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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.

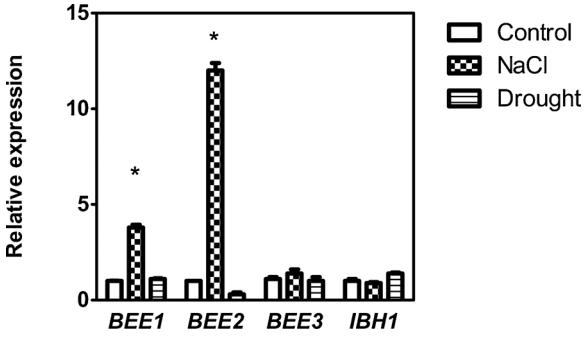

Fig. 1. *BEE1* and *BEE2* expression is induced by salinity in Arabidopsis plants. The relative transcript levels of *BEE1*, *BEE2* and *BEE3* were measured in rosette leaves of 25 day-old WT (Col-0) plants after 48 h of salinity and drought stress treatments. *BEEs* transcript abundance was measured 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