The function of OsbHLH068 is partially redundant with its homolog, AtbHLH112, in the regulation of the salt stress response but has opposite functions to control flowering in *Arabidopsis*

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Abstract

**Key message**  The homologous genes OsbHLH068 and AtbHLH112 have partially redundant functions in the regulation of the stress response but opposite functions to control flowering in *Arabidopsis*.

**Abstract**  The transcription factor (TF) basic/Helix-Loop-Helix (bHLH) is important for plant growth, development, and stress responses. OsbHLH068, which is a homologous gene of AtbHLH112 that is up-regulated under drought and salt stresses, as indicated by previous microarray data analysis. However, the intrinsic function of OsbHLH068 remains unknown. In the present study, we characterized the function and compared the role of OsbHLH068 with that of its homolog, AtbHLH112. Histochemical GUS staining indicated that OsbHLH068 and AtbHLH112 share a similar expression pattern in transgenic *Arabidopsis* during the juvenile-to-adult phase transition. Heterologous overexpression of OsbHLH068 in *Arabidopsis* delays seed germination, decreases salt-induced H\(_2\)O\(_2\) accumulation, and promotes root elongation, whereas AtbHLH112 knock-out mutant displays an opposite phenotype. Both OsbHLH068-overexpressing transgenic *Arabidopsis* seedlings and the Atbhlh112 mutant display a late-flowering phenotype. Moreover, the expression of OsbHLH068-GFP driven by an AtbHLH112 promoter can compensate for the germination deficiency in the Atbhlh112 mutant, but the delayed-flowering phenotype tends to be more severe. Further analysis by microarray and qPCR indicated that the expression of FT is down-regulated in both OsbHLH068-overexpressing *Arabidopsis* plants and Atbhlh112 mutant plants, whereas SOC1 but not FT is highly expressed in AtbHLH112-overexpressing *Arabidopsis* plants. A comparative transcriptomic analysis also showed that several stress-responsive genes, such as AtERF15 and AtPUB23, were affected in both OsbHLH068- and AtbHLH112-overexpressing transgenic *Arabidopsis* plants. Thus, we propose that OsbHLH068 and AtbHLH112 share partially redundant functions in the regulation of abiotic stress responses but have opposite functions to control flowering in *Arabidopsis*, presumably due to the evolutionary functional divergence of homolog-encoded proteins.

**Keywords**  Transcription factor · OsbHLH068 · AtbHLH112 · Salt stress · Flowering

Introduction

The transcription factor (TF) basic/Helix-Loop-Helix (bHLH) protein comprises a large family in plants (Feller et al. 2011). To date, at least 162 *AtbHLHs* and 167 *OsbHLHs* have been identified and can be categorized into 25 subfamilies based on their bHLH domain (Bailey et al. 2003; Heim et al. 2003; Li et al. 2006; Toledo-Ortiz et al. 2011).
A typical bHLH domain normally contains two functionally distinct regions: a basic region for DNA binding that recognizes the target cis-acting element, known as E-box (5'-CANNTG-3'), and an HLH region for protein homo- or hetero-dimerization (Feller et al. 2011; Heim et al. 2003; Murre et al. 1989; Pires and Dolan 2010). Notably, a few atypical members known as HLHs lack the basic region. Thus, the HLH–bHLH heterodimeric complex can disrupt the bHLH–bHLH interaction and prevent DNA binding. Based on the dimeric forms, plant bHLH can play a dual role in regulating growth and development. For example, the POSITIVE REGULATOR OF GRAIN LENGTH 1 (PGL1)-ANTAGONIST OF PGL1 (APG) heterodimer, an OsHLH–OsbHLH complex, increases grain length and weight, whereas the APG homodimer decreases grain length and weight (Heang and Sassa 2012). Moreover, plant bHLHs are also involved in many aspects of growth and development, including Z-box binding factor 1 (ZBF1/AtbHLH6), dysfunctional tapetum 1 (DYT1/AtbHLH22), and root hairless 1 (RHL1/OsbHLH3), which are involved in blue light-mediated seedling development, anther development, and root hair development, respectively (Cui et al. 2016; Ding et al. 2009; Maurya et al. 2015). However, the physiological and regulatory roles of most bHLHs in either Arabidopsis or rice remain poorly understood.

Although plants are sessile organisms that cannot escape from deleterious environments, they have already developed and established an exquisite regulatory mechanism to address the changes in their surrounding environment. For example, the inducer of CBF expression 1 (ICE1)-C-repeat binding factor 3/dehydration-responsive element-binding protein 1 A (CBF3/DREB1A) transcriptional cascade, a well-known bHLH–APETALA2/ethylene-responsive factor (AP2/ERF) regulatory pathway, contributes to cold tolerance in Arabidopsis and rice, which illustrates the importance of bHLH in plant stress responses (Chinnusamy et al. 2003; Ito et al. 2006). In fact, AtbHLH116/ICE1 positively regulates the expression of CBF3/DREB1A [AtERF#031 (At4g25480)], which confers freezing tolerance through an ABA-independent pathway (Chinnusamy et al. 2003). In addition to AtbHLH116/ICE1, several plant bHLHs are also involved in abiotic stress responses. For example, OrbHLH1 and OrbHLH2, two ICE-like proteins in wild rice (Oryza rufipogon), confer salt stress tolerance in transgenic Arabidopsis plants through an ICE/CBF-independent and an ABA-independent pathway, respectively (Li et al. 2010; Zhou et al. 2009). OsbHLH062 activates the expression of JA-responsive genes to confer salt stress tolerance, while OsbHLH062 represses the expression of JA-responsive genes when interacting with JASMONATE-ZIM-DOMAIN PROTEIN 9 (OsJAZ9) and NOVEL INTERACTOR OF JAZ (OsNINJA) (Wu et al. 2015). NaCl-induced expression of AtbHLH092 confers tolerance to salt and osmotic stresses in Arabidopsis through a pathway that is partially dependent on ABA and SALT OVERLY SENSITIVE 2 (SOS2) (Jiang et al. 2009). In response to the high ambient temperature, AtbHLH9/PHYTOCHROME INTERACTING FACTOR 4 (PIF4) directly activates the expression of YUCCA8 and TAA1, two auxin biosynthetic genes, which in turn triggers hypocotyl elongation in Arabidopsis (for a review, see Proveniers and van Zanten 2013). AtbHLH122 improves drought and osmotic tolerance through direct repression of CYP707A3, an ABA catabolic gene (Liu et al. 2014). Interestingly, AtbHLH112, which belongs to the F subfamily, confers abiotic stress tolerance by increasing proline levels and enhancing reactive oxygen species (ROS) scavenging ability; however, overexpression of AtbHLH112 suppresses lateral root emergence (Liu et al. 2015; Wang et al. 2014). These studies reveal that some bHLHs, such as AtbHLH9/PIF4 and AtbHLH112, are not only involved in the stress response but also play a pleiotropic regulatory role for optimal plant growth and development.

