/bee2/bee3 exhibits drought tolerance during the vegetative stage

BEE1, *BEE2* and *BEE3* genes were shown to be functionally redundant in the control of developmental and hormone-induced responses [9]. To further investigate putative roles of these genes in abiotic stress responses, WT and triple *bee1/bee2/bee3* mutant plants were subjected to drought stress and salinity treatments during the vegetative stage. We applied a mild drought stress by controlling the pot weight on 60% of its water capacity, whereas salinity stress was achieved by watering with increasing concentrations of NaCl solution every 5 days as described in Methods. Such treatments did not cause death and the general aspect of the plants looked rather healthy during the treatment (Fig. 2A). During the drought stress the *bee1/bee2/bee3* mutant lost less water than control plants, indicating an enhanced tolerance (Fig. 2B). Total chlorophyll content of the *bee1/bee2/bee3* mutant was similar to that of WT after drought stress treatment (data not shown). Conversely, we found an enhanced tolerance to salinity stress of the *bee1/bee2/bee3* mutant plants, revealed by a sustained inhibition of senescence and accompanied with a higher concentration of chlorophyll content in leaf tissues compared to the WT (Fig. 3A and B). These results indicated a negative regulation of drought and salinity tolerance responses by BEE TFs.

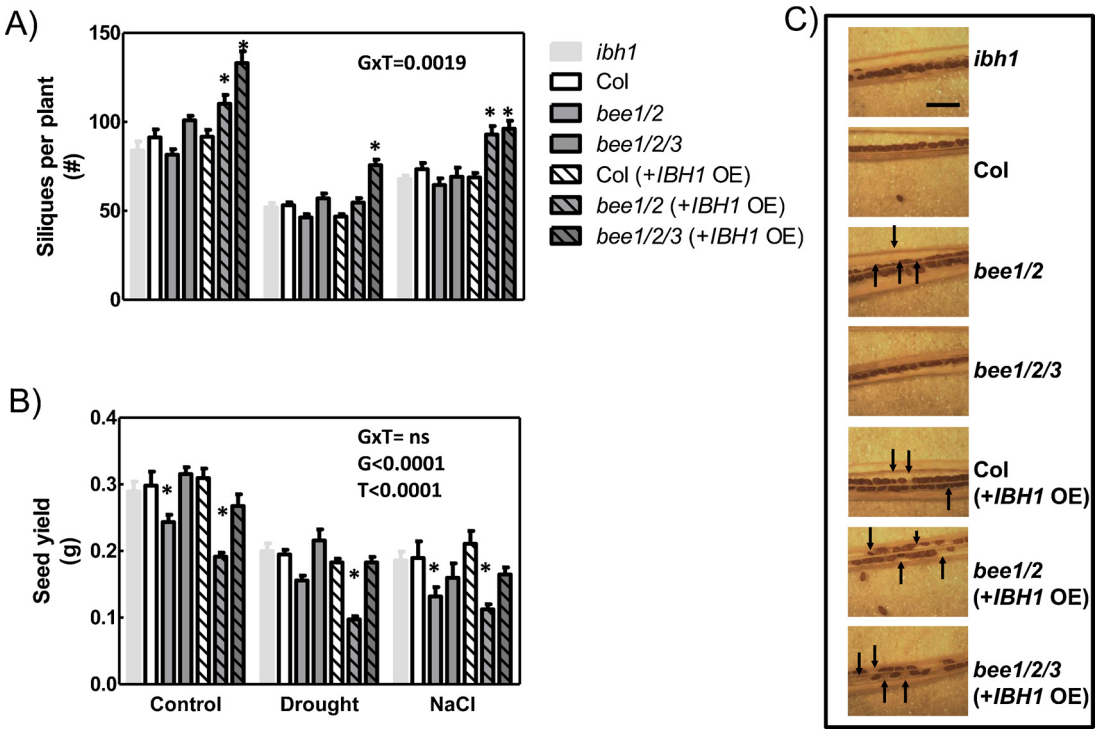

Fig. 6. Ectopic expression of *IBH1* affects plant yield components. (A) Total silique number was scored in the plants of the following genotypes: Col-0, *ibh1*, *bee1/bee2* and *bee1/bee2/bee3* and those overexpressing *IBH1* in Col-0, *bee1/bee2* and *bee1/bee2/bee3*. (B) Seed yield measured in grams of total seeds per pot. (C) Representative picture showing seed arrange within the silique of each genotype. Black arrows point to aborted seeds. Total silique number and seed yield was measured in 8 replicates. In all cases, thin bars represent standard errors. Asterisks indicate significant differences between means within the treatment ($P < 0.05$, Bonferroni posttests). G states for Genotype and T for treatment, whereas GxT is the interaction of such factors.

