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The IbPYL8-IbbHLH66-IbbHLH118 complex mediates the abscisic acid–dependent drought response in sweet potato

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Summary

• Drought limits crop development and yields. bHLH (basic helix-loop-helix) transcription factors play critical roles in regulating the drought response in many plants, but their roles in this process in sweet potato are unknown.

• Here, we report that two bHLH proteins, IbbHLH118 and IbbHLH66, play opposite roles in the abscisic acid (ABA)–mediated drought response in sweet potato. ABA treatment repressed *IbbHLH118* expression but induced *IbbHLH66* expression in the drought-tolerant sweet potato line Xushu55-2. Overexpressing *IbbHLH118* reduced drought tolerance, whereas overexpressing *IbbHLH66* enhanced drought tolerance, in sweet potato.

• IbbHLH118 directly binds to the E-boxes in the promoters of *ABA-insensitive 5* (*IbABI5*), *ABA-responsive element binding factor 2* (*IbABF2*), and *tonoplast intrinsic protein 1* (*IbTIP1*) to suppress their transcription. IbbHLH118 forms homodimers with itself or heterodimers with IbbHLH66. Both of the IbbHLHs interact with the ABA receptor IbPYL8. ABA accumulates under drought stress, promoting the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex. This complex interferes with IbbHLH118's repression of ABA-responsive genes, thereby activating ABA responses and enhancing drought tolerance.

• These findings shed light on the role of the IbPYL8-IbbHLH66-IbbHLH118 complex in the ABA-dependent drought response of sweet potato and identify candidate genes for developing elite crop varieties with enhanced drought tolerance.

Key words: ABA, drought, *IbPYL8*, *IbbHLH66*, *IbbHLH118*, *IbABI5*, *IbABF2*, sweet potato.

Introduction

Sweet potato (*Ipomoea batatas*) is an economically important root and tuber crop that is widely used as an industrial and bioenergy resource worldwide. This crop is mainly planted on marginal lands (Jata *et al*., 2011). Extreme or prolonged drought conditions lead to significant reductions in sweet potato yield, prompting the need to improve the drought tolerance of this crop (Motsa *et al*., 2015). Genetic engineering is an effective approach for improving drought tolerance in sweet potato (Zhai *et al*., 2016; Kang *et al*., 2018; Mbinda *et al*., 2019; Zhang *et al*., 2019; Zhang *et al*., 2021). However, the transcriptional regulatory mechanisms underlying sweet potato's response to drought stress remain largely unknown.

Abscisic acid (ABA) is a crucial phytohormone involved in plant responses to drought stress (Fujita *et al*., 2006). This phytohormone plays essential roles in integrating a wide range of stress signals and regulating multiple downstream stress responses (Assmann & Jegla, 2016). ABA biosynthesis and signaling have been well studied in plants. Key enzymes involved in ABA biosynthesis include zeaxanthin epoxidase (ZEP), 9-cis-epoxycarotenoid dioxygenase (NCED), and aldehyde oxidase (AAO) (Xiong & Zhu, 2003). In the ABA signaling pathway, ABA binds to its receptor Pyrabactin resistance 1/PYR-like (PYR/PYL), forming the ABA-PYR/PYL complex. This complex interacts with ABA-insensitive (ABI)-clade protein phosphatase 2Cs (PP2Cs) and represses their phosphatase activity, consequently releasing activated Snf1-related Kinase 2s (SnRK2s) to phosphorylate downstream ABA-bound transcription factors (ABFs) to promote ABA responses (Tuteja *et al*., 2007; Sun *et al*., 2011; Danquah *et al*., 2014).

The basic helix-loop-helix (bHLH) superfamily, the second largest transcription factor (TF) family, is widely present in eukaryotes (Pires & Dolan, 2010). bHLH TFs are classified into six subgroups, A, B, C, D, E, and F, based on their phylogenetic relationships and DNA binding functions; most plant bHLH proteins belong to subgroups A and B. Subgroup A members specifically bind to the E-box core sequence in the promoters of their target genes, but subgroup B members preferentially bind to the G-box sequence (Li *et al*., 2021; Atchley *et al*., 1997). bHLH proteins usually consist of approximately 60 amino acids with two functionally distinct regions: the basic region, which contains 13–17 primarily

basic amino acids for DNA binding; and the HLH region, which enables the formation of homodimers or heterodimers with one or several different partners (Tian *et al*., 2019). Therefore, bHLH proteins usually function by DNA binding and dimerization (Martínez-García *et al*., 2000; Hao *et al*., 2021).

bHLH TFs are important regulators of plant growth and development, including seed germination (Penfield *et al*., 2005; Oh *et al*., 2006; Groszmann *et al*., 2010), flowering (Kumar *et al*., 2012; Ito *et al*., 2012; Sharma *et al*., 2016; Wang *et al*., 2017), cell fate determination (Menand *et al*., 2007; Yi *et al*., 2010; Chen *et al*., 2011; Qi *et al*., 2015), anthocyanin biosynthesis (Zhao *et al*., 2019; Liu *et al*., 2021), environmental responses (Yuan *et al*., 2008; Balazadeh *et al*., 2010; Guan *et al*., 2013; Tanabe *et al*., 2018), and signaling pathways of phytohormones such as auxin (IAA), jasmonate acid (JA), and ABA (Varaud *et al*., 2011; Schweizer *et al*., 2013; Li *et al*., 2019). Although several bHLH proteins, such as AtbHLH68, AtbHLH112, AtbHLH122, and ZmPTF1, have been reported to mediate abiotic stress responses by regulating the ABA signaling pathway in plants such as *Arabidopsis*, maize, and peanut (Liu *et al*., 2014; Liu *et al*., 2015; Le *et al*., 2017; Li *et al*., 2019; Li *et al*., 2021), the biological functions and regulatory mechanisms of bHLH proteins in the drought response of sweet potato remain unclear.

In this study, we demonstrate that two bHLH proteins, IbbHLH118 and IbbHLH66, play opposite roles in the ABA-mediated drought stress responses of sweet potato. ABA promotes the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, which activates the expression of ABA-responsive genes, thereby enhancing ABA signaling and drought adaptation. These findings provide novel insights into the regulatory mechanisms of bHLH TFs in plants.

Materials and Methods

Plant materials

All the plant materials are stored in lab stock. The drought-tolerant sweet potato (*Ipomoea batatas* (L.) Lam.) line 'Xushu55-2' (Zhu *et al*., 2019, reported by our lab), the drought-sensitive sweet potato variety 'Lizixiang' (Zhang *et al*., 2017, reported by our lab), and the

tobacco (*Nicotiana tabacum* L.) cultivar 'Wisconsin38 (W38)' were cultivated in the field, greenhouse, or growth chamber at China Agricultural University, Beijing, China. Xushu55-2 was employed for cloning *IbbHLH118*, *IbbHLH66*, *IbPYL8*, and *IbTIP1*. Lizixiang and W38 was used to characterize their functions. *In vitro*–grown transgenic sweet potato Xushu55-2 and Lizixiang plants were cultured on Murashige and Skoog (MS) medium at 27 ± 1°C under a photoperiod consisting of 13 h of cool-white fluorescent light at 54 μmol m^{-2} s^{-1} and 11 h of darkness.

DNA sequencing and analysis

Genomic DNA (OminiPlant RNA Kit) and total RNA (Fast Plasmid Miniprep Kit) were extracted from fresh leaves of Xushu55-2 plants. The genomic DNA and cDNA sequences were amplified using primers listed in Table S1. The conserved domains were searched using InterPro (http://www.ebi.ac.uk/interpro/). Multiple sequence alignment was performed using DNAMAN software (Lynnon-BioSoft, San Ramon, CA, USA). Phylogenetic analysis was conducted using the neighbor-joining method in MEGA11.0 with 1,000 bootstrap iterations (Tamura *et al*., 2021). The exon-intron structures of genes were analyzed using the Splign program (https://www.ncbi.nlm.nih.gov/sutils/splign). The *cis* elements in the promoter regions were analyzed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression analysis

The leaves of 4-week-old *in vitro*–grown Xushu55-2 and Lizixiang plants were sampled at 0, 0.5, 1, 3, 6, and 12 h after treatment with 20% polyethylene glycol (PEG) 6000, 100 μM ABA, or 200 mM hydrogen peroxide (H_2O_2) in half-strength Hoagland solution. Total RNA was extracted from leaf, stem, and root tissues of 4-week-old *in vitro*–grown Xushu55-2 plants and from leaf, stem, petiole, storage root, and fibrous root tissues of 2-month-old field-grown Xushu55-2 plants using the TRIzol method (CWBIO). The transcript levels were measured using quantitative reverse-transcription PCR (qRT-PCR). The sweet potato *ACTIN* (AY905538) gene was used as an internal control (Table S1).

Promoter activity assay

The promoter sequence of *IbbHLH118* of Lizixiang or Xushu55-2 was inserted into the pMDC162 vector. The plasmids were separately transformed into the sweet potato protoplasts, *Nicotiana benthamiana*, and tobacco cv. W38 by *Agrobacterium*-mediated transformation according to Horsch *et al.* (1985). Four-week-old transgenic tobacco plants were cultured separately in half-strength Hoagland solution with PEG6000 (10%) or ABA (100 μM) for 24 and 48 h. β-Glucuronidase (GUS) activity in leaves was measured as described by Jefferson (1987). Three independent biological replicates were performed.

Subcellular localization

The entire *IbbHLH118*, *IbbHLH66*, and *IbPYL8* coding regions without the stop codon were cloned into pCAMBIA1300. The constructs and the membrane marker PIP2A-mCherry were transformed into *N. benthamiana* leaf epidermal cells by *Agrobacterium*-mediated infiltration. The fluorescent signals were detected using a confocal laser-scanning microscope (LSM880, Zeiss).

Transcriptional activation assay

The full-length coding sequence of *IbbHLH118* or fragments encoding amino acids 1–175 and 176–298 and the full-length coding sequence of *IbbHLH66* or fragments encoding amino acids 1–100, 101–350, and 351–465 were inserted into the pGBKT7 vector. These constructs, pGBKT7-53 (positive control), and pGBKT7-Lam (negative control) were transferred into yeast strain AH109 according to the Yeast Protocols Handbook (Clontech). The transformed yeast colonies were cultured on SD/-Trp medium for 2 days and streaked onto SD/-Trp/-His/-Ade medium.

Transgenic plant generation

The *35S:IbbHLH118-GFP*, *35S:IbbHLH66-GFP*, *35S:IbPYL8-GFP*, and *35S:IbTIP1-GFP* (pCAMBIA1300) vectors were transfected into *Agrobacterium* strain EHA105. In addition, a pair of forward and reverse nonconserved fragments of *IbbHLH118* were inserted into the plant RNA interference (RNAi) vector pCAMBIA1300-35SI-X and was transfected into *Agrobacterium* strain EHA105. Transformation and plant regeneration were performed using embryogenic suspension cultures of the drought-sensitive variety

Lizixiang, or transformed into W38 via *A. tumefaciens*-mediated transformation (Liu *et al.*, 2001; Zhang *et al.*, 2020).

The pTRV2-*IbbHLH66*, pTRV2-*IbABI5*, pTRV2-*IbABF2*, pTRV2, and pTRV1 were transferred into *Agrobacterium* strain EHA105 for tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) in the drought-sensitive sweet potato variety 'Lizixiang'. The VIGS and VWT plants were generated by *Agrobacterium*-mediated transformation (Yan *et al.*, 2012). The transgenic plants transiently overexpressing *IbbHLH66* or *IbPYL8* in *IbbHLH118*-OE lines were generated by *Agrobacterium*-mediated vacuum infiltration (Bi & Zhang, 2014).