Flowering time is one of the major yield traits correlated with grain production in cereal crops. The precise timing of flowering in plants is coordinately controlled by endogenous and environmental factors, including physiological maturity, accumulated temperature, and day length. In Arabidopsis, a facultative long-day (LD) plant, flowering time is determined by the autonomous, vernalization, photoperiod, and gibberellin pathways (Mouradov et al. 2002), during which photoperiod is the predominant cue controlling flowering time (Andrés and Coupland 2012; Song et al. 2015). These four major pathways integrat-egulately regulate the expression of floral meristem identity genes, such as FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), and LEAFY (LFY), to trigger floral initiation (Wigge et al. 2005). Compared to Arabidopsis, rice is a facultative short-day (SD) plant and thus flowers earlier under SDs than LDs (Hayama et al. 2003). In rice, two FT homologous genes, Heading date 3a (Hd3a) and RICE FLOWERING LOCUS T 1 (RFT1), encode a ‘florigen’ that promotes flowering (Komiya et al. 2008, 2009; Tamaki et al. 2007; Tsuji et al. 2013). The expression of Hd3a and RFT1 is regulated by Early heading date 1 (Ehd1) and Heading date 1 (Hd1) (Doi et al. 2004). In fact, Ehd1 activates the expression of Hd3a and RFT1 under both SDs and LDs, whereas Hd1 functions as a transcriptional activator of Hd3a under SDs but not under LDs (Hayama et al. 2003; Ishikawa et al. 2011). Recently, several plant bHLHs have also been found to be involved in the control of flowering. Four FLOWERING BHLH (FBH1-4) proteins directly activate CONSTANS (CO) expression for photoperiodic flowering in Arabidopsis (Ito et al. 2012). Under SDs, NO FLOWERING IN SHORT DAY (NFL) interacts with the GA signaling pathway (Sharma et al. 2016). In
Results

Salt enhances OsbHLH068 expression

To determine which uncharacterized OsbHLH members may also be involved in regulating salt stress responses, we analyzed publicly available microarray data (GSE6901), and several abiotic stress-responsive OsbHLHs with an unknown function were identified (Fig. 1a). Among them, the expression of OsbHLH068 was up-regulated under either drought- or salt-treated conditions. Notably, OsbHLH068 and AtbHLH112 have been categorized as members of the F subfamily (Li et al. 2006). Phylogenetic analysis also revealed that OsbHLH068 was highly homologous to AtbHLH112 based on the sequence similarity within the bHLH domains (Supplementary Fig. S1).

To verify whether the expression of OsbHLH068 was in fact up-regulated under salt stress conditions, histochemical staining using OsbHLH068p::GUS transgenic rice was performed. Under 0 mM NaCl conditions, GUS was slightly expressed in the aerial tissues of 14-day-old transgenic seedlings; however, it was enhanced in response to 200 mM NaCl (Fig. 1b). Additionally, the expression pattern of OsbHLH068 was similar to that of OsSOS1, a well-known salt-responsive gene that was up-regulated under 200 mM NaCl conditions in Nipponbare and Tainung 67 seedlings (Fig. 1c, d). These data show that OsbHLH068, an AthHLH112 homolog, is a salt-responsive gene. Incidentally, we also investigated the spatiotemporal expression of OsbHLH068 in rice. As shown in Fig. 2a, b, the GUS signals were localized to the embryo of germinated transgenic seeds and in the terrestrial tissues of transgenic seedlings. Additionally, GUS signals were also observed in flag leaves, lemma nerves, and anthers during reproductive growth (Fig. 2c).