3.3. IBH1 could be interacting with BEEs to improve drought and salinity tolerance

Different lines of evidence have shown that IBH1, an atypical bHLH TF lacking the DNA binding domain, is able to interact with BEE2 and other bHLH proteins of the same clade [11,12]. To analyze whether the drought tolerance observed in the *bee1/bee2/bee3* mutant was the sole effect of BEEs mutation or somehow the effect of IBH1 capturing BEE2, we obtained transgenic plants overexpressing *IBH1* in the WT, the double *bee1/bee2*, or the *bee1/bee2/bee3* mutant backgrounds. The *ibh1* mutant and the double *bee1/bee2* mutant were used as complementary controls to the experiment. A mild drought stress was applied by stopping watering until the pots weight reached 60% of their water capacity. After 10 days of this treatment, WT, *ibh1* and *bee1/bee2* mutant plants showed a strong sensitivity to the treatment whereas *bee1/bee2/bee3* mutant looked rather healthy (Supplementary Fig. S1). Notably, *IBH1* overexpression on all these genotypes not only rescued the drought sensitiveness in Col-0 plants but also improved the *bee1/bee2/bee3* mutant performance in front of such stress (Fig. 4A–C and Supplementary Fig. S1). Since low water potential promotes cellular damage, and despite mechanisms to protect photosynthesis-related apparatus, chlorophyll content in green tissues diminishes after prolonged abiotic stresses like drought and salinity [21,22]. In order to estimate the ratio of the remaining green tissues in the pot, we performed a leaf densitometry between rosette green area vs total rosette area. The leaf densitometry of drought stress plants indicated that the *bee1/bee2/bee3* mutant as well as *IBH1* OE both in Col-0, *bee1/bee2* or *bee1/bee2/bee3* backgrounds increased this index, whereas *ibh1*, *bee1/bee2* mutants were as sensitive as WT plants to the treatment (Fig. 4A). As a complementary assay to estimate the level of drought stress of these plants, we performed a leaf rehydration assay on detached leaves. Considering water loss during the treatment, *IBH1* OE and *bee1/bee2/bee3* plants lost less water than controls and even less than the double *bee1/bee2* mutant (Fig. 4B). This differential response in water uptake was consistent with the increased leaf densitometry of *bee1/bee2/bee3* compared to WT leaves (Fig. 4A and B).

In parallel, we performed salinity assays to explore the contribution of BEEs and IBH1 to the salt tolerance response. Interestingly, the different genotypes showed marked variations on NaCl toxicity symptoms. WT plants, as well as *ibh1* and *bee1/bee2* mutant plants, showed a strong sensitivity to the NaCl treatment (Supplementary Fig. S1). These genotypes developed large chlorotic areas that translated into reduced levels of total leaf chlorophyll 15 days after treatment initiation (Fig. 4C). On the contrary, the triple *bee1/bee2/bee3* mutant showed an enhanced tolerance to salinity, that it was even improved with the overexpression of IBH1 (Fig. 4C and Supplementary Fig. S1). Yet, the overexpression of *IBH1* in Col-0 and *bee1/bee2* plants also enhanced the tolerance to the salinity treatment including fewer toxicity symptoms and higher chlorophyll content in leaves compared to WT (Fig. 4C and Supplementary Fig. S1). Taken together, these results indicate that *IBH1* overexpression in Arabidopsis plants also plays a role in the response to salinity.