Drought tolerance assays

The conditions for the drought treatments were established based on stress adaptability of transgenic plants. The *IbbHLH118* (4 w), *IbbHLH66* (4 w), *IbTIP1* (4 w), *IbbHLH66*-VIGS (2 w), *IbABI5*-VIGS (2 w), *IbABF2*-VIGS (2 w), and the wild type (WT) plants were grown on MS medium containing 30% PEG. Three independent biological replicates were taken. *IbPYL8* transgenic tobacco plants were grown on 1/2 MS medium containing 10% PEG for 4 weeks. Three independent biological replicates were taken.

Cuttings (~20 cm) from field-grown transgenic and WT plants were cultured in Hoagland solution containing 15% (*IbbHLH118*-OE lines) or 30% (*IbbHLH66*-OE lines) PEG, transferred to Hoagland solution, and cultured for 2 weeks. The *IbbHLH118*-OE, *IbbHLH66* (*IbbHLH118*-OE), *IbPYL8*/*IbbHLH66* (*IbbHLH118*-OE) and WT plants were cultured in Hoagland solution with or without 20% PEG for 6 h. Three independent biological replicates were taken. Further, cuttings were planted in a transplanting box in a greenhouse and grown without watering for 4 (*IbbHLH118*-OE lines) or 6 (*IbbHLH118*-RNAi and *IbbHLH66*-OE lines) weeks. Three independent biological replicates were taken.

Cuttings of *IbbHLH118*-OE and WT plants were planted in a greenhouse and grown without watering for 3 months. For normal condition, the soil moisture was maintained at approximately 65–75% for 3 months. Twenty independent biological replicates were taken. At harvest, the aboveground weight (AW) and storage root (belowground) weight (BW) of five consecutive plants from each genotype/treatment were measured.

Stomatal aperture assay

The leaves of field-grown transgenic and WT plants were incubated in stomatal opening solution (50 mM KCl, 10 mM MES-KOH, and 10 mM $CaCl_2$, pH 6.1) for 3 h and transferred to stomatal opening solution containing 20μM ABA, followed by incubation for 2 h. Eighty stomata were randomly selected and measured using a fluorescence microscope (Revolve, Echo, USA).

Measurement of drought tolerance indices

The 3,3'-Diaminobenzidine (DAB) staining and nitro blue tetrazolium (NBT) staining were performed according to Zhang *et al*. (2022). The superoxide dismutase (SOD) and peroxidase (POD) activities and ABA (Ruixinbio, Quanzhou, China), H_2O_2, proline, and malondialdehyde (MDA) contents in the leaves of transgenic and WT plants were measured using assay kits (Comin Biotechnology Co. Ltd., Suzhou, China). The photosynthesis rate, stomatal conductance, and transpiration rate were measured according to Zhang *et al*. (2019). For the measurement of the relative electrical conductivity (REC), ten leaf discs (1 cm diameter) from each line were placed in 10 ml of distilled water, vacuumed for 10 min, and then surged for 1 h to measure the initial electric conductivity (S_1). The materials were boiled for 10 min and then cooled to room temperature to measure the final electric conductivity (S_2). The distilled water was used as a blank control and its electric conductivity (S_0) was measured. REC was calculated as $REC = (S_1 - S_0) / (S_2 - S_0) \times 100$.

Yeast two-hybrid (Y2H) assay

The full-length *IbbHLH118*, *IbbHLH66*, and *IbPYL8* sequences were cloned into pGADT7. The sequences encoding amino acid residues 1–175 of *IbbHLH118* and amino acid residues 101–350 of *IbbHLH66* were cloned into pGBKT7 (Table S1). These constructs were transferred into yeast strain AH109. Positive clones were selected on SD/-Ade/-His/-Leu/-Trp/+3AT/+x-α-ga1 medium with or without 100 μM ABA at 30°C according to the Yeast Protocols Handbook (Clontech).

Coimmunoprecipitation (CoIP) assay

The HA-IbbHLH118-FLAG, HA-IbPYL8-FLAG, IbbHLH118-GFP, and IbbHLH66-GFP

vectors were transiently expressed in *N. benthamiana* leaves. Total proteins were extracted from the leaves using extraction buffer (Zhang *et al*., 2020). The total proteins were mixed with HA agarose beads (B26201, BIMAKE) and incubated at 4°C for 4 h. The agarose was washed at least five times with extraction buffer and boiled in 5× SDS loading buffer for 15 min to separate the proteins from the agarose beads. The proteins were detected using polyclonal anti-HA (1:10000, H3663, SIGMA) and anti-GFP antibodies (1:10000, BE2002, EASYBIO).

Bimolecular fluorescence complementation (BiFC) assay

The full-length *IbbHLH118*, *IbbHLH66*, and *IbPYL8* sequences were cloned into the pSPYNE-35S vector and fused to the N-terminus of yellow fluorescent protein (nYFP), and the full-length *IbbHLH118*, *IbbHLH66*, and *IbPYL8* sequences were cloned into pSPYCE-35S and fused to the C-terminus of YFP (cYFP; Walter *et al*., 2004) (Table S1). These constructs were introduced into *N. benthamiana* leaves by *Agrobacterium*-mediated infiltration. The yellow fluorescence signal was observed using a confocal laser-scanning microscope (LSM880, Zeiss).

Firefly luciferase complementation imaging (LCI) assay

The full-length *IbbHLH118* and *IbbHLH66* sequences were cloned into the C-terminus–encoding regions, and the full-length *IbPYL8* sequences were cloned into N-terminus–encoding regions of the luciferase, respectively (Chen *et al*., 2008) (Table S1). These constructs were coinfiltrated into *N. benthamiana*, and the infiltrated leaves were analyzed for LUC activity at 48 h after infiltration using chemiluminescence imaging (LB985, Berthold) and enzyme-labeled instrument (Glomax Discover, Promega).

Immunoblot analysis

The HA-IbbHLH118-FLAG, IbbHLH66-Myc, and IbPYL8-GFP vectors were transiently expressed in *N. benthamiana* leaves with or without 100 μM ABA treatment. Total proteins were extracted and detected using polyclonal anti-HA (1:10000, H3663, SIGMA), anti-Myc (1:10000, M4439, SIGMA), and anti-GFP antibodies (1:10000, BE2002, EASYBIO), respectively.

Yeast one-hybrid assay

The coding sequences of *IbbHLH118*, *IbbHLH66*, and *IbPYL8* were fused to the activation domain of the pB42AD vector. The *IbNCED3*, *IbNCED5*, *IbABI5*, *IbABF2*, and *IbTIP1* promoters were separately inserted into the pLacZi2μ vector to drive LacZ reporter expression. These effector and reporter plasmids were co-transformed into yeast strain EGY48, which were cultured on SD/-Trp/-Ura/+x-ga1 medium to screen positive clones.

Dual-luciferase assay

The full-length *IbbHLH118*, *IbbHLH66*, and *IbPYL8* coding sequences were inserted into pGreenII 62-SK driven by the CaMV 35S promoter. The *IbABI5*, *IbABF2*, and *IbTIP1* promoter sequences were cloned into pGreenII 0800-LUC. Sweetpotato protoplasts were isolated and used for the dual-luciferase assays as described previously (Zhang *et al*., 2020). The Firefly LUC and Renilla luciferase (REN) activity levels were measured using the Dual-Luciferase Reporter Assay System (Glomax Discover, Promega). Three independent biological replicates were taken.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed according to the method of Zhang *et al*. (2020) with minor modifications. The pCold-SUMO-*IbbHLH118*, pCold-SUMO-*IbbHLH66*, and pCold-SUMO-*IbPYL8* constructs were transferred into competent *E. coli* strain Transetta (DE3) cells to produce the 6His-IbbHLH118, 6His-IbbHLH66, and 6His-IbPYL8 proteins. These proteins were treated with SUMO protease to remove the SUMO proteins. Probes labeled with or without biotin at their 5' ends were used as binding or competitive probes.

Chromatin immunoprecipitation (ChIP) assay

The leaves of OE-X9 and OE-a5 plants were used for the ChIP assays according to Zhang *et al*. (2020). Anti-GFP (1:5000, BE2002, EASYBIO) antibodies were used to immunoprecipitate the protein-DNA complex, and the precipitated DNA was recovered. An equal amount of chromatin sample without antibody precipitation was used as an input control. ChIP DNA was analyzed by qPCR, and the ChIP values were normalized against the values of the respective input. The primers used for ChIP-qPCR are listed in Table S1.

The experiment was independently repeated three times with similar results.

Results

Differential expression of *IbbHLH118* in drought-tolerant and -sensitive germplasms

To identify potential regulators of the drought response in sweet potato, we analyzed the expression of bHLH TF family genes using the transcriptomes of several sweet potato varieties under drought stress (Lau *et al*., 2018; Zhu *et al*., 2019; Arisha *et al*., 2020). *IbbHLH118* was differentially expressed in drought-tolerant versus -sensitive germplasms. We performed qRT-PCR to detect the relative transcript levels of *IbbHLH118* in the drought-tolerant sweet potato line Xushu55-2 and drought-sensitive sweet potato variety Lizixiang under various stress conditions. Under PEG, ABA, and H_2O_2 treatment, the expression of *IbbHLH118* was suppressed almost 0.54-fold (at 1 h), 0.18-fold (at 1 h), and 0.14-fold (at 6 h) in Xushu55-2, but induced almost 6.75-fold (at 6 h), 3.75-fold (at 1 h), and 2.16-fold (at 6 h) in Lizixiang, respectively (Fig. 1a, b, c). In addition, *IbbHLH118* was highly expressed in the leaves of 4-week-old *in vitro*–grown (Fig. S1a) and 2-month-old field-grown Xushu55-2 plants (Fig. S1b).

The 897-bp open reading frame (ORF) of *IbbHLH118* encodes a protein of 298 amino acids with a predicted molecular weight of 33.54 kDa. IbbHLH118, belonging to subgroup A of the bHLH TF family, contains one conserved bHLH domain and is most closely related to its homolog in *Arabidopsis*, AtbHLH118 (Fig. 1d, e). The genomic sequence of *IbbHLH118* contains three exons and two introns, and its length is similar to that of its *Arabidopsis* homolog but is shorter than the homologous genes in the other plants (Fig. 1f).

The *IbbHLH118* promoter regions in Xushu55-2 and Lizixiang both contain various abiotic stress–responsive elements, such as ACGT-containing ABA response elements (ABREs; Sonal *et al*., 2014), MYB binding sites (MBSs; Karkute *et al*., 2018), and long terminal repeats (LTRs; Wu *et al*., 2019) (Fig. S1c). More abiotic stress–responsive elements, such as TCA- and ABRE-elements are present in the *IbbHLH118* promoter of Lizixiang (Fig. S1c). The *IbbHLH118* promoter of Xushu55-2 contains an $(ACGT)_{N15}(ACGT)$ *cis*-element, which may act as a negative regulator leading to reduced

promoter activity (Armstrong *et al*., 1992; Horn & Boutros, 2010; Mehrotra *et al*., 2013). Consistent with that, the *GUS* expression and GUS activity driven by the *IbbHLH118* promoter of Xushu55-2 were significantly lower than those driven in Lizixiang (Fig S1d, e). We further generated transgenic tobacco plants expressing GUS driven by the *IbbHLH118* promoter of Lizixiang. Histochemical staining showed that the leaves exhibited higher GUS activity than stems or roots, and the promoter activity was significantly induced by PEG and ABA treatment in leaves (Fig. S1f, g). Collectively, these results indicate that *IbbHLH118* is involved in drought and ABA responses in sweet potato.

IbbHLH118 is a nuclear and cell membrane–localized transcriptional activator

We examined the subcellular localization of IbbHLH118 by transiently expressing the IbbHLH118-GFP fusion protein in *N. benthamiana* epidermal cells. Analysis of the fluorescent signal indicated that IbbHLH118 was localized to the nucleus and cell membrane (Fig. 1g).