Overexpression of OsbHLH068 confers salt tolerance in Arabidopsis

To investigate whether OsbHLH068 plays a positive regulatory role in the salt stress response similar to that of its homolog, AtbHLH112, we compared the seed germination properties of Atbhlh112, Col-0, two OsbHLH068-overexpressing transgenic Arabidopsis lines [35S::OsbHLH068 (6) and (9)], and three independent complemented transformants [AtbHLH112p::GFP-OsbHLH068, AtbHLH112p::OsbHLH068-GFP (10), and (23)] sown on basal medium containing 0 or 100 mM NaCl. Genetic identification of homozygous Atbhlh112 mutant lines (SALK_033618 and _148540), OsbHLH068-overexpressing transgenic Arabidopsis lines, and complemented transformants was performed by genomic DNA genotyping (Supplementary Fig. S2 and S3). Similar to the ABA over-accumulating 35S::LiNCED3 transformant, two independent OsbHLH068-overexpressing transgenic Arabidopsis lines had a lower germination rate in the presence of either 0 or 100 mM NaCl on day 2 compared with the corresponding Col-0 (Fig. 3a). The germination rate of the Atbhlh112 mutant was not significantly different from that of Col-0 in the presence of 0 mM NaCl on day 2 but increased in the mutant compared with Col-0 under 100 mM NaCl conditions (82% and 60%, respectively). On day 3, almost all of the seeds had germinated under 0 mM NaCl conditions; however, two OsbHLH068-overexpressing transgenic lines and the 35S::LiNCED3 transformant still exhibited a lower germination rate under 100 mM NaCl conditions (Supplementary Fig. S4A). Notably, the germination rates of the three independent complemented transformants ranged from approximately 63–72% under 100 mM NaCl conditions on day 2; these rates were similar to the corresponding levels in Col-0 (Fig. 3a). These results indicate that OsbHLH068 can replace the functional role of AtbHLH112 in the regulation of seed germination. On 200 mM NaCl-containing medium, the Atbhlh112 had a higher germination rate than the corresponding Col-0 at days 2 and 3 after seeding, whereas the germination rates of two OsbHLH068-overexpressing transgenic lines were lower than that of the corresponding Col-0 at days 2, 3, 4, and 5 after seeding (Supplementary Fig. S4B). Seed germination reached
Fig. 1 Salt-enhanced OsbHLH068 expression. a Abiotic stress-responsive OsbHLHs. Red and green indicate increased and decreased gene expression, respectively. The scale bar shows log₂-fold changes. C, cold treatment; D, drought treatment; H, heat treatment; S, salt treatment. Tos17, rice Tos17 insertion mutant database; TRIM, Taiwan rice insert mutant database. b Histochemical staining of the aerial tissues in OsbHLH068p::GUS transgenic Tainung 67 (Oryza sativa L. spp. japonica cv. Tainung 67) seedlings. c The expression pattern of OsbHLH068 in Nipponbare (Oryza sativa L. spp. japonica cv. Nipponbare) and Tainung 67 aerial tissues by RT-PCR. The Arabic numerals represent the individual rice plants. d Quantification of OsSOS1 and OsbHLH068 mRNA levels in Nipponbare and Tainung 67 aerial tissues by qPCR. The values are the mean ± SD of two independent experiments, each performed in triplicate. *P < 0.05; **P < 0.01, Student’s t-test. Seedlings used for GUS staining (b), RT-PCR (c), and qPCR (d) assays were grown on basal medium for 13 days and then transferred to basal medium containing 0 or 200 mM NaCl for an additional day.
approximately 100% in each genotype at day 6, reflecting that seed germination delay in OsbHLH068-overexpressing transgenic lines was not due to poor seed vitality. Although Atbhlh112 displayed early germination on 200 mM NaCl-containing medium, more bleached Atbhlh112 seedlings were observed after prolonged culture; however, the late-germinating OsbHLH068-overexpressing transgenic lines had a lower bleached seedling frequency than both Col-0 and Atbhlh112 (Supplementary Fig. S4C, D). Additionally, further investigations revealed that the two OsbHLH068-overexpressing transgenic lines had a lower level of both malondialdehyde (MDA, a lipid peroxidation marker) and hydrogen peroxide (H$_2$O$_2$, a ROS) than the corresponding Col-0 under salt (250 mM NaCl) treatment conditions, whereas the Atbhlh112 mutant had a relatively high level of both MDA and H$_2$O$_2$ (Fig. 3b). Moreover, the levels of both MDA and H$_2$O$_2$ in the complemented transformants were similar to the corresponding levels in Col-0 under salt treatment conditions. Taken together, these data show that OsbHLH068 plays a similar role to that of AtbHLH112 in the regulation of the plant response to salt stress.

In addition to the salt stress response, previous studies have shown that overexpression of AtbHLH112 suppresses lateral root emergence and salt-inhibited root growth (Liu...
(A) Germination rate (%): 0 mM NaCl vs. 100 mM NaCl for various genotypes.

(B) MDA content (nmol gDW⁻¹): 0 mM NaCl vs. 250 mM NaCl for various genotypes.

(C) Root length (cm): 0 mM NaCl vs. 100 mM NaCl for various genotypes.

(D) Phenotypic images showing growth under NaCl stress for different genotypes.

(E) H₂O₂ content (μmol gDW⁻¹): 0 mM NaCl vs. 250 mM NaCl for various genotypes.

(F) Fresh and dry weights (mg): Comparison between different genotypes under NaCl stress.

Note: Data points are represented as mean ± standard error. Statistical significance is indicated by asterisks: * for p < 0.05, ** for p < 0.01.
The seed germination rates in the Atnced3 mutant, 35S::LnfNCED3 transformant, Atbhlh112 mutant, Col-0, two OsbHLH068-overexpressing transgenic Arabidopsis lines and three complemented transformants on day 2. The levels of MDA (upper panel) and H2O2 (lower panel) in the Atbhlh112 mutant, Col-0, two OsbHLH068-overexpressing transgenic Arabidopsis lines, and three complemented transformants. c The root lengths of the Atnced3 mutant, 35S::LnfNCED3 transformant, Atbhlh112 mutant, Col-0, and two OsbHLH068-overexpressing transgenic Arabidopsis lines. d Phenotypic comparison of the 15-day-old Atnced3 mutant, 35S::LnfNCED3 transformant, Atbhlh112 mutant, Col-0, and two OsbHLH068-overexpressing transgenic Arabidopsis lines grown under LD conditions. e The fresh and dry weights of Atnced3 mutant, 35S::LnfNCED3 transformant, Atbhlh112 mutant, Col-0, and two OsbHLH068-overexpressing transgenic Arabidopsis lines. *P < 0.05; **P < 0.01, Student’s t-test. The ABA-deficient mutant, Atnced3, and the ABA over-accumulating 35S::LnfNCED3 transformant were used as negative and positive controls, respectively. Atbhlh112 mutant, SALK_148540 et al. 2015; Wang et al. 2014). Therefore, we also investigated whether overexpression of OsbHLH068 affects root development. Indeed, two independent OsbHLH068-overexpressing transgenic Arabidopsis lines and the 35S::LnfNCED3 transformant had a longer primary root compared with the corresponding Col-0 seedlings under either 0 or 100 mM NaCl conditions on day 5, whereas the primary roots of both the Atbhlh112 and ABA-deficient Atnced3 mutants were shorter (Fig. 3c). Additionally, the fresh and dry weights of OsbHLH068-overexpressing transgenic Arabidopsis aerial tissues were less than those of Col-0 when grown in soil on day 15 (Fig. 3d, e).