To further evaluate the functional role of IBH1 and BEE using standardized experiments, we evaluated plant survival after severe abiotic stress, both in drought and salinity assays. In the case of drought, we stopped watering the plants for two weeks to rehydrate them for another week. At this point, we recorded plant survival as a ratio between livings vs. dead plants. WT plants did not survive this drought treatment (Fig. 5, Table 1 and Supplementary Fig. S2B). A similar effect was observed for *ibh1* and *bee1/bee2* mutant plants (Fig. 5, Table 1 and Supplementary Fig. S2B). On the other side, the *bee1/bee2/bee3* showed an enhanced survival rate compared to WT (Table 1). In coincidence with the results obtained for mild drought treatment, the overexpression of *IBH1* improved plant performance in all genetic backgrounds (Fig. 5, Table 1 and Supplementary Fig. S2B). However, the impact of *IBH1* overexpression on the *bee1/bee2* background was

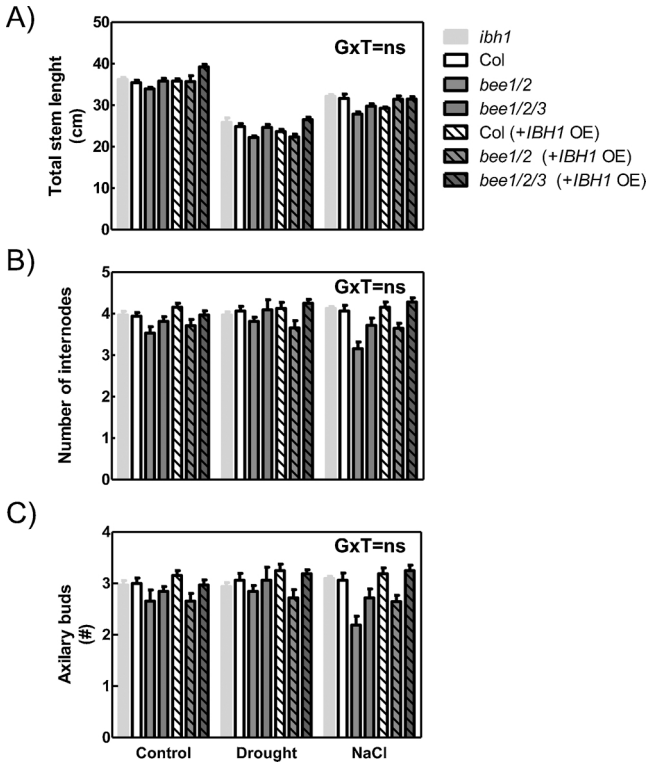

Fig. 7. Morphological characterization of plants with ectopic expression of *IBH1*. (A) Total stem length of the following plant genotypes: Col-0, *ibh1*, *bee1/bee2* and *bee1/bee2/bee3* and those overexpressing *IBH1* in Col-0, *bee1/bee2* and *bee1/bee2/bee3*. (B) Number of internodes in the main floral stem. (C) Number of axillary buds recorded on 40 day old plants. Morphological parameters were recorded in independent 8 replicates. In all cases, thin bars represent standard error. Asterisks indicate significant differences between means within the treatment ($P < 0.05$, Bonferroni posttests). G states for Genotype and T for treatment, whereas GxT is the interaction of the factors.