To explore whether IbbHLH118 harbors transcriptional activation activity, we separately inserted three fragments encoding the full-length IbbHLH118 protein, amino acids 1–175, and amino acids 176–298 of this protein into the GAL4 pGBKT7 vector and separately transformed the fusion constructs into yeast cells. Yeast colonies harboring either BD-IbbHLH118 or BD-176-298 grew well and turned blue on SD medium lacking Trp, His, and Ade and containing X-α-gal (Fig. S1h). These results indicate that IbbHLH118 is a nuclear- and cell membrane–localized transcriptional activator.

Knockdown of *IbbHLH118* enhances drought tolerance in sweet potato

To explore how *IbbHLH118* affects the drought response in sweet potato, we generated 15 overexpression (designated as OE-X1 to OE-X15) and 5 knockdown (designated as Ri-X1 to Ri-X5) lines from cell aggregates of the drought-sensitive sweet potato variety Lizixiang via *Agrobacterium tumefaciens*-mediated transformation (Fig. S2). After examining the expression levels of *IbbHLH118* in these transgenic lines, we selected three overexpression (OE-X4, 6, and 9) and three knockdown (Ri-X2, 3, and 5) lines for further study.

We planted the transgenic and the WT plants on MS culture medium containing 30%

PEG for *in vitro* assays. Under PEG treatment, the *IbbHLH118*-RNAi lines exhibited significantly stronger growth and rooting and higher FW and DW than WT plants, while the *IbbHLH118*-OE lines displayed opposite changes (Fig. 2a, b; Table S2).

The transgenic and WT plants were then transferred to soil in the greenhouse or the field. We cultured cuttings of the transgenic and WT plants in a transplanting box and subjected them to drought stress. Under normal conditions, the *IbbHLH118*-OE plants showed shorter stems and roots compared with WT plants, but no obvious morphological differences were observed in *IbbHLH118*-RNAi plants (Fig. 2c, d, f, g; Table S3). Under drought conditions, the *IbbHLH118*-RNAi lines exhibited better growth and rooting and greater FW and DW, whereas the *IbbHLH118*-OE lines became brown and dried earlier than WT plants (Fig. 2c-h; S3). These results indicate that knockdown of *IbbHLH118* enhances the drought tolerance of sweet potato.

ABA stimulates stomatal closure to maintain osmotic pressure in plants in response to drought stress (Munemasa *et al.*, 2015). We therefore quantified endogenous ABA levels in the transgenic plants. Under drought stress, the ABA contents were significantly lower in *IbbHLH118*-OE but higher in *IbbHLH118*-RNAi versus WT plants (Fig. 3a). We then examined whether exogenous ABA treatment would affect the stomatal aperture of *IbbHLH118* transgenic plants. The *IbbHLH118*-OE lines exhibited reduced but *IbbHLH118*-RNAi lines exhibited increased ABA-induced stomatal closure compared to WT plants (Fig. 3b, c). These results indicated that knockdown of *IbbHLH118* led to increased ABA accumulation and a sharp response to ABA.

Drought stress causes excessive reactive oxygen species (ROS) generation, resulting in oxidative damage to plants (Foyer 2018; Sharma *et al.*, 2012). Proline acts as an osmoticum and a ROS scavenger under drought stress (Ghosh *et al.*, 2022). DAB and NBT staining and H_2O_2 measurement revealed that the *IbbHLH118*-RNAi plants accumulated less H_2O_2 and superoxide anion radical (O^{2-}) than the WT (Fig. 3d-h). Moreover, significantly higher POD and SOD activities and proline contents were detected in *IbbHLH118*-RNAi versus WT plants (Fig. 3i-k; Table S4). By contrast, the *IbbHLH118*-OE lines showed the opposite pattern for the respective physiological indices. These results indicate that knockdown of *IbbHLH118* activated the ROS scavenging system of sweet potato.

IbbHLH118 forms homodimers or heterodimers

To better understand the regulatory mechanisms of *IbbHLH118*-mediated drought and ABA responses, we used amino acids 1–175 of IbbHLH118 as a bait to screen a yeast Y2H library constructed using RNA from sweet potato leaves. Two bHLH proteins, IbbHLH118 itself and IbbHLH66, were identified as interacting proteins of IbbHLH118 (Fig. 4a). A transcriptional activation assay showed that IbbHLH66 is a transcriptional activator (Fig. 4b), but it could not form a homodimer with itself (Fig. 4a).

Next, we performed BiFC and CoIP assays to verify the interaction of IbbHLH118 with itself and with IbbHLH66. IbbHLH118 indeed formed homodimers as well as heterodimers with IbbHLH66 in plant cells, and both pairs interacted in the nucleus and cell membranes (Fig. 4c-e). We then investigated the subcellular localization of IbbHLH66 in *N. benthamiana* leaf epidermal cells. IbbHLH66 localized to the nucleus and cell membranes (Fig. 4f), which matches the subcellular localization of IbbHLH118 and the sites of the interaction between IbbHLH118 and IbbHLH66 (Fig. 1g; 4c).

The 1,395-bp ORF of *IbbHLH66* encodes a protein of 465 amino acids with a predicted molecular weight of 48.5 kDa. IbbHLH66, also belonging to subgroup A of the bHLH TF family, contains one conserved bHLH domain and is most closely related to its *Arabidopsis* homolog, AtbHLH66 (Fig. S4a, b). *IbbHLH66* contains seven exons and six introns, whereas *AtbHLH66* contains four exons and three introns (Fig. S4c). Under PEG treatment, the expression of *IbbHLH66* was induced to higher levels in Xushu55-2 than in Lizixiang (Fig. 4g). *IbbHLH66* was upregulated 7.93-fold (at 6 h), 5.42-fold (at 6 h), and 9.56-fold (at 6 h) in Xushu55-2 under PEG, ABA, and H_2O_2 treatment, respectively (Fig. 4h). These results indicate that IbbHLH118 forms homodimers with itself or forms heterodimers with the drought- and ABA-responsive protein IbbHLH66 in sweet potato.

***IbbHLH66* enhances drought tolerance in sweet potato**

To study the role of *IbbHLH66* in drought tolerance, we overexpressed this gene in sweet potato (Fig. S5) and selected five lines with high *IbbHLH66* transcript levels, as determined by qRT-PCR (OE-a1 to a5; Fig. S5j), for drought tolerance assays. The five overexpression lines and the WT were planted on MS culture medium containing 30% PEG for the *in vitro*

assays. Notably, *IbbHLH66*-OE plants exhibited significantly better growth and rooting than WT plants (Fig. 5a, b; Table S2).

Then, three randomly selected overexpression lines (OEa3, a4, and a5) and WT plants were transferred to soil and grew in the greenhouse or field (Fig. S5e, f). The cuttings of these lines and the WT were cultured in half-strength Hoagland solution containing 30% PEG for 3 weeks, followed by standard Hoagland solution for 2 weeks. Under PEG stress, the transgenic plants formed new leaves and longer roots, while the WT plants died (Fig. 5c-e; S6; Table S3). Finally, we grew OEa3, a4, a5, and WT plants in a transplanting box and subjected them to drought stress. The *IbbHLH66*-OE plants exhibited better growth and rooting and greater FW and DW than the WT, with higher photosynthetic rates and transpiration rates, while the WT plants turned brown and died sooner (Fig. 6a, b).

Under drought stress, the ABA contents were significantly higher in *IbbHLH66*-OE plants than in WT plants (Fig. 6c). Upon exogenous ABA treatment, the *IbbHLH66*-OE plants were more sensitive to ABA-induced changes in stomatal aperture than WT plants (Fig. 6d, e). In addition, DAB and NBT staining and H_2O_2 measurement revealed that the *IbbHLH66*-OE plants accumulated less H_2O_2 and O^{2-} than the WT under drought stress (Fig. 6f-j). Upon exposure to drought stress, the SOD activities and proline contents were significantly higher in *IbbHLH66*-OE versus WT plants, while the MDA contents were significantly lower in these lines (Fig. 6k-m; Table S5).

We further examined knockdown phenotypes of *IbbHLH66* by VIGS. qRT-PCR analysis showed that *IbbHLH66* was significantly reduced in *IbbHLH66*-silenced sweet potato leaves (Fig. S7b, e), indicating that *IbbHLH66* was effectively silenced in sweet potato. After treatment with 30% PEG for 14 days, the VWT plants exhibited better growth with a lower browning rate than the *IbbHLH66*-VIGS plants (Fig. S7). These results indicate that overexpressing *IbbHLH66* led to increased, whereas knockdown of *IbbHLH66* resulted in decreased drought tolerance in sweet potato.

ABA promotes IbPYL8-IbbHLH66-IbbHLH118 complex formation

To explore the possible interacting partners of IbbHLH66 involved in ABA-mediated drought response in sweet potato, we screened the yeast two-hybrid library. Because the 1-

100 and 351-465 amino acid residues of IbbHLH66 were required for its trans-activation activity in yeast (Fig. 4b), we used 101–350 amino acid residues of IbbHLH66, which included a bHLH domain, as the bait in Y2H screens. The ABA receptor IbPYL8 was identified as an interacting partner of IbbHLH66 (Fig. 7a; S8a). The Y2H assays demonstrated that IbbHLH118 also interacts with IbPYL8 (Fig. 7a; S8a). We then performed CoIP and BiFC assays to verify the interaction of IbPYL8 with IbbHLH66 or with IbbHLH118. IbbHLH66 and IbbHLH118 both interacted with IbPYL8 in plant cells, and both pairs interacted in the nucleus and cell membranes (Fig. 7b-d). These three proteins formed an IbPYL8-IbbHLH66-IbbHLH118 ternary complex.

The PYL8 was reported to mediate ABA perception, and in turn ABA specifically stabilizes PYL8 and induces its accumulation in plant (Belda-Palazon *et al.*, 2018; Garcia-Maquilon *et al.*, 2021). Therefore, we examined whether exogenous ABA treatment would affect the interactions of IbPYL8 by Y2H and LCI assays. Notably, ABA treatment enhanced the interactions of IbPYL8 with IbbHLH66 or IbbHLH118 in both yeast and *N. benthamiana* (Fig. 7a, e, f; S8a, b). Further, IbbHLH118, IbbHLH66, and IbPYL8 protein levels were determined after transiently expressing for different combinations in *N. benthamiana*. The protein levels of IbbHLH66 and IbPYL8 were induced, but IbbHLH118 was repressed after exogenous treatment with 100 μM of ABA. Being consistent with this trend, inside the IbPYL8-IbbHLH66-IbbHLH118 ternary complex, IbbHLH66 and IbPYL8 protein increased, but IbbHLH118 protein decreased after ABA treatment (Fig. 7g). Collectively, these results indicate that a ternary complex formed by IbbHLH66 and IbbHLH118 with the ABA receptor IbPYL8 functions in the ABA-dependent drought response in sweet potato.

***IbPYL8* enhances drought tolerance in transgenic tobacco plants**

Further qRT-PCR analysis showed that *IbPYL8* was significantly induced by PEG (3.18-fold at 1 h), ABA (2.46-fold at 6 h), and H_2O_2 (2.12-fold at 3 h) treatment in Xushu55-2 (Fig. S8c). Subcellular localization analysis indicated that IbPYL8-GFP was located in the nucleus and cell membranes (Fig. S8d). To investigate the role *IbPYL8* in drought tolerance, we generated transgenic tobacco (*N. tabacum*) plants overexpressing *IbPYL8* and

challenged them with drought stress. *In vitro*–grown *IbPYL8*-OE plants showed better growth than the wild type W38 when grown on 1/2 MS medium containing 10% PEG (Fig. S9a).