OsbHLH068 and AtbHLH112 act oppositely to control flowering in Arabidopsis

Because OsbHLH068 exerts a pleiotropic effect on many aspects of growth and development in Arabidopsis during the vegetative stage, we subsequently investigated whether OsbHLH068-overexpressing transgenic Arabidopsis had a different phenotype during the reproductive stage. As shown in Fig. 4a, b, the time to flowering in Col-0 was approximately 17 days when grown under LD conditions but was delayed to 21 and 19 days in the 35S::OsbHLH068 (6) and (9) transformants, respectively. Additionally, the flowering times of 35S::OsbHLH068 (6) and the (9) transformants were also delayed when grown under SD conditions (Fig. 4a, b). To validate that the delayed flowering time was not a side effect derived from the constitutive expression of OsbHLH068 in transgenic Arabidopsis seedlings, the spatiotemporal expression of OsbHLH068 was investigated by histochemical staining using OsbHLH068::GUS transgenic Arabidopsis. As shown in Fig. 4c, GUS was expressed in transgenic seedlings during the transition from the vegetative (14 days old) to the reproductive (17 days old) stage. GUS signals were localized only to the cotyledon and its axis in 14-day-old transgenic Arabidopsis seedlings but were also detected in the upper true leaves of 17-day-old transgenic seedlings (Supplementary Fig. S5A). Microscopic observation revealed that GUS signals were localized to the florets, hydathodes, leaf veins, trichome bases, and vascular bundles of the inflorescence in 17-day-old transgenic seedlings (Supplementary Fig. S5B). Because day length plays a key role in determining the flowering time of Arabidopsis, we further examined whether the expression of OsbHLH068 was affected by illumination. As shown in Fig. 4d and Supplementary Fig. S6, the GUS signals in transgenic seedlings decayed when grown in the dark compared with the signals in plants grown under LD conditions. These data suggest that light-induced OsbHLH068 plays a negative role in regulating flowering time in Arabidopsis. Notably, both Atbhlh112 mutants (SALK_033618 and _148540) also displayed a late-flowering phenotype under LD conditions (Fig. 4a, b; Supplementary Fig. S7). These data suggest that OsbHLH068 and AtbHLH112 may act oppositely to control flowering time in Arabidopsis.

To confirm that OsbHLH068 and AtbHLH112 act oppositely to control flowering time, we subsequently investigated the flowering times of Atbhlh112, Col-0, and three complemented transformants under LD conditions. Indeed, the flowering times of the three complemented transformants were more severely delayed than Atbhlh112 when compared to Col-0 seedlings under LD conditions (Fig. 5a, b). Additionally, GUS driven by a 2.2-kb AtbHLH112 native promoter, as used in the complemented transformants expressing GFP-OsbHLH068 or OsbHLH068-GFP, was expressed in the AtbHLH112::GUS transformant during the transition from the vegetative (14 days old) to the reproductive (17 days old) stage (Fig. 5c). Similar to OsbHLH068::GUS transgenic Arabidopsis plants, the GUS signals were localized to the cotyledon and its axis in 14-day-old AtbHLH112::GUS seedlings and presented in the upper true leaves of 17-day-old AtbHLH112::GUS seedlings (Supplementary Fig. S5A vs. S8A). Microscopic observation revealed GUS signal localization in the florets, leaf veins, and trichome bases of 17-day-old AtbHLH112::GUS seedlings (Supplementary Fig. S8B). Incidentally, both the GFP-OsbHLH068 and OsbHLH068-GFP fusion proteins were localized to the nucleus of root cells in the complemented transformants (Fig. 5d). Taken together, these data indicate that OsbHLH068 and AtbHLH112 share a similar expression pattern during the phase transition from vegetative to reproductive growth but act oppositely to control the flowering time.
Transcriptomic analysis of OsbHLH068-overexpressing transgenic Arabidopsis plants

To better understand the molecular mechanism of OsbHLH068 in the regulation of the salt stress response and flowering, we conducted a comparative transcriptomic analysis of OsbHLH068-overexpressing transgenic Arabidopsis (35S::OsbHLH068 (9)) and Col-0 seedlings. After background correction and normalization, a total of 568 differentially expressed probes (DEPs) were identified. Among these, 168 DEPs were up-regulated while 400 DEPs were down-regulated in OsbHLH068-overexpressing trans- formants (Figs. 6a; Supplementary Data 1). As expected, several well-known stress-responsive genes were found among these DEPs, including MYB domain protein 4 (MYB4), protein phosphatase 2 C 5 (PP2C5), CBF2, and 1-aminocyclopentane-1-carboxylate synthase 11 (ACS11). Notably, almost none of the flowering-related genes, such as LFY, SOC1, and FT, were detected as DEPs. However, the log2-fold changes in SOC1 and FT were approximately −0.25 and −0.79, respectively, with a P-value of at least < 0.05 based on Student’s t-test (Fig. 6c, Supplementary Data 1). Thus, a qPCR assay was performed to verify the microarray data. As shown in Fig. 6b, d, the mRNA levels of MYB4 and PP2C5 were higher in OsbHLH068-overexpressing trans-formants than in Col-0, while the mRNA levels of CBF2, ACS11, SOC1, and FT were lower in OsbHLH068-overexpressing trans-formants than in Col-0. The results obtained by qPCR were consistent with the microarray data. In fact, the qPCR data also revealed that the late-flowering Atbhlh112 mutant had a relatively low level of both FT and APETALA1 (API) expression and a relatively high level of FLOWERING LOCUS C (FLC) expression, which were similar to those seen in the OsbHLH068-over- expressing trans-formants (Fig. 6d; Supplementary Fig. S9).