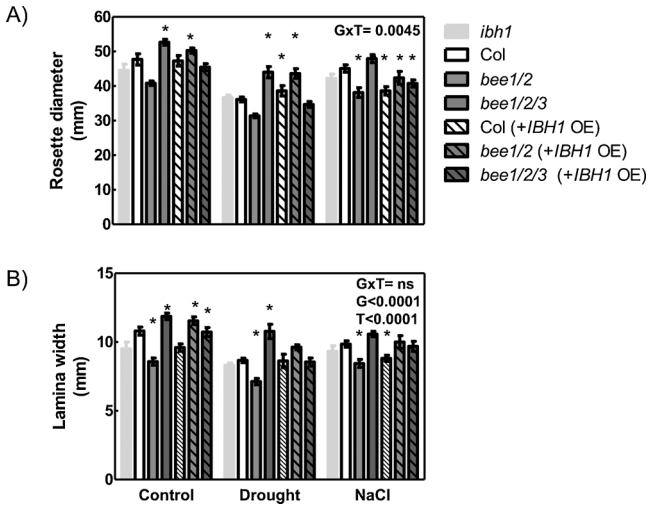

Fig. 8. Rosette diameter and leaf width is not associated to the enhanced tolerance to abiotic stress of some genotypes. Rosette diameter (A) and leaf lamina width (B) of the following plant genotypes: Col-0, *ibh1*, *bee1/bee2* and *bee1/bee2/bee3* and those overexpressing *IBH1* in Col-0, *bee1/bee2* and *bee1/bee2/bee3*. All measurements were recorded as stated in Material and Methods. Morphological parameters were recorded in 8 independent replicates. In all cases, thin bars represent standard error. Asterisks indicate significant differences between means within the treatment ($P < 0.05$, Bonferroni posttests). G states for Genotype and T for treatment, whereas GxT is the interaction of the factors.

lower compared to the rest of the genotypes (Table 1).

In the case of salinity treatment, plant survival rate was in agreement with the behavior previously observed (Fig. 5, Table 1 and Supplementary Fig. S2C). Whereas WT, *ibh1* and *bee1/bee2* plants showed evident signs of leaf senescence (Supplementary Fig. S2C), the *bee1/bee2/bee3* genotype and the OE lines of *IBH1* showed and enhanced tolerance to salinity (Supplementary Fig. S2C). In a similar way, *bee1/bee2/bee3* and *IBH1 OE* lines showed higher survival rate than WT plants (Table 1). Taken altogether, the results suggest that the overexpression of *IBH1* as well the lack of the three *BEE* genes improves plant tolerance to drought and salinity.

3.4. IBH1 OE and bee1/bee2/bee3 mutant plants are stress tolerant but yielded less than controls

To understand if the enhanced tolerance of the triple mutant and *IBH1* OE plants could be translated into a higher seed yield, we scored number of siliques and seed yield per plant. Surprisingly, the silique number and seed yield of the *bee1/bee2/bee3* and *IBH1* OE plants was significantly different from that of WT after mild drought and salinity treatments as well as in control conditions (Fig. 6 and Supplementary Fig. S3). The number of siliques of *IBH1* OE both on the *bee1/bee2* or *bee1/bee2/bee3* mutant backgrounds, but not in Col-0 background, was larger than in WT plants, both in standard or salinity stress conditions (Fig. 6A and Supplementary Fig. S3A). For drought stress only the triple *bee1/bee2/bee3* (+*IBH1* OE) exhibited a better performance compared with other genotypes. All other genotypes (*bee1/bee2/bee3*, *ibh1* and *bee1/bee2*) did not present significant differences in this parameter. Notably, this augmented silique number did not render more seeds in any condition (control or stress) (Fig. 6B and Supplementary Fig. S3B). On the contrary, less seed yield was obtained from *IBH1 OE* lines compared with WT. To further investigate this point, we analyzed siliques under the microscope and these observations indicated the presence of aborted seeds in the *IBH1* OE plants, explaining their reduced yield (Fig. 6C).

3.5. Rosette and lamina width are affected by the transcription factors BEE1, BEE2 and BEE3

To understand if the enhanced tolerance of the mutant and OE plants (*bee1/bee2* and *bee1/bee2/bee3* mutants with ectopic expression of *IBH1*) was related to morphological traits associated to water loss, we performed a thorough phenotypic characterization of adult plants subjected to drought or salinity stress, respectively. Stem length, internode number and axillary buds did not significantly differ between Col-0 and *ibh1*, bee1/bee2, and *bee1/bee2/bee3* mutants, neither with *IBH1* OE on Col-0, *bee1/bee2* or *bee1/bee2/bee3* backgrounds (Fig. 7A–C). Stem length was reduced in water-stressed plants as well as in salinity treated ones but the reduction was similar for all the genotypes (Fig. 7A–C). Considering rosette area and lamina width, some differences were detected between genotypes. The triple mutant *bee1/bee2/bee3* exhibited larger rosettes and wider laminas upon control condition and drought whereas no significant differences were observed upon salinity treatment (Fig. 8A–B).