In addition, we measured higher ABA and proline contents, and POD and SOD activities, but lower MDA and H_2O_2 contents in the *IbPYL8*-OE lines compared to W38 (Fig. S9b-g). These results indicate that *IbPYL8* is a positive regulator against drought stress, likely by ABA signaling and ROS scavenging in plants.

IbbHLH118 directly targets ABA signaling–related genes *IbABI5* and *IbABF2*

Since IbbHLH118 and IbbHLH66 are involved in the ABA-mediated drought response, we examined the expression levels of key genes involved in ABA biosynthesis and signaling in the transgenic plants. Under normal and drought conditions, key genes related to ABA biosynthesis (*IbNCED3* and *IbNCED5*) and ABA signaling (*IbABI5* and *IbABF2*) were significantly downregulated in *IbbHLH118*-OE plants but significantly upregulated in *IbbHLH66*-OE plants compared to the WT (Fig. 8a, b; S10).

To investigate whether IbbHLH118 and IbbHLH66 directly regulate these genes, we conducted Y1H assays. Neither IbbHLH118 nor IbbHLH66 bound to the promoter region of *IbNCED3* or *IbNCED5*. IbbHLH118 directly bound to the promoter regions of *IbABI5* and *IbABF2* to drive *LacZ* reporter gene expression in yeast cells, whereas IbbHLH66 did not (Fig. 8c, d). Therefore, IbbHLH118, but not IbbHLH66, directly targets and represses the key ABA signaling genes *IbABI5* and *IbABF2* that induce the ABA response in sweet potato.

We further explored the function of *IbABI5* and *IbABF2* in drought response using VIGS. qRT-PCR analysis showed that *IbABI5* and *IbABF2* were significantly reduced in gene-silenced sweet potato leaves during PEG stress (Fig. S11e, k). After treatment with 30% PEG for 14 days, the *IbABI5*-VIGS and *IbABF2*-VIGS plants exhibited worse growth with a higher browning rate than the VWT plants (Fig. S11). These results indicate that *IbABI5* and *IbABF2* function as positive regulators to drought tolerance in sweet potato.

IbbHLH66 inhibits the DNA binding activity of IbbHLH118

Since bHLHs usually function as dimers to bind to their target DNAs (Toledo-Ortiz *et al.*, 2003), we asked how IbbHLH66 and IbPYL8 affect the transcriptional activity of *IbbHLH118*. We performed transient dual-luciferase assays using sweetpotato protoplasts and a reporter construct in which the expression of the *LUC* reporter gene was driven by the *IbABI5* or *IbABF2* promoter. LUC activity analysis indicated that IbbHLH118 directly suppressed the *IbABI5* and *IbABF2* promoters, whereas IbbHLH66 activated these promoters (Fig. 8e, f; S12a, b). When IbbHLH66 or IbbHLH66 and IbPYL8 were co-expressed with IbbHLH118, *LUC* expression significantly gradually increased, whereas the addition of IbPYL8 alone had no effect on its expression, indicating that IbbHLH66 inhibits the function of IbbHLH118.

IbbHLH118, a subgroup A bHLH protein, specifically binds to E-box elements in its target gene promoters (Dennis *et al.*, 2019). Further ChIP-qPCR and EMSA assays indicated that IbbHLH118, but not IbbHLH66, could directly target *IbABI5* and *IbABF2* to suppress their expression by binding to particular E-boxes in their promoters (Fig. 8g-j). However, the addition of IbbHLH66 inhibited the DNA binding activity of IbbHLH118 to *IbABI5* and *IbABF2* (Fig. 8g-j). These results suggested that IbbHLH66 suppresses the inhibitory activity of IbbHLH118 towards *IbABI5* and *IbABF2*, thereby leading to their activation.

To further verify the regulation mode of IbPYL8-IbbHLH66-IbbHLH118 complex in sweet potato, we transiently overexpressed *IbbHLH66*, or *IbbHLH66* and *IbPYL8* into the *IbbHLH118*-OE lines (OE-X4 and OE-X6), and detected the transcript levels of *IbABI5* and *IbABF2* under normal or PEG treatment. The results showed that the expressions of *IbABI5* and *IbABF2* were gradually upregulated with the sequential overexpression of IbbHLH118, IbbHLH66-IbbHLH118, and IbPYL8-IbbHLH66-IbbHLH118 (Fig. S13). Collectively, our data demonstrate that under drought stress, IbPYL8-IbbHLH66-IbbHLH118 complex interferes with IbbHLH118's repression of *IbABI5* and *IbABF2*, thereby promoting ABA signaling and drought tolerance in sweet potato.

The IbPYL8-IbbHLH66-IbbHLH118 complex targets the ABA-responsive gene *IbTIP1*

Aquaporins respond to ABA, and are usually involved in helping maintain a balance of cellular water levels by modifying membrane permeability and stomatal opening (Kaldenhoff *et al.*, 2008; Maurel *et al.*, 2021). We identified *tonoplast intrinsic protein 1* (*IbTIP1*), encoding an aquaporin, whose expression level was significantly downregulated in *IbbHLH118*-OE plants but significantly upregulated in *IbbHLH66*-OE plants (Fig. 9a, b). The Y1H assay revealed that IbbHLH118 directly bound to the promoter region of *IbTIP1* to drive *LacZ* reporter gene expression in yeast cells, but IbbHLH66 did not (Fig. 9c). Transient dual-luciferase assays indicated that IbbHLH118 suppressed, but IbbHLH66 activated, the *IbTIP1* promoter. When IbbHLH66 or IbbHLH66 and IbPYL8 were co-expressed with IbbHLH118, *IbTIP1* promoter activity significantly gradually increased (Fig. 9d; S12c). Further ChIP-qPCR and EMSA assays showed that IbbHLH118, but not IbbHLH66, directly targets *IbTIP1* by binding to the E-box element in its promoter, but the addition of IbbHLH66 or IbbHLH66 and IbPYL8 abolished this binding (Fig. 9e-g). These results indicate that IbbHLH66 suppresses the inhibitory activity of IbbHLH118 towards *IbTIP1*, thereby leading to its activation.

In drought-tolerant sweet potato line Xushu55-2, *IbTIP1* was significantly induced by almost 1.96-fold (at 3 h), 1.59-fold (at 1 h), and 2.06-fold (at 6 h) under PEG, ABA, and H_2O_2 treatment, respectively (Fig. S14a), and this gene was highly expressed in leaves and stems (Fig. S14b). To investigate the role of *IbTIP1* in drought tolerance, we overexpressed it in sweet potato (Fig. S15) and selected five lines with high *IbTIP1* transcript levels, as determined by qRT-PCR (OE-t3, t4, t6, t9, and t12; Fig. S15f), for a drought tolerance assay. Under 30% PEG and drought treatment, the *IbTIP1*-OE plants exhibited significantly better growth and rooting and lower relative electrical conductivity compared to WT plants (Fig. 9h-j; S16a-d). Upon exogenous ABA treatment, the *IbTIP1*-OE plants were more sensitive to ABA-induced changes in stomatal aperture than WT plants (Fig. S16e, f). Together, these results indicate that under drought stress, ABA promotes the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, which targets the ABA-responsive gene *IbTIP1* and activates its expression, thereby reducing membrane damage and enhancing drought tolerance in sweet potato (Fig. S13).

Discussion

Drought causes oxidative stress and metabolic and osmotic damage in plants and inhibits cell growth and photosynthesis (Fàbregas and Fernie *et al*., 2019). Plants have evolved complex regulatory hormonal signaling networks to respond and adapt to drought conditions. ABA has emerged as a crucial regulator of the drought response (Li *et al*., 2021). bHLH TFs are involved in regulating ABA signaling to help plants cope with drought stress (Hao *et al*., 2021). *Arabidopsis* plants overexpressing *AtbHLH68* displayed significantly increased tolerance to drought stress, likely due to enhanced sensitivity to ABA and increased ABA contents (Le *et al*., 2017). Overexpression of the bHLH TF gene *ZmPTF1* in maize activated ABA-mediated stress responses, thereby increasing drought tolerance (Li *et al*., 2019). Heterologous expression of *Myrothamnus flabellifolia bHLH38* in *Arabidopsis* improved drought tolerance and increased stomatal closure in response to mannitol and ABA (Qiu *et al*., 2020). Overexpressing *AhHLH112* improved drought tolerance in peanut, along with increased ABA accumulation (Li *et al*., 2021). However, the biological functions and regulatory mechanisms of bHLH proteins in sweet potato remain unclear.

In this study, we showed that IbbHLH118 forms homodimers with itself and heterodimers with IbbHLH66. These two proteins play different roles in the ABA-mediated drought response. ABA treatment repressed *IbbHLH118* expression but significantly induced *IbbHLH66* expression in the drought-tolerant sweet potato line Xushu55-2 (Fig. 1a-c; 4g, h). Overexpressing *IbbHLH118* reduced drought tolerance, whereas overexpressing *IbbHLH66* enhanced drought tolerance in sweet potato (Fig. 2; 5). In *Arabidopsis*, *AtbHLH66* was involved in root development by regulating root epidermis growth (Lin *et al*., 2015), and *AtbHLH118* was involved in cell division orientation during vascular development (Smet, 2018).

Drought triggers ABA accumulation in plant tissues. The accumulated ABA is sensed by PYL proteins to initiate the ABA signaling cascade, promoting the expression of key ABA-responsive factors such as ABIs and ABFs, which regulate the ABA response, leading to drought tolerance (Daszkowska-Golec 2016). *AtPYL8*-overexpressing *Arabidopsis* plants

were hypersensitive to ABA and exhibited high degrees of stomatal closure in response to ABA (Lim *et al.*, 2013). In date palm (*Phoenix dactylifera*), the PdPYL8-like receptor Pd27 accumulated after ABA treatment, and *Pd27*-overexpressing plants were more efficient than the WT in reducing transpiration under a negative soil water potential, leading to enhanced drought tolerance (Garcia-Maquilon *et al.*, 2021). *HvABI5* is involved in the ABA-dependent drought response in barley (Collin *et al.*, 2021). Overexpressing *AtABF2* altered ABA sensitivity, dehydration tolerance, and the expression levels of ABA-regulated genes in *Arabidopsis* (Kim *et al.*, 2004).

In the current study, IbbHLH66, which positively regulates the drought response, did not directly target the ABA-responsive genes *IbABI5* and *IbABF2* (Fig. 8d-j). By contrast, IbbHLH118, which negatively regulates the drought response, directly bound to the E-box elements in the promoters of these two genes, repressing their transcription (Fig. 8d-j). We propose that in sweet potato, IbbHLH118 forms homodimers with itself or heterodimers with IbbHLH66 (Fig. 4a-e), and IbbHLH66 suppresses the inhibitory activity of IbbHLH118 (Fig. 8e, f, i, j). In addition, IbPYL8, a positive regulator to drought stress (Fig. S9), interacts with IbbHLH66 and IbbHLH118 to form the IbPYL8-IbbHLH66-IbbHLH118 complex (Fig. 7a-f). Under drought stress, accumulated ABA promotes and enhances the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, interfering with IbbHLH118's repression of *IbABI5* and *IbABF2*, thereby promoting ABA signaling and drought tolerance (Fig. 7a, e-g; 8e, f; 10).