Common differentially expressed genes between OsbHLH068- and AtbHLH112-overexpressing Arabidopsis

Because OsbHLH068 and AtbHLH112 share a highly similar bHLH domain, we assumed that both TFs should commonly regulate the expression of certain downstream genes, especially when they were constitutively expressed in the same genetic background. Therefore, we further comparatively analyzed the publicly available microarray data, GSE84087 and GSE60260, to identify the common targets between OsbHLH068- and AtbHLH112-overexpressing transgenic Arabidopsis. After background correction and normalization of AtbHLH112-related microarray data (GSE60260), a total of 11,616 DEPs consisting of 6632 up-regulated and 4984 down-regulated DEPs were identified (Supplementary Data 2). Notably, a total of 358 DEPs were commonly presented in both OsbHLH068- and AtbHLH112-overexpressing transgenic Arabidopsis (Fig. 7), of which 61 and 263 DEPs were identically up- and down-regulated, respectively, in both transgenic plants. Additionally, the 61 common up-regulated DEPs can be re-lected in 52 genes (denoted as common up-regulated DEGs) based on TIGR annotation, while the 263 common down-regulated DEPs can be represented in 206 genes (designated as common down-regulated DEGs), including CBF2 and ACS11 (Supplementary Data 3). Gene ontology analysis revealed that the major ontological categories of the commonly regulated DEGs were ‘response to stimulus’ and ‘transcription regulator activity’ of ‘biological process’ and ‘molecular function’, respectively (Supplementary Fig. S10). Incidentally, further promoter analysis indicated that 51 and 194 common up- and down-regulated DEGs, respectively, contain at least one E-box element in their 1-kb promoter region (Supplementary Data 4).

Discussion

Partial functional redundancy of AtbHLH112 and OsbHLH068 in the stress response

The phytohormone abscisic acid (ABA) plays an important role in regulating seed maturation, dormancy, stomatal closure, and abiotic stress responses (Gubler et al. 2005; Karssen et al. 1983; Leung and Giraudat 1998; MacRobbie 1998; Seiler et al. 2011). In fact, ABA-mediated seed dormancy is an adaptive mechanism that maintains viable seeds in a quiescent state and leads to the escape from or avoidance of a stressful environment (Seo et al. 2006). Indeed, overexpression of ABA biosynthetic genes, such as nine-cis-epoxy-carotenoid dioxygenase 3 (AtNCED3) and ABA2, delays seed germination and confers drought/salt resistance.
stress tolerance in transgenic *Arabidopsis* plants (Cheng et al. 2002; Iuchi et al. 2001; Lin et al. 2007). In contrast, ABA-deficient mutants, such as *aba1*, *aba2*, and *aba4*, have a rapid seed germination phenotype but are susceptible to osmotic stress (Lin et al. 2007; North et al. 2007). Additionally, root architecture alteration is another adaptive mechanism for plants to survive under stress conditions (Price et al. 1997; Serraj et al. 2004; Uga et al. 2013). In rice, drought-resistant varieties usually develop a deeper root system so that the roots can take up water from deeper soil layers to manage water deficiency (Gowda et al. 2011; Price et al. 1997; Uga et al. 2013). As mentioned in a previous study, AtbHLH112 confers abiotic stress tolerance by enhancing the ROS scavenging ability and promotes primary root growth under salt-treated conditions (Liu et al. 2015). In this study, heterologous overexpression of *OsbHLH068*, an *AtbHLH112* homolog, in *Arabidopsis* delayed seed germination, decreased the accumulation of MDA and $H_2O_2$, and enhanced primary root elongation under salt-treated conditions, whereas the *Atbhlh112* mutant displayed a rapid seed germination phenotype, a relatively high level of MDA and $H_2O_2$, and a short root length phenotype (Fig. 3a–c and Supplementary Fig. S4). Notably, the seed germination and root elongation properties of *OsbHLH068*-overexpressing transgenic *Arabidopsis* plants and the *Atbhlh112* mutant were highly similar to those of the ABA

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**Fig. 5** Complementary expression of *GFP-OsbHLH068* or *OsbHLH068-GFP* causes a more severe late-flowering phenotype in the *Atbhlh112* mutant background grown under LD conditions. 

**a** Phenotypic comparison of Col-0, the *Atbhlh112* mutant, and the three complemented transformants. The seedlings were grown in soil for 19 days. 

**b** The bolting time of Col-0, the *Atbhlh112* mutant, and the three complemented transformants. The values are the mean ± SD of two independent experiments, each with ten replicates. 

**c** Histochemical staining of the aerial tissues in *AtbHLH112p::GUS* transgenic seedlings during vegetative, transitional, and reproductive stages. Seedlings were grown in soil for the indicated period of time. Scale bar 1 cm. Enlarged versions of each black disc-labeled image are shown in Supplementary Fig. S8A. 

**d** Subcellular localization of GFP-*OsbHLH068* (left panel) and OsbHLH068-GFP (right panel) proteins in complemented transformants. *Atbhlh112* mutant, SALK_148540

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**D**

|   |   |   |
|---|---|---|
| **AtbHLH112p::GFP-OsbHLH068** | **AtbHLH112p::OsbHLH068-GFP (23)** |
| **GFP** | **GFP** |   |
| Bright Field | Bright Field |   |
| Merged | Merged |   |

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**Notes:** 

*P* < 0.05; 