4. Discussion

Genes from the bHLH family control different aspects of plant growth and development. There are several reports showing that phylogenetically related bHLHs play redundant roles which were revealed only with the use of high order mutants. That was the case of *BEE* genes that were shown to play a redundant role in the regulation of the shade avoidance response together with *BIM* genes [23]. The genetic combination of *bee* and *bim* mutations also demonstrated that BIM2 is required for plant viability in the absence of other *BIM* and *BEE* genes [23]. One of the *BEE* genes, *BEE2*, was shown to interact with an

atypical bHLH factor called IBH1 [11,12]. This may be relevant for *BEE* gene function since these atypical bHLH factors inhibit the DNA binding activity of canonical bHLH through dimerization [10,11]. Since we found that the expression of *BEE1* and *BEE2* is strongly induced by NaCl, we studied the contribution of these genes to abiotic stress responses. The induction of *BEE1* and *BEE2* expression upon abiotic stress is puzzling since it was previously reported that the expression of these genes was repressed by ABA treatment, a central hormone in the plant response to abiotic stress [9]. Still, these results are not directly comparable since the experimental conditions were overall different such as Petri-dished grown seedlings versus soil grown adult plants, early versus late molecular response, among others. In the context of our initial hypothesis, we tested the tolerance to abiotic stress of the *bee1/bee2* and *bee1/bee2/bee3* mutant. The *bee1/bee2/bee3* mutant plants were more tolerant to NaCl treatment and drought stress than WT plants (Figs. 2–4). These traits were accompanied by a lower water intake of detached leaves in response to drought and higher concentration of total chlorophyll of NaCl treated leaves (Figs. 2 and 3). Notably, this improved performance to abiotic stress was absent in *bee1/bee2* plants (Fig. 4). This result is consistent with previous observations, in which the hypocotyl growth response of single and double *bee* mutants was indistinguishable from that of the WT [9]. Since it was reported that BEE2 directly interacts with IBH1 [11,12], we wondered if IBH1 could alter in a synergistic or redundant way the role of *BEE2*-related genes in abiotic stress responses. Several reports showed that the degree of stunted growth phenotype of *IBH1* OE rosettes was associated to the expression level of *IBH1* [10,15,24]. The underlying molecular mechanism is related to the ability of IBH1 to titter down ACE1 and other cell elongation factors inducing limited expansion of the leaf lamina. However, the role of IBH1 in abiotic stress is still unknown. To answer this question, we ectopically expressed *IBH1* in genetic backgrounds lacking functional *BEE* genes. In order to avoid deleterious effects of *IBH1* overexpression on rosette growth, we screened for transgenic plants with no visible growth retardation or inhibition of lamina expansion. Interestingly, *IBH1* OE plants showed an enhanced tolerance to NaCl and drought stress, and this improvement was present in all genetic backgrounds including Col-0, *bee1/bee2* and *bee1/bee2/bee3* (Fig. 4). Nevertheless, the leaf rehydration assay on detached leaves suggested that the water loss of *IBH1* OE in the *bee1/bee2/bee3* background was significantly lower than that of controls, including those in the background of *bee1/bee2/bee3* mutant (Fig. 4). Survival assays showed the singular case of the *IBH1* OE in the *bee1/bee2* background that were significantly more sensitive than other *IBH1* OE lines to a severe drought treatment (Fig. 5 and Table 1). Since *IBH1* is able to enhance plant tolerance to abiotic stress in the *bee1/bee2/bee3*, we propose a model where IBH1 enhances this agronomic trait through different pathways, one of them involving the *BEE* genes which were shown here to play an unknown role regulating plant abiotic responses. It was recently proposed that an ectopic expression of a dominant negative version of *IBH1* could be used to reduce cell size of tobacco plants and this fact improves the production of recombinant proteins in plants [24]. We can conclude that the ectopic expression of *IBH1* at low levels might be used as a novel biotechnological tool to improve tolerance to abiotic stress without penalization in biomass production. It is tempting to speculate that the use of a promoter unable to drive the expression in flowers could overcome seed yield penalization. Further work is currently in course to corroborate this hypothesis.

Author contributions

JEM and RLC conceived and designed research. JEM and GMP conducted experiments and analyzed data. JEM and RLC wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2018.03.024.

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