Accumulating evidence indicates that bHLH TFs usually function as binary or ternary complexes that bind to target DNA (Zhang *et al.*, 2021). The bHLH proteins MyoD, SREBP-2, and Max form homodimers and function in transcriptional regulation (Ma *et al.*, 1994; Parraga *et al.*, 1998; Grandori *et al.*, 2000). The bHLH TFs MYC2, MYC3 (bHLH5), and MYC4 (bHLH4) form homodimers and bind to the G-boxes in the promoters of genes in the JA signaling pathway (Fernández-Calvo *et al.*, 2011; Schweizer *et al.*, 2013). Several bHLHs were reported to form heterodimers with other proteins (Heim *et al.*, 2003). In *Arabidopsis*, MYC3 interacts with Jasmonate ZIM-domain proteins (JAZs) to mediate JA responses (Cheng *et al.*, 2011). In blueberry, the MYB-bHLH-WD40 regulatory complex controls anthocyanidin biosynthesis during fruit development (An *et al.*, 2012). In

Artemisia annua, AabHLH1 interacts with AaMYB3 to regulate the accumulation of procyanidine (Li *et al*., 2019). In *Arabidopsis*, AtbHLH104 interacts with another bHLH protein, IAA-LEUCINE RESISTANT3 (ILR3), to modulate iron homeostasis (Zhang *et al*., 2015). Here, we demonstrated that IbbHLH118 forms homodimers, but IbbHLH66 does not (Fig. 4a, c). Both IbbHLH118 and IbbHLH66 form heterodimers with IbPYL8 and play important roles in regulating the ABA-mediated drought response (Fig. 7; 10).

The IbPYL8-IbbHLH66-IbbHLH118 complex is also involved in the induction of other ABA-responsive genes in sweet potato under drought conditions. Our study showed that IbbHLH118 directly bound to the E-box element in the *IbTIP1* promoter to inhibit its expression (Fig. 9c-g). Under drought stress, ABA promotes the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, which targets the ABA-responsive gene *IbTIP1* and activates its expression (Fig. S14a). In plants, aquaporins play vital roles in cellular water and osmotic homeostasis under both normal and water deficit conditions (Ding *et al*., 2016; Kayum *et al*., 2017). Aquaporin genes are usually induced or suppressed by ABA in plants, and involved in regulating water efflux and stomatal closure (Zhu *et al*., 2005; Guo *et al*., 2006; Maurel *et al*., 2021). In *Eucalyptus grandis*, *EgTIP2* promoter activity was induced by mannitol treatment (Rodrigues *et al*., 2013). *HvTIP1;1* and *HvTIP1;2* play important roles in the adaptation of barley to drought stress conditions (Kurowska *et al*., 2019). However, the functions and regulatory mechanisms of most *TIPs* in plants are still unclear. Here, we showed that *IbTIP1* was highly expressed in the leaves and stems of the drought-tolerant sweet potato line Xushu55-2 and was significantly induced by PEG, ABA, and H_2O_2 treatment (Fig. S14a). Overexpressing *IbTIP1* reduced membrane damage and enhanced ABA-mediated drought tolerance in sweet potato (Fig. 9h-j; S16a-d).

To adapt to harsh environments, plants have evolved elaborate mechanisms involving the stress-responsive phytohormones ABA and JA (Peleg & Blumwald, 2011), the ROS scavengers PODs and SODs (Li *et al*., 2015), and the osmoprotectant proline (Kavi & Sreenivasulu, 2014). Under drought stress, the ABA contents, SOD activity, proline contents, photosynthetic rate, stomatal conductance, and transpiration rate were higher, whereas H_2O_2 and MDA contents were lower in *IbbHLH66*-OE plants compared to the WT (Fig. 6). In addition, the leaves of *IbbHLH66*-OE plants were more sensitive than the WT

to ABA-induced changes in stomatal aperture (Fig. 6d, e); *IbbHLH118*-OE plants showed the opposite patterns (Fig. 3). These data indicate that IbbHLH66 and IbbHLH118 have opposite regulatory effects on the physiological responses of sweet potato plants to drought stress, with IbbHLH66 functioning as a positive regulator and IbbHLH118 functioning as a negative regulator of these responses (Fig. 10).

In summary, we elucidated the regulatory mechanism underlying the role of the IbPYL8-IbbHLH66-IbbHLH118 complex in sweet potato's response to drought stress. Under drought, accumulated ABA is sensed by IbPYL8 and promotes the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, which relieves IbbHLH118's repression of ABA-responsive genes, such as *IbABI5*, *IbABF2*, and *IbTIP1*, thereby promoting ABA signaling and drought tolerance. Our study provides insights into the roles of bHLH TFs in regulating ABA and drought responses in plants.

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Author contributions

QL, LX, HZhang, and HZhai conceived and designed the research. LX, ZW, SX, and YW performed the experiments. ZW, LX, HZhang, SH, SG, and NZ analyzed the data. LX and ZW wrote the paper. LX, HZhang, and QL revised the paper. All authors read and approved the final version of the paper. LX and ZW contributed equally to this work.

Data availability

The data that support the findings of this study are available in the Supporting Information of this article.

References

An XH, Tian Y, Chen KQ, Wang XF, Hao YJ. 2012. The apple WD40 protein MdTTG1

interacts with bHLH but not MYB proteins to regulate anthocyanin accumulation. *Journal of Plant Physiology* **169**: 710-717.

Armstrong GA, Weisshaar B, Hahlbrock K. 1992. Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. *The Plant Cell* **4**: 525-537.

Arisha MH, Ahmad MQ, Tang W, Liu Y, Yan H, Kou M, Wang X, Zhang Y, Li Q. 2020. RNA-sequencing analysis revealed genes associated drought stress responses of different durations in hexaploid sweet potato. *Scientific Reports* **10**: 1-17.

Assmann SM, Jegla T. 2016. Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO_2. *Current Opinion in Plant Biology* **33**: 157–167.

Atchley WR, Fitch WM. 1997. A natural classification of the basic helix–loop–helix class of transcription factors. *Proceedings of the National Academy of Sciences, USA* **94**: 5172-5176.

Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Kohler B, Mueller-Roeber B. 2010. A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. *The Plant Journal* **62**: 250-264.

Belda-Palazon B, Gonzalez-Garcia MP, Lozano-Juste J, Coego A, Antoni R, Julian J, Peirats-Llobet M, Rodriguez L, Berbel A, Dietrich D *et al*. 2018. PYL8 mediates ABA perception in the root through non-cell-autonomous and ligand-stabilization–based mechanisms. *Proceedings of the National Academy of Sciences, USA* **115**: E11857-E11863.

Bi H, Zhang P. 2014. Agroinfection of sweet potato by vacuum infiltration of an infectious sweepovirus. *Virologica Sinica* **29**: 148-154.

Chen H, Zou Y, Shang Y, Lin H, Wang Y, Cai R, Tang X, Zhou J. 2008. Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiology* **146**: 368.

Chen Q, Sun J, Zhai Q, Zhou W, Qi L, Xu L, Wang B, Chen R, Jiang H, Qi J *et al.* 2011. The basic helix-loop-helix transcription factor MYC2 directly

represses *plethora* expression during jasmonate-mediated modulation of the root stem cell niche in *Arabidopsis*. *The Plant Cell* **23**: 3335-3352.

Cheng Z, Sun L, Qi T, Zhang B, Peng W, Liu Y, Xie D. 2011. The bHLH transcription factor MYC3 interacts with the jasmonate ZIM-domain proteins to mediate jasmonate response in *Arabidopsis*. *Molecular Plant* **4:** 279-288.

Collin A, Daszkowska-Golec A, Szarejko I. 2021. Updates on the role of abscisic acid insensitive 5 (ABI5) and abscisic acid-responsive element binding factors (ABFs) in ABA signaling in different developmental stages in plants. *Cells* **10**: 1996.

Danquah A, de Zelicourt A, Colcombet J, Hirt H. 2014. The role of ABA and MAPK signaling pathways in plant abiotic stress responses. *Biotechnology Advances* **32**: 40-52.

Daszkowska-Golec A. 2016. The role of abscisic acid in drought stress: how ABA helps plants to cope with drought stress. *Drought Stress Tolerance in Plants* **2016**: 123-151.

Dennis DJ, Han S, Schuurmans C. 2019. bHLH transcription factors in neural development, disease, and reprogramming. *Brain Research* **1705**: 48-65.

Ding L, Li Y, Wang Y, Gao L, Wang M, Chaumont F, Shen Q, Guo S. 2016. Root ABA accumulation enhances rice seedling drought tolerance under ammonium supply: interaction with aquaporins. *Frontiers in Plant Science* **7**: 1206.

Fàbregas N, Fernie AR. 2019. The metabolic response to drought. *Journal of Experimental Botany* **70**: 1077-1085.

Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM *et al*. 2011. The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *The Plant Cell* **23**: 701-715.

Foyer CH. 2018. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environmental and Experimental Botany* **154**: 134-142.

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion*

in Plant Biology **9**: 436–442.

Garcia-Maquilon I, Coego A, Lozano-Juste J, Messerer M, de Ollas C, Julian J, Ruiz-Partida R, Pizzio G, Belda-Palazón B, Gomez-Cadenas A *et al*. 2021. PYL8 ABA receptors of *Phoenix dactylifera* play a crucial role in response to abiotic stress and are stabilized by ABA. *Journal of Experimental Botany* **72**: 757-774.

Ghosh UK, Islam MN, Siddiqui MN, Cao X, Khan MAR. 2022. Proline, a multifaceted signalling molecule in plant responses to abiotic stress: understanding the physiological mechanisms. *Plant Biology* **24**: 227-239.

Grandori C, Cowley SM, James LP, Eisenman RN. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annual Review of Cell and Developmental Biology* **16**: 653-699.

Groszmann M, Bylstra Y, Lampugnani ER, Smyth DR. 2010. Regulation of tissue-specific expression of *SPATULA*, a bHLH gene involved in carpel development, seedling germination, and lateral organ growth in *Arabidopsis*. *Journal of Experimental Botany* **61**: 1495-1508.

Guan Q, Wu J, Yue X, Zhang Y, Zhu J. 2013. A nuclear calcium-sensing pathway is critical for gene regulation and salt stress tolerance in *Arabidopsis*. *PLoS Genetics* **9**: e1003755.

Guo L, Wang ZY, Lin H, Cui WE, Chen J, Li M, Chen Zl, Qu LJ, Gu H. 2006. Expression and functional analysis of the rice plasma-membrane intrinsic protein gene family. *Cell Research* **16**: 277-286.

Hao Y, Zong X, Ren P, Qian Y, & Fu A. 2021. Basic helix-loop-helix (bHLH) transcription factors regulate a wide range of functions in *Arabidopsis*. *International Journal of Molecular Sciences* **22**: 7152.

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC. 2003. The basic helix–loop–helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Molecular Biology and Evolution* **20**: 735-747.

Horn T, Boutros M. 2010. E-RNAi: a web application for the multi-species design of RNAi reagents—2010 update. *Nucleic Acids Research* **38**: W332-W339.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. 1985. A simple

and general method for transferring genes into plants. *Science* **227**: 1229–1231.

Ito S, Song YH, Josephson-Day AR, Miller RJ, Breton G, Olmstead RG, Imaizumi T. 2012. FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator *constans* in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **109**: 3582-3587.

Jata SK, Nedunchezhian M, Misra SR. 2011. The triple 'f' (food, fodder and fuel) crop sweet potato [*Ipomoea batatas* (L.) Lam.]. *Orissa Review* **5**: 82-92.

Jefferson RA. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5**: 387-405.

Kaldenhoff R, Ribas-Carbo M, Sans JF, Lovisolo C, Heckwolf M, Uehlein N. 2008. Aquaporins and plant water balance. *Plant, Cell and Environment* **31**: 658-666.

Kang, C, He S, Zhai H, Li R, Zhao N, Liu Q. 2018. A sweetpotato auxin response factor gene (*IbARF5*) is involved in carotenoid biosynthesis and salt and drought tolerance in transgenic *Arabidopsis*. *Frontiers in Plant Science* **9**: 1307.