**P** < 0.01, Student’s t-test.
over-accumulating 35S::LfNCED3 transformant and the ABA-deficient mutant, Atnced3, respectively. Furthermore, complementary expression of either GFP-OsbHLH068 or OsbHLH068-GFP driven by a 2.2-kb AtbHLH112 promoter could restore, partially or completely, the early germination and $\text{H}_2\text{O}_2$ over-accumulation of the Atbhlh112 mutant to normal germination and accumulation, respectively (Fig. 3a, b). These data showed that the regulatory role of AtbHLH112 in seed germination and $\text{H}_2\text{O}_2$ scavenging could be replaced by OsbHLH068, presumably due to the conserved function between Arabidopsis (dicot) and rice (monocot). Additionally, comparative transcriptomic analysis indicated that 52 up-regulated and 206 down-regulated DEGs were commonly presented in OsbHLH068- and AtbHLH112-overexpressing transgenic Arabidopsis plants (Supplementary Data 3), of which 51 common up-regulated DEGs and 194 common down-regulated DEGs had at least one E-box element (5′-CANNTG-3′) in their 1-kb
promoter region, including several well-known stress-responsive genes [e.g., *AtERF15* (At2g31230), *AtPUB23* (At2g35930) and *WRKY48* (At5g49520)] (Supplementary Data 4). *AtERF15*, a commonly up-regulated DEG, plays a positive role in regulating ABA-mediated drought tolerance, whereas *plant U-box 23* (*AtPUB23*), a commonly down-regulated DEG, negatively regulates ABA-mediated drought stress responses (Lee et al. 2015; Seo et al. 2012). The osmotic stress- and pathogen-induced *AtWRKY48*, a common down-regulated DEG, functions as a negative regulator of defense-related genes, such as PRs, and basal resistance to the bacterial pathogen *Pseudomonas syringae* (Xing et al. 2008). Taken together, our data reveal that *OsbHLH068* plays a role similar to that of *AtbHLH112* in the regulation of abiotic stress responses, presumably due to the partially functional redundancy of these homologous genes.

**Functional divergence of AtbHLH112 and OsbHLH068 in flowering control**

Homologous gene-encoded proteins usually play a similar role in regulating plant developmental and physiological processes. For example, the CBFs/DREBs, which belong to the AP2/ERF family, play a positive regulatory role in the cold tolerance of evolutionarily diverse plant species, including *Arabidopsis* and rice (for a review, see Chinnsamy et al. 2010). Notably, both *OsbHLH068* and *AtbHLH112* are members of the F subfamily (Li et al. 2006). In the F subfamily, both the *OsbHLH068* domain and the *AtbHLH112* domain share a high sequence similarity (Supplementary Fig. S1). Additionally, *OsbHLH068* and *AtbHLH112* are expressed in the upper true leaves of transgenic *Arabidopsis* seedlings in a nearly overlapping pattern during the juvenile-to-adult phase (Fig. 4c vs. 5c, Supplementary Fig. S5 vs. S8). These biological characteristics suggest that *OsbHLH068* and *AtbHLH112* should play similar roles in regulating plant growth and development, especially during the phase transition process. Indeed, heterologous overexpression of *OsbHLH068* in *Arabidopsis* delayed flowering with a relatively low level of *SOC1*, *FT*, or *AP1* expression and a relatively high level of *FLC* expression under LD conditions (Figs. 4a, b, 6c, d; Supplementary Fig. S9). Surprisingly, two *Atbhlh112* mutant lines (SALK_033618 and _148540) also displayed a late-flowering phenotype under LD conditions (Fig. 4a, b; Supplementary Fig. S7). Interestingly, the late-flowering *Atbhlh112* mutant (SALK_148540) exhibited a relatively low level of both *FT* and *AP1* expression and a relatively high level of *FLC* expression under LD conditions, whereas constitutive expression of *AtbHLH112* in *Arabidopsis* up-regulated the expression of *SOC1* but not *FT* (Fig. 6d; Supplementary Fig. S9 and Data 2). Inconceivably, complementary expression of GFP-*OsbHLH068* or *OsbHLH068*-GFP driven by a 2.2-kb *AtbHLH112* promoter in the late-flowering *Atbhlh112* mutant delayed the flowering time more severely under LD conditions (Fig. 5a, b). The opposite effect of *OsbHLH068* and *AtbHLH112* on flowering control did not appear to be caused by the differential transcriptional activity because complementary expression of GFP-*OsbHLH068* or *OsbHLH068*-GFP driven by a 2.2-kb *AtbHLH112* promoter in the *Atbhlh112* mutant partially or completely restored the early seed germination and H$_2$O$_2$ over-accumulation to normal levels (Fig. 3a, c). Therefore, we assume that *OsbHLH068* and *AtbHLH112* act oppositely in flowering control, presumably due to the divergent evolution of plant flowering.