Karkute SG, Gujjar RS, Rai A, Akhtar M, Singh M, Singh B. 2018. Genome wide expression analysis of WRKY genes in tomato (*Solanum lycopersicum*) under drought stress. *Plant Gene* **13**: 8-17.

Kavi Kishor PB, & Sreenivasulu N. 2014. Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? *Plant, Cell and Environment* **37**: 300-311.

Kayum M, Park JI, Nath UK, Biswas MK, Kim HT, Nou IS. 2017. Genome-wide expression profiling of aquaporin genes confer responses to abiotic and biotic stresses in Brassica rapa. *BMC Plant Biology* **17**: 1-18.

Kim S, Kang JY, Cho DI, Park JH, Kim SY. 2004. ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *The Plant Journal* **40**: 75-87.

Kumar SV, Lucyshyn D, Jaeger KE, Alós E, Alvey E, Harberd NP, Wigge PA. 2012. Transcription factor PIF4 controls the thermosensory activation of flowering. *Nature* **48**: 242-245.

Kurowska MM, Wiecha K, Gajek K, Szarejko I. 2019. Drought stress and re-watering

affect the abundance of TIP aquaporin transcripts in barley. *PloS One* **14**: e0226423.

Lau KH, del Rosario Herrera M, Crisovan E, Wu S, Fei Z, Khan MA, Robin Buell C, Gemenet DC. 2018. Transcriptomic analysis of sweet potato under dehydration stress identifies candidate genes for drought tolerance. *Plant Direct* **2**: e00092.

Le Hir, Castelain M, Chakraborti D, Moritz T, Dinant S, Bellini C. 2017. At*bHLH68* transcription factor contributes to the regulation of ABA homeostasis and drought stress tolerance in *Arabidopsis thaliana*. *Physiologia Plantarum* **160**: 312-327.

Li C, Qiu J, Huang S, Yin J, Yang G. 2019. AaMYB3 interacts with AabHLH1 to regulate proanthocyanidin accumulation in *Anthurium andraeanum* (Hort.)—another strategy to modulate pigmentation. *Horticulture Research* ***6***: 14.

Li C, Yan C, Sun Q, Wang J, Yuan C, Mou Y, Shan S, Zhao X. 2021. The bHLH transcription factor *AhbHLH112* improves the drought tolerance of peanut. *BMC Plant Biology* **21**: 1-12.

Li JB, Luan YS, Liu Z. 2015. *SpWRKY1* mediates resistance to *Phytophthora infestans* and tolerance to salt and drought stress by modulating reactive oxygen species homeostasis and expression of defense-related genes in tomato. *Plant Cell, Tissue and Organ Culture* **123**: 67-81.

Li J, Li X, Han P, Liu H, Gong J, Zhou W, Shi B, Liu A, Xu L. 2021. Genome-wide investigation of *bHLH* genes and expression analysis under different biotic and abiotic stresses in *Helianthus annuus* L. *International Journal of Biological Macromolecules* **189**: 72-83.

Li L, Hao X, Liu H, Wang W, Fu X, Ma Y, Shen Q, Chen M, Tang K. 2019. Jasmonic acid-responsive AabHLH1 positively regulates artemisinin biosynthesis in *Artemisia annua*. *Biotechnology and Applied Biochemistry* **66**: 369-375.

Li Q, Xu F, Chen Z, Teng Z, Sun K, Li X, Yu J, Zhang G, Liang Y, Huang X *et al*. 2021. Synergistic interplay of ABA and BR signal in regulating plant growth and adaptation. *Nature Plants* **7**: 1108-1118.

Li Z, Liu C, Zhang Y, Wang B, Ran Q, Zhang J. 2019. The bHLH family member ZmPTF1 regulates drought tolerance in maize by promoting root development and

abscisic acid synthesis. *Journal of Experimental Botany* **70**: 5471-5486.

Lim CW, Baek W, Han SW, Lee SC. 2013. *Arabidopsis* PYL8 plays an important role for ABA signaling and drought stress responses. *The Plant Pathology Journal* **29**: 471.

Lin Q, Ohashi Y, Kato M, Tsuge T, Gu H, Qu LJ, Aoyama T. 2015. GLABRA2 directly suppresses basic helix-loop-helix transcription factor genes with diverse functions in root hair development. *The Plant Cell* **27**: 2894-2906.

Liu Q, Zhai H, Wang Y, Zhang D. 2001. Efficient plant regeneration from embryogenic suspension cultures of sweetpotato. *In Vitro Cellular & Developmental Biology - Plant* **37**: 564–567.

Liu W, Tai H, Li S, Gao W, Zhao M, Xie C, Li WX. 2014. *bHLH122* is important for drought and osmotic stress resistance in *Arabidopsis* and in the repression of ABA catabolism. *New Phytologist* **201**: 1192-1204.

Liu Y, Ji X, Nie X, Qu M, Zheng L, Tan Z, Zhang H, Huo L, Liu S, Zhang B *et al.* 2015. *Arabidopsis* AtbHLH112 regulates the expression of genes involved in abiotic stress tolerance by binding to their E-box and GCG-box motifs. *New Phytologist* **207**: 692-709.

Liu Y, Ma K, Qi Y, Lv G, Ren X, Liu Z, Ma F. 2021. Transcriptional regulation of anthocyanin synthesis by MYB-bHLH-WDR complexes in Kiwifruit (*Actinidia chinensis*). *Journal of Agricultural and Food Chemistry* **69**: 3677-3691.

Ma PC, Rould MA, Weintraub H, Pabo CO. 1994. Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* **77**: 451-459.

Martínez-García JF, Huq E, Quail PH. 2000. Direct targeting of light signals to a promoter element-bound transcription factor. *Science* **288**: 859-863.

Maurel C, Tournaire-Roux C, Verdoucq L, Santoni V. 2021. Hormonal and environmental signaling pathways target membrane water transport. *Plant Physiology* **187**: 2056-2070.

Mbinda W, Ombori O, Dixelius C, Oduor R. 2018. *Xerophyta viscosa* aldose reductase, XvAld1, enhances drought tolerance in transgenic sweetpotato. *Molecular Biotechnology* **60**: 203-214.

Mehrotra R, Sethi S, Zutshi I, Bhalothia P, Mehrotra S. 2013. Patterns and evolution of ACGT repeat cis-element landscape across four plant genomes. *BMC Genomics* **14**: 1-11.

Menand B, Yi K, Jouannic S, Hoffmann L, Ryan E, Linstead P, G. Schaefer D, Dolan L. 2007. An ancient mechanism controls the development of cells with a rooting function in land plants. *Science* **316**: 1477-1480.

Motsa NM, Modi AT, Mabhaudhi T. 2015. Sweet potato *(Ipomoea batatas* L.) as a drought tolerant and food security crop. *South African Journal of Science* **111**: 1-8.

Munemasa S, Hauser F, Park J, Waadt R, Brandt B, Schroeder, JI. 2015. Mechanisms of abscisic acid-mediated control of stomatal aperture. *Current Opinion in Plant Biology* **28**: 154-162.

Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung WI, Choi G. 2006. Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in *Arabidopsis*. *The Plant Journal* **47**: 124-139.

Parraga A, Bellsolell L, Ferre-D'Amare AR, Burley SK. 1998. Co-crystal structure of sterol regulatory element binding protein 1a at 2.3 Å resolution. *Structure* **6**: 661-672.

Peleg Z, Blumwald E. 2011. Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology* **14**: 290-295.

Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA. 2005. Cold and light control seed germination through the bHLH transcription factor SPATULA. *Current Biology* **15**: 1998-2006.

Pires N, Dolan L. 2010. Origin and diversification of basic-helix-loop-helix proteins in plants. *Molecular Biology and Evolution* **27**: 862-874.

Qi T, Huang H, Song S, Xie D. 2015. Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in *Arabidopsis*. *The Plant Cell* **27**: 1620-1633.

Qiu JR, Huang Z, Xiang XY, Xu WX, Wang JT, Chen J, Song L, XiaoY, Li X, Ma J *et al*. 2020. MfbHLH38, a *Myrothamnus flabellifolia* bHLH transcription factor, confers tolerance to drought and salinity stresses in *Arabidopsis*. *BMC Plant Biology* **20**: 1-14.

Rodrigues MI, Bravo JP, Sassaki FT, Severino FE, Maia IG. 2013. The tonoplast intrinsic aquaporin (TIP) subfamily of *Eucalyptus grandis*: Characterization of *EgTIP2*, a root-specific and osmotic stress-responsive gene. *Plant Science* **213**: 106-113.

Schweizer F, Fernández-Calvo P, Zander M, Diez-Diaz M, Fonseca S, Glauser G, G. Lewsey M, R. Ecker J, Solano R, Reymond P. 2013. *Arabidopsis* basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. *The Plant Cell* **25**: 3117-3132.

Sharma P, Jha AB, Dubey RS, Pessarakli M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany* **2012**: 2012.

Sharma N, Xin R, Kim DH, Sung S, Lange T, Huq E. 2016. No flowering in short day (NFL) is a bHLH transcription factor that promotes flowering specifically under short-day conditions in *Arabidopsis*. *Development* **143**: 682-690.

Smet WMS. 2018. Control of cell division orientation during vascular development in *Arabidopsis thaliana* (Doctoral dissertation, Wageningen University and Research, Wageningen, the Netherlands).

Sonal M, Aparna S, Swati U, Sanchita, Pooja S, Seema S, Ujjal J, Phukan, Abha M, Feroz K *et al*. 2014. *Retracted*: identification, occurrence, and validation of DRE and ABRE *cis*-regulatory motifs in the promoter regions of genes of *Arabidopsis thaliana*. *Journal of Integrative Plant Biology* **4**: 388-398.

Sun L, Wang YP, Pei C, Ren J, Ji K, Qian L, Ping L, Dai S-J, Ping L. 2011. Transcriptional regulation of *SlPYL*, *SlPP2C*, and *SlSnRK2* gene families encoding ABA signal core components during tomato fruit development and drought stress. *Journal of Experimental Botany* **62**: 5659-5669.

Tamura K, Stecher G, Kumar S. 2021. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution* **7**: 38.

Tanabe N, Noshi M, Mori D, Nozawa K, Tamoi M, Shigeoka S. 2019. The basic helix-loop-helix transcription factor, bHLH11 functions in the iron-uptake system in *Arabidopsis thaliana*. *Journal of Plant Research* **132**: 93-105.

Tian S, Li L, Wei M, Yang F. 2019. Genome-wide analysis of basic helix–loop–helix superfamily members related to anthocyanin biosynthesis in eggplant (*Solanum melongena* L.). *PeerJ* **7**: e7768.

Toledo-Ortiz G, Huq E, Quail PH. 2003. The *Arabidopsis* basic/helix-loop-helix transcription factor family. *The Plant Cell* **15**: 1749-1770.

Tuteja N. 2007. Abscisic acid and abiotic stress signaling. *Plant Signaling & Behavior* **2**: 135–138.

Varaud E, Brioudes F, Szécsi J, Leroux J, Brown S, Perrot-Rechenmann C, Bendahmane M. 2011. Auxin response factor 8 regulates *Arabidopsis* petal growth by interacting with the bHLH transcription factor BIGPETALp. *The Plant Cell* **23**: 973-983.

Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oecking C *et al*. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *The Plant Journal* **40**: 428–438.

Wang H, Li Y, Pan J, Lou D, Hu Y, Yu D. 2017. The bHLH transcription factors MYC2, MYC3, and MYC4 are required for jasmonate-mediated inhibition of flowering in *Arabidopsis*. *Molecular Plant* **10**: 1461-1464.

Wu C, Zheng CY, Ji GS, Jiang P. 2019. Synergistic effects of HSE and LTR elements from *hsp70* gene promoter of *Ulva prolifera* (Ulvophyceae, Chlorophyta) upon temperature induction. *Journal of Phycology* **3**: 55.

Xiong L, Zhu JK. 2003. Regulation of abscisic acid biosynthesis. *Plant Physiology* **133**: 29–36.

Yan HX, Fu DQ, Zhu BZ, Liu HP, Shen XY, Luo YB. 2012. Sprout vacuum-infiltration: a simple and efficient agroinoculation method for virus-induced gene silencing in diverse solanaceous species. *Plant Cell Reports* **31**: 1713-1722.

Yi K, Menand B, Bell E, Dolan L. 2010. A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nature Genetics* **42**: 264-267.

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling HQ. 2008. FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron

homeostasis in *Arabidopsis*. *Cell Research* **18**: 385-397.

Zhai H, Wang F, Si Z, Huo J, Xing L, An Y, He S, Liu Q. 2016. A *myo*-inositol-1-phosphate synthase gene, *IbMIPS1*, enhances salt and drought tolerance and stem nematode resistance in transgenic sweet potato. *Plant Biotechnology Journal* **14**: 592-602.

Zhang H, Zhang Q, Wang Y, Li Y, Zhai H, Liu Q, He S. 2017. Characterization of salt tolerance and fusarium wilt resistance of a sweetpotato mutant. *Journal of Integrative Agriculture* **16**: 1946-1955.

Zhang H, Gao X, Zhi Y, Li X, Zhang Q, Niu J, Wang J, Zhai H, Zhao N, Li J *et al.* 2019. A non-tandem CCCH-type zinc-finger protein, IbC3H18, functions as a nuclear transcriptional activator and enhances abiotic stress tolerance in sweet potato. *New Phytologist* **223**: 1918-1936.

Zhang H, Zhang Q, Zhai H, Gao S, Yang L, Wang Z, Xu Y, Huo J, Ren Z, Zhao N *et al*. 2020. IbBBX24 promotes the jasmonic acid pathway and enhances fusarium wilt resistance in sweet potato. *The Plant Cell* **32**: 1102-1123.

Zhang H, Wang Z, Li X, Gao X, Dai Z, Cui Y, Zhi Y, Liu Q, Zhai H, Gao S *et al*. 2022. The IbBBX24–IbTOE3–IbPRX17 module enhances abiotic stress tolerance by scavenging reactive oxygen species in sweet potato. *New Phytologist* **233**: 1133-1152.

Zhang Y, Mitsuda N, Yoshizumi T, Horii Y, Oshima Y, Ohme-Takagi M, Matsui M, Kakimoto T. 2021. Two types of bHLH transcription factor determine the competence of the pericycle for lateral root initiation. *Nature Plants* **7**: 633-643.

Zhang J, Liu B, Li M, Feng D, Jin H, Wang P, Liu J, Xiong F, Wang J, Wang HB. 2015. The bHLH transcription factor bHLH104 interacts with IAA-leucine resistant 3 and modulates iron homeostasis in *Arabidopsis*. *The Plant Cell* **27**: 787-805.

Zhao M, Li J, Zhu L, Chang P, Li L, Zhang L. 2019. Identification and characterization of MYB-bHLH-WD40 regulatory complex members controlling anthocyanidin biosynthesis in blueberry fruits development. *Genes* **10**: 496.

Zhu C, Schraut D, Hartung W, Schäffner AR. 2005. Differential responses of maize *MIP* genes to salt stress and ABA. *Journal of Experimental Botany* **56**: 2971-2981.

Zhu H, Zhou Y, Zhai H, He S, Zhao N, Liu Q. 2019. Transcriptome profiling reveals

insights into the molecular mechanism of drought tolerance in sweetpotato. *Journal of Integrative Agriculture* **1**: 9-23.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Tissue-specific expression, the promoter characterization and transcriptional activation assays of IbbHLH118.

Fig. S2 Production of *IbbHLH118* transgenic sweet potato plants.

Fig. S3 Detailed time-course analysis of *IbbHLH118* transgenic and WT sweet potato plants grown in transplanting boxes without stress (Normal) or subjected to drought stress for the indicated times (weeks).

Fig. S4 Sequence, expression, and genomic structure analysis of *IbbHLH66* in sweet potato and other plants.

Fig. S5 Production of *IbbHLH66*-OE transgenic sweet potato plants.

Fig. S6 Detailed time-course analysis of *IbbHLH66* transgenic and WT sweet potato plants grown in Hoagland solution with or without 30% PEG for the indicated times (weeks).

Fig. S7 Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) of *IbbHLH66* in the drought-sensitive sweet potato variety 'Lizixiang' reduces drought tolerance of sweet potato.

Fig. S8 Y2H analysis, expression analysis, and subcellular localization of IbPYL8.

Fig. S9 *IbPYL8* overexpression enhances drought tolerance in transgenic tobacco plants.

Fig. S10 Relative expression of *IbbHLH118, IbbHLH66, IbNCED3*, and *IbNCED5* in *IbbHLH118* and *IbbHLH66* transgenic and WT sweet potato plants.

Fig. S11 TRV-based VIGS of *IbABI5* and *IbABF2* in the drought-sensitive sweet potato variety 'Lizixiang' reduce drought tolerance of sweet potato.

Figure Legends

Fig. 1 Expression analysis, sequence analysis, and subcellular localization of IbbHLH118. (a-c) Expression analysis of *IbbHLH118* in 4-week-old *in vitro*–grown sweet potato line Xushu55-2 and variety Lizixiang upon exposure to 20% PEG, 100 μM abscisic acid (ABA), or 200 mM H_2O_2 over a 24-h period. The sweet potato *ACTIN* gene was used as a reference. The expression at 0 h in each treatment was considered as "1". Data are shown as mean ± SD (n = 3). (d) Multiple protein sequence alignment of IbbHLH118 and other plant bHLHs, with conserved amino acids shaded in different colors. The entire black line represents the conserved bHLH domain. (e) Phylogenetic analysis of bHLH proteins from *I. batatas* (IbbHLH118) and other plants using the neighbor-joining method in MEGA6.0 with 1000 bootstrap iterations. The numbers at the nodes of the tree indicate bootstrap values from 1000 replicates. (f) Comparison of the genomic structures of *IbbHLH118* and other plant *bHLHs*. Boxes indicate exons, and lines indicate introns. (g) Subcellular localization of IbbHLH118. *N. benthamiana* leaf epidermal cells were transformed with the fusion construct (IbbHLH118-GFP) and the membrane marker PIP2-mCherry. Bars = 20 μm.

Fig. 2 Knockdown of *IbbHLH118* enhances drought tolerance in sweet potato. (a, b) Responses and plant weight of *IbbHLH118* transgenic and wild-type (WT) sweet potato plants grown for 4 weeks on MS medium under normal condition or subjected to 30% PEG. Bars = 10 cm. FW, fresh weight; DW, dry weight. (c-e) Responses and plant weight of 2-month-old field-grown *IbbHLH118*-OE and WT sweet potato plants grown in transplanting boxes under normal condition or subjected to drought stress for 4 weeks. Bars = 10 cm. (f-h) Responses and plant weight of 1-month-old field-grown *IbbHLH118*-RNAi and WT plants grown in a transplanting box under normal condition or subjected to drought stress for 6 weeks. Bars = 10 cm. A time-course of the phenotypes of *IbbHLH118* transgenic and WT plants under normal and drought conditions are shown in Supporting Information Fig. S3. All data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test.

Fig. 3 Knockdown of *IbbHLH118* activates abscisic acid (ABA) signaling pathway and reactive oxygen species (ROS) scavenging under drought stress in sweet potato. (a) ABA content in the leaves of 4 weeks *IbbHLH118* transgenic and WT plants under normal condition or subjected to 30% PEG. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test. (b, c) Stomatal apertures of 2-month-old field-grown *IbbHLH118* transgenic and WT plants under normal condition or treated with 20 μM ABA for 2 h. Bar = 10 μm. Data are presented as the means ± SD (n = 80). **, P < 0.01; Student's t-test. (d, e) DAB staining (Bars = 1 cm), (f) H_2O_2 content, (g, h) NBT staining, (i) POD activity, (j) SOD activity, and (k) proline content in leaves of 4-week-old *IbbHLH118* transgenic and WT plants under normal condition or subjected to 30% PEG. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test.

Fig. 4 IbbHLH118 forms homodimers with itself, or forms heterodimers with the drought- and ABA-responsive protein IbbHLH66. (a) Y2H analysis showing that IbbHLH118 interacts with itself or IbbHLH66. aa, amino acid. IbbHLH118$^{1-175}$ contains IbbHLH118 aa residues 1 to 175, and IbbHLH66$^{101-350}$ contains IbbHLH66 aa residues 101 to 350, both without transcriptional activation activity. Yeast cells were plated onto SD/-Ade/-His/-Leu/-Trp + 3 mM 3AT medium to screen for possible interactions. (b) Transcriptional activation assay of IbbHLH66. Fusion proteins between the GAL4 DNA binding domain and different portions of IbbHLH66 were produced in yeast strain Y2H Gold. pGBKT7-Lam was used as a negative control, while pGBKT7-53 was used as a positive control. The positive transformants were streaked onto SD medium –Trp –His –Ade +X-a-gal. (c)

BiFC analysis showing that IbbHLH118 interacts with itself or IbbHLH66 in *N. benthamiana* leaf epidermal cells. Bars = 20 μm. (d, e) Co-IP analysis showing that IbbHLH118 interacts with itself or IbbHLH66 *in vivo*. *, non-specific protein band. (f) Subcellular localization of IbbHLH66. *N. benthamiana* leaf epidermal cells were transformed with the fusion construct (IbbHLH66-GFP) and the membrane marker PIP2-mCherry. Bars = 20 μm. (g) Expression analysis of *IbbHLH66* in 4-week-old *in vitro*–grown Xushu55-2 and Lizixiang upon exposure to 20% PEG over a 12-h period. The sweet potato *ACTIN* gene was used as a reference. The expression at 0 h in each treatment was considered as "1". Data are shown as mean ± SD (n = 3). (h) Expression analysis of *IbbHLH66* in 4-week-old *in vitro*–grown Xushu55-2 upon exposure to 20% PEG, 100 μM ABA, or 200 mM H_2O_2 over a 12-h period. The expression at 0 h in each treatment was considered as "1". Data are shown as mean ± SD (n = 3). **, P < 0.01; Student's t-test.

Fig. 5 Overexpression of *IbbHLH66* enhances drought tolerance in sweet potato. (a, b) Responses and plant weight of *IbbHLH66* transgenic and WT sweet potato plants grown for 4 weeks on MS medium under normal condition or subjected to 30% PEG. Bars = 10 cm. FW, fresh weight; DW, dry weight. (c-e) Responses and plant weight of 2-month-old field-grown *IbbHLH66*-OE and WT sweet potato plants grown hydroponically in half-strength Hoagland solution alone (Normal) or with the addition of 30% PEG6000 for 3 weeks. Bars = 10 cm. (f-h) Responses and plant weight of 2-month-old field-grown *IbbHLH66*-OE and WT sweet potato plants grown in transplanting boxes under normal condition or subjected to drought stress for 6 weeks. Bars = 10 cm. All data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test.

Fig. 6 Overexpression of *IbbHLH66* activates ABA signaling pathway and ROS scavenging under drought stress in sweet potato. (a) Photosynthetic rate, (b) transpiration rate, (c) ABA content in leaves of *IbbHLH66*-OE transgenic and WT plants under normal condition or drought stress for 5 weeks. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test. (d, e) Stomatal apertures of 2-month-old field-grown *IbbHLH66* transgenic and WT plants under normal condition or treated with 20 μM ABA for 2 h. Bar = 10 μm. Data are presented as the means ± SD (n = 80). **, P < 0.01; Student's t-test. (f) H_2O_2 content, (g, h) DAB staining (Bars = 1 cm), (i, j) NBT staining, (k) SOD activity, (l) proline content, and (m) MDA content in leaves of *IbbHLH66* transgenic and WT plants under normal condition or drought stress for 5 weeks. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test.