Divergent evolution is a pathway involved in speciation that contributes to the species abundance and diversity of biological systems. Gene duplication, followed by the functional divergence of paralog-encoded proteins, is considered to be a major driving force in the evolution of biological diversity (for review, see Kondrashov et al. 2002; Ohno 1970). For example, *ATX1* and *ATX2*, two *Arabidopsis thaliana TRITHORAX* homologous genes derived from a segmental duplication, belong to the same clade as the sister paralogs (Alvarez-Venegas and Avramova 2002; Baumbusch et al. 2001). Although the structure of *ATX1* is similar to that of *ATX2*, *ATX1* and *ATX2* have opposite biochemical activities (Saleh et al. 2008): *ATX1* trimethylates K4 of histone H3, whereas *ATX2* dimethylates it. They activate and inactivate the transcriptional expression of common target genes, including *WRKY70*, a TF gene involved in the regulation of the disease response. In addition to *ATX1* and *ATX2*, the *ARABIDOPSIS MYOTUBULARIN1* (*AtMTM1*) and *AtMTM2* homologs also originated from a segmental
duplication and encode catalytically active enzymes with a similar domain architecture and a conserved biochemically active catalytic site (Ding et al. 2012). However, AtMTM1 elevates the cellular level of phosphatidylinositol 5-phosphate (PtdIns5P) in response to dehydration stress, but the function of AtMTM2 remains unclear. More importantly, AtMTM1-mediated PtdIns5P can bind to the PHD domain of ATX1 and then repress the methylation activity of ATX1 (Alvarez-Venegas et al. 2006a, b; Ndamukong et al. 2010). These cases illustrate that the functional divergence of paralog-encoded proteins confers, at least in part, the biochemical and biological diversity of plants. To identify flowering-related genes in monocot rice, a facultative SD plant, the transcriptional expression of two FT homologous genes, Hd3a and RFT1 paralogs, is activated by Ehd1 in the vascular tissues of leaf blades under both SDs and LDs (Doi et al. 2004). However, the transcriptional expression of Hd3a is repressed by Hd1, a CO homolog, under LDs (Kojima et al. 2002). Notably, no Ehd1 homolog was found in Arabidopsis. In contrast, the dicot Arabidopsis is classified as a facultative LD plant. Transcriptional expression of FT is activated by CO in the vascular tissues of leaf blades under LDs (Kardailsky et al. 1999). Interestingly, the transcriptional regulation of homologous Hd3a and FT by Hd1 and CO, two homologous zinc-finger TFs, also displays an opposite relationship in the flowering control between rice and Arabidopsis, respectively, under LDs. These data indicate that functional divergence of homologous flowering-related genes was present during the divergent evolution of rice and Arabidopsis. The present data indicate that OsbHLH068 and AtbHLH112 have a similar transcriptional pattern during the juvenile-to-adult phase transition, but both OsbHLH068-overexpressing transgenic Arabidopsis plants and the Atbhlh112 mutants show a late-flowering phenotype with a relatively low level of FT expression under LDs. Notably, constitutive expression of AtbHLH112 in transgenic Arabidopsis seedlings resulted in a relatively high expression level of SOC1 but no difference from that of FT (Supplementary Data 2). Further promoter analysis revealed that at least 5, 3, 4, and 6 E-box elements were located in the promoter region within 1 kb upstream of the FT, SOC1, Hd3a, and RFT transcription start sites, respectively (Supplementary Table 1). Thus, we conclude that OsbHLH068 and AtbHLH112, two homologous TFs, have an opposite effect on the transcriptional regulation of downstream homologous flowering genes between rice and Arabidopsis, which is similar to the effect observed for Hd1 and CO in the transcriptional regulation of Hd3a and FT, respectively. Additionally, AtbHLH112 seems to play a fine-tuning role in regulating flowering time by interacting with the different proteins in Arabidopsis. Taken together, we propose that the opposite roles of OsbHLH068 and AtbHLH112 in flowering control, as well as Hd1 and CO, are to be at least partially incorporated into the divergent evolution of rice and Arabidopsis (for a summary, see Fig. 8).

**A putative role of OsbHLH068 in salt stress response and flowering**

Flowering is important for plants to complete the life cycle. However, the timing of flowering is highly susceptible to environmental conditions, particularly when the plant is exposed to abiotic stresses. In fact, many studies have shown that the effects of abiotic stress on flowering control are different in various plant species. For example, the flowering of Arabidopsis plants is accelerated by drought and delayed by salinity (Kazan and Lyons 2016; Riboni et al. 2014), whereas the flowering of rice plants is delayed by drought (Galbiati et al. 2016). Notably, previous studies have also shown that certain stress-inducible TFs play important roles in connecting environmental factors to flowering, including CmMYB2 and OsABF1. Heterologous overexpression of CmMYB2, an abiotic stress-inducible R2R3-MYB TF in chrysanthemum, improves osmotic-stress tolerance and delays flowering time in transgenic Arabidopsis plants (Shan et al. 2012). In rice, a drought-inducible bZIP TF, OsABF1, acts as a negative regulator of floral transition upon water deficit (Zhang et al. 2016). In this study, our data reveal that heterologous overexpression of OsbHLH068, a salt-inducible TF, in Arabidopsis results in decreased salt-dependent accumulation of either MDA or H$_2$O$_2$, increased root length, and delayed flowering. In
fact, the OsbHLH068-overexpressing transgenic rice plants also displayed a late-flowering phenotype with a relatively low level of Hd3a and RFT1 expression (unpublished data). Thus, we speculate that OsbHLH068 may play a pivotal role in linking the salt stress response to rice flowering.

Materials and methods

Plant materials and growth conditions

In Arabidopsis, the SALK_033618 and _148540 lines are AtbHLH112 knock-out mutants, which have been documented in a previous study (Wang et al. 2014). These mutants were obtained from the Arabidopsis Biological Resource Center (ABRC, OH). All mutants were derived from the Columbia-0 (Col-0) accession. Seeds were treated with 1.2% (v/v) commercial bleach for 15 min, rinsed twice with sterile water for 10 min each, and subsequently stored at 4°C in the dark for 3 days. Sterilized and cold-pretreated seeds were sown on agar plates or in pots containing disinfected Tref substrate (Jiffy). Seedlings were grown at 24°C under LD (16-h light/8-h dark cycle) or SD (8-h light/16-h dark cycle) conditions with a light intensity of approximately 100 μE/s/m². For the germination and root elongation tests, the basal agar medium was composed of half-strength MS salts (Murashige and Skoog 1962), B5 organic compounds (Gamborg et al. 1968), 0.05% MES [2-(N-morpholino)ethane sulfonic acid monohydrate], and 1% sucrose. The Atnced3 mutant and 35S::LfNCED3 transformant were obtained from Dr. Wan-Hsing Cheng (Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan) and have been used in previous studies (Chen et al. 2011; Wan and Li 2006). In rice, Oryza sativa L. cv. Nipponbare and Oryza sativa L. cv. Tainung 67 were investigated in the present analysis. The seeds were sterilized and imbibed at 37°C for 2 days in the dark. After imbibition, the germinated seeds were grown on a wired stand in beakers at 28°C under LD conditions (16-h light/8-h dark cycle) with a light intensity of approximately 100 μE/s/m². The basal medium was Kimura B solution (Yoshida et al. 1976).

Transgene constructs

The full-length coding sequences of OsbHLH068 and GFP, with or without a stop codon, were PCR-amplified and cloned into the pGEM-T Easy vector (Promega). These fragments were then subcloned into a binary vector, pCAMBIA-1300, where their expression was driven by either a 2.2-kb AbhHLH112 native promoter (AbhHLH112p::GFP-OsbHLH068 and AbhHLH112p::OsbHLH068-GFP) or a CaMV 35 S promoter (35S::OsbHLH068). After sequencing, the AbhHLH112p::GFP-OsbHLH068 and AbhHLH112p::OsbHLH068-GFP constructs were subsequently transformed into the Abhhlh112 (SALK_148540) mutant by the floral dipping method for functional complementation and protein subcellular localization assays, and the 35S::OsbHLH068 construct was introduced into Columbia-0 for functional analysis. Additionally, both 1.2-kb OsbHLH068 and 2.2-kb AbhHLH112 native promoters were used to replace the 35 S promoter to drive the expression of the β-glucuronidase (GUS) reporter gene in the binary vector pCAMBIA-1305.1. After sequencing, the OsbHLH068p::GUS and AbhHLH112p::GUS constructs were subsequently transformed into Col-0 and/or Oryza sativa L. cv. Tainung 67 via an Agrobacterium-mediated method for analyzing spatiotemporal gene expression.

Phenotypic comparisons and plant weight measurements

For the phenotypic comparisons, cold-pretreated seeds from each type of Arabidopsis plant were grown in pots containing disinfected Tref substrate (Jiffy). For the plant weight measurements, seedlings were grown under LD conditions. The aerial parts of the 15-day-old plants were excised and the detached rosette leaves were used to measure the fresh weight. These detached tissues were subsequently vacuum dried at least overnight to measure the dry weight. All fresh and dry weights are the mean ± SD of two independent experiments, each with 6 biological replicates (each with an average value of two technical replicates).

Germination and root elongation tests

For the germination and root elongation tests, cold-pretreated seeds of each type of Arabidopsis plant were sown on agar plates containing basal agar medium with or without 100 mM NaCl. Germination was defined as the point when radicle emergence first started to exceed seed coat. All germination rates are the mean ± SE of 4 independent biological replicates. Each biological replicate contains at least 35 seeds that were harvested from an individual Arabidopsis plant. The root length was measured after vertical growth for 5 days on agar plates. All root lengths are the mean ± SE of 20 independent biological replicates.

MDA and H2O2 determinations

For the MDA and H2O2 assays, cold-pretreated seeds of each type of Arabidopsis plant were grown in pots containing disinfected Tref substrate (Jiffy) under LD conditions. After growing for 12 days in pots, the seedlings were watered with fresh water containing 0 or 250 mM NaCl for an additional 3 days and then harvested for analysis.
The MDA and H₂O₂ levels were measured according to methods described by Heath and Packer (1968), Jana and Choudhuri (1981), respectively.

RNA extraction and cDNA synthesis

Total RNA was extracted from the various plant tissues using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. To avoid genomic DNA contamination, total RNA was treated with Turbo DNA-free™ DNase (Ambion) following the manufacturer’s instructions. The DNase-treated total RNA was subjected to cDNA synthesis using the SuperScript™ III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions.

Reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR)

The initial amount of template cDNA in each RT-PCR and qPCR reaction was 25 and 10 ng, respectively. The qPCR reactions were performed using an ABI 7500 system with the SYBR® Green PCR Master Mix Kit [Applied Biosystems (ABI)]. The 2−ΔCt was used to show a difference between the target gene and internal control. OsACTIN1 was used as an internal control for qPCR normalization. The primer sequences used are listed in Supplementary Table 2.

Spatiotemporal gene expression and protein subcellular localization

Samples were soaked in fixation buffer (0.3% formaldehyde, 10 mM MES hydrate, 0.3 M mannitol, and 2 mM dithiothreitol, pH 5.6) for 30 min before staining. After fixation, the samples were rinsed twice with 50 mM sodium phosphate (pH 7.0) and then submerged in a staining solution [50 mM sodium phosphate dibasic, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 2 mM dithiothreitol, and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt), pH 7.0]. The staining assay was conducted under ambient conditions. For the protein subcellular localization assay, GFP fluorescence was detected with spectral settings of 500–540 nm for emission and 488 nm for excitation using a Zeiss LSM 510 Meta confocal microscope.

Microarray and data analysis

Total RNA was amplified and labeled using a Low-Input Quick Amp Labeling Kit, One-Color (Agilent, USA), according to the manufacturer’s instructions. Cyanine 3 (Cy3)-labeled cRNA was fragmented by incubation at 60°C for 30 min. After fragmentation, Cy3-labeled cRNA was pooled and hybridized to the Agilent Arabidopsis V4 Oligo 4×44 K Microarray as suggested by the manufacturer. The array image was analyzed using Feature Extraction software version 10.7.1.1 with default settings. The gene expression data are available under accession number GSE84087. Additionally, the microarray dataset (GSE60260) was obtained from the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/). To identify DEPs, the raw microarray data were analyzed with the Bioconductor Limma package (Ritchie et al. 2015). The raw data were background-corrected using the ‘normexp’ method and then normalized using the ‘quantile’ method. Each up- or down-regulated DEP had a log₂-fold change > or <1, respectively, with a P-value < 0.05 based on Student’s t-test. Each DEP-annotated gene is listed and described in the corresponding supplemental data. Common DEG ontology graphical analysis was conducted using the agriGO database (Du et al. 2010).

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Author Contributions HCC was responsible for the design of experiments, the data assemble and the manuscript writing. CCH carried out microarray analysis and other studies. PCL helped in conducting experiments. YLY and YWW offer great help in microarray data processing and integration. WHC and MHL were responsible for providing the experimental facilities and propagating the plant materials, respectively. MCC is the corresponding person for giving final approval of the version to be submitted. All authors have read and approved the submission of final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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