Fig. 7 Interaction of IbPYL8 with IbbHLH66 or IbbHLH118 *in vitro* and *in vivo*. (a) Y2H analysis showing that IbPYL8 interacts with IbbHLH66 or IbbHLH118. IbbHLH66$^{101\text{-}350}$ contains IbbHLH66 amino acid residues 101 to 350, whereas IbbHLH118$^{1\text{-}175}$ contains IbbHLH118 amino acid residues 1 to 175, both without transcriptional activation activity. Yeast cells were plated onto SD/-Ade/-His/-Leu/-Trp + 3 mM 3AT medium to screen for possible interactions. (b, c) Co-IP analysis showing that IbPYL8 interacts with IbbHLH66 or IbbHLH118 *in vivo*. (d) BiFC analysis showing that IbPYL8 interacts with IbbHLH66 or IbbHLH118 in *N. benthamiana* leaf epidermal cells. Bars = 20 μm. (e) ABA treatment enhanced the interactions of IbPYL8 with IbbHLH66 or IbbHLH118 in yeast. Yeast cells were plated onto SD/-Ade/-His/-Leu/-Trp + 3 mM 3AT + 100 μM ABA medium. (f) LCI assay showing that ABA treatment enhanced the interactions of IbPYL8 with IbbHLH66 or IbbHLH118 in *N. benthamiana*. The N-terminus of LUC was fused to IbPYL8, and the C-terminus of LUC was fused to IbbHLH66 and IbbHLH118, respectively. The LUC activities were detected two days later. For ABA treatment,

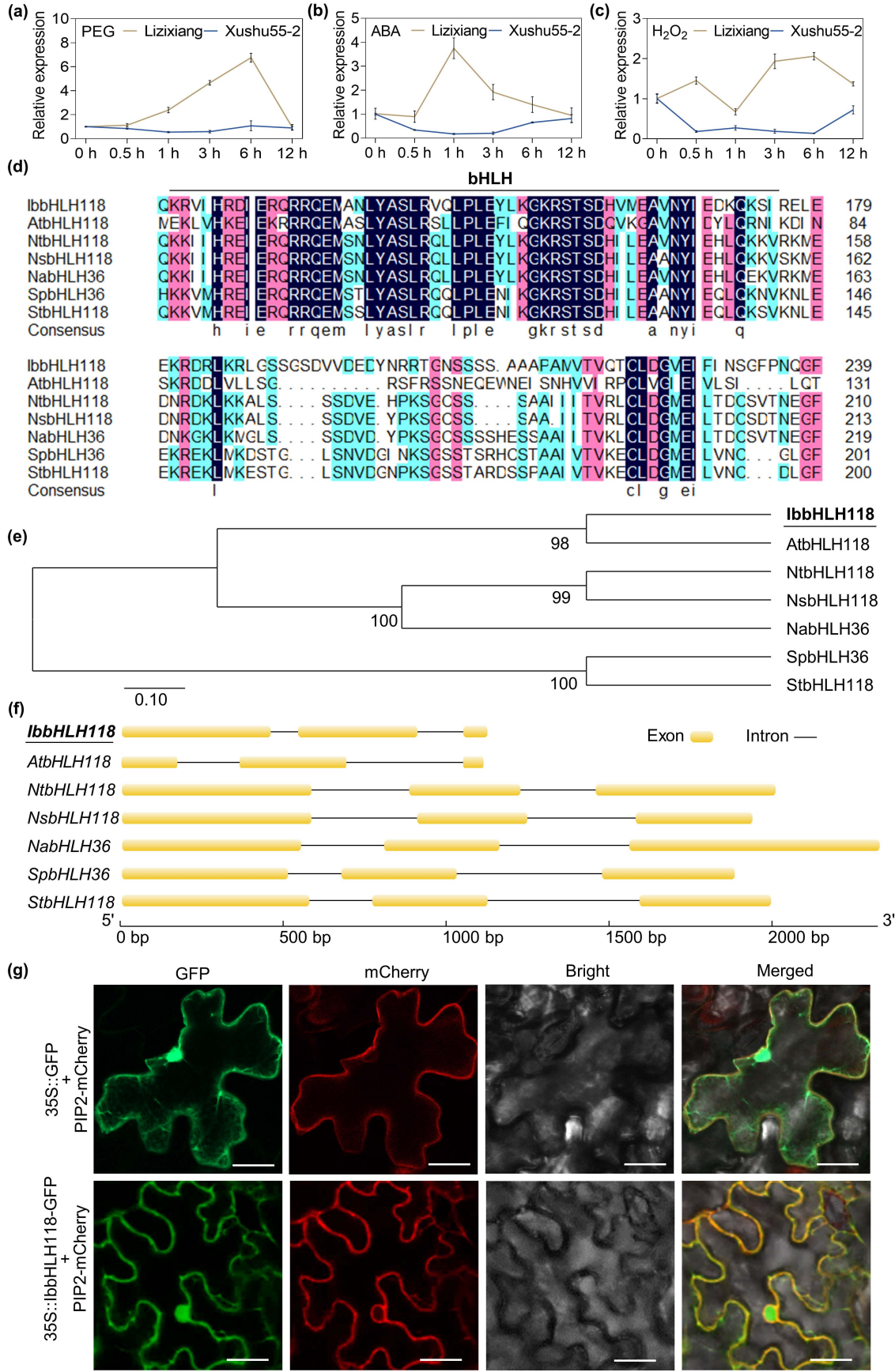
the tobacco leaves were sprayed with 100 μM ABA. The error bars indicate ± SD (n = 3). **, P < 0.01; Student's t-test. (g) Immunoblots showing that ABA induced the accumulation of IbbHLH66 and IbPYL8 protein, but repressed the accumulation of IbbHLH118 protein, under both conditions of alone expression or as components of IbPYL8-IbbHLH66-IbbHLH118 complex. Anti-ACTIN was used as a sample loading control.

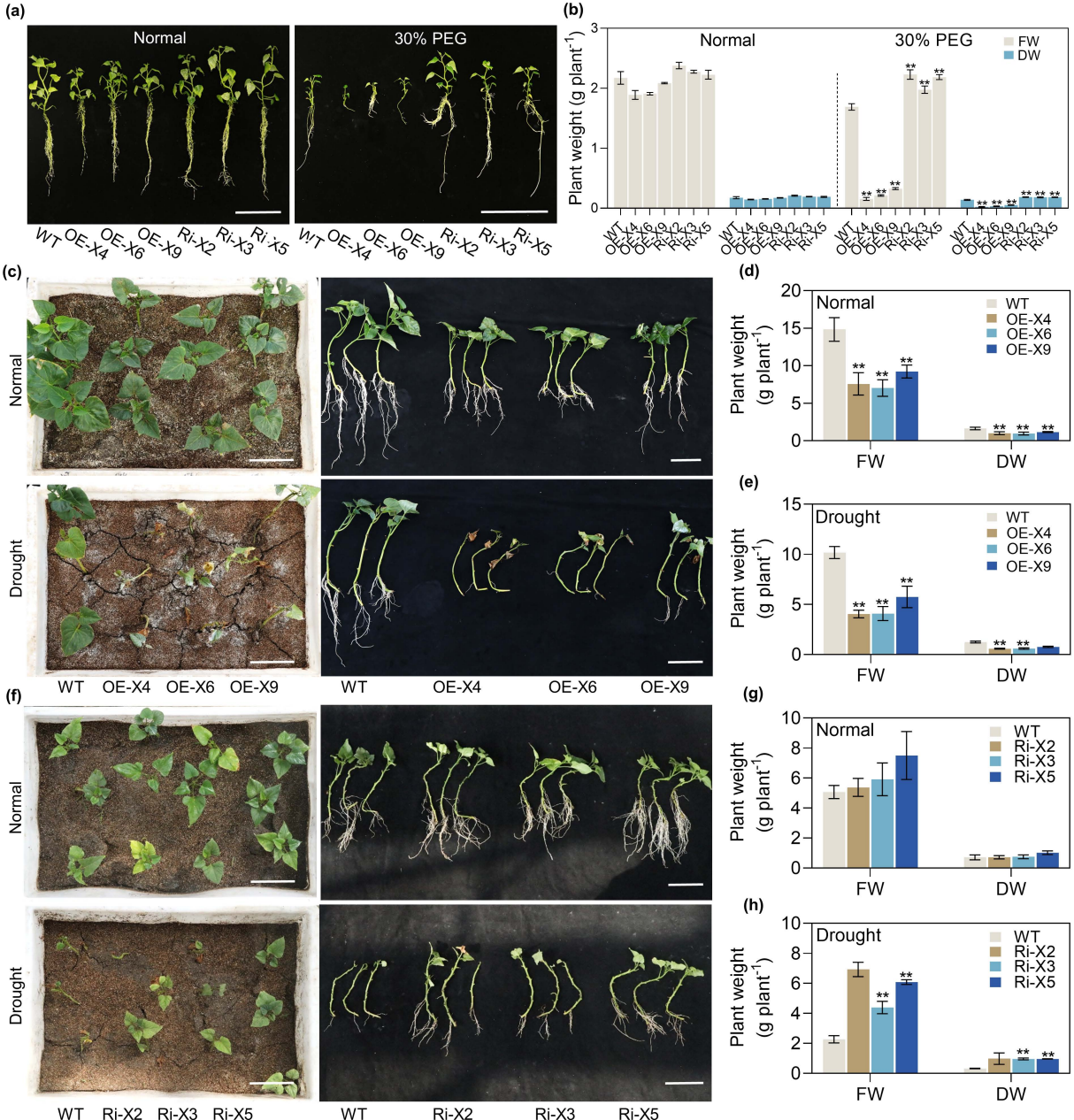
Fig. 8 The IbPYL8-IbbHLH66-IbbHLH118 complex interferes with IbbHLH118's repression of ABA-responsive genes *IbABI5* and *IbABF2* in sweet potato. (a, b) Expression analysis of *IbABI5* and *IbABF2* in 4-week-old *IbbHLH118* and *IbbHLH66* transgenic and WT plants under normal condition or subjected to 30% PEG. The values were determined by RT-qPCR from three biological replicates consisting of pools of three plants. The error bars indicate ± SD (n = 3). **, P < 0.01; Student's t-test. (c, d) Y1H assays showing that IbbHLH118 binding to the *IbABI5* and *IbABF2* promoters. (e, f) Dual-LUC assays showing that IbbHLH118 suppressed the *IbABI5* and *IbABF2* promoters, but the IbPYL8-IbbHLH66-IbbHLH118 complex interferes with IbbHLH118's repression of *IbABI5* and *IbABF2*. Data are shown as mean ± SD (n = 3). Different letters indicate significant differences for each treatment at P < 0.05 based on Student's t-test. (g, h) ChIP-qPCR analysis using 35S:IbbHLH118-GFP, 35S:IbbHLH66-GFP, and 35S:GFP plants with anti-GFP antibody, which showed that IbbHLH118 could directly bind to the *IbABI5* and *IbABF2* promoters, but IbbHLH66 could not. The *ACTIN* promoter was used as an internal reference for ChIP-qPCR. Data are shown as mean ± SD (n = 3). (**) Significant difference from 35S: GFP at P < 0.01 based on Student's t-test. (i, j) EMSA showing that IbbHLH118, but not IbbHLH66, could directly target *IbABI5* and *IbABF2* by binding to E-boxes in their promoters. The addition of IbbHLH66 inhibited the DNA binding activity of IbbHLH118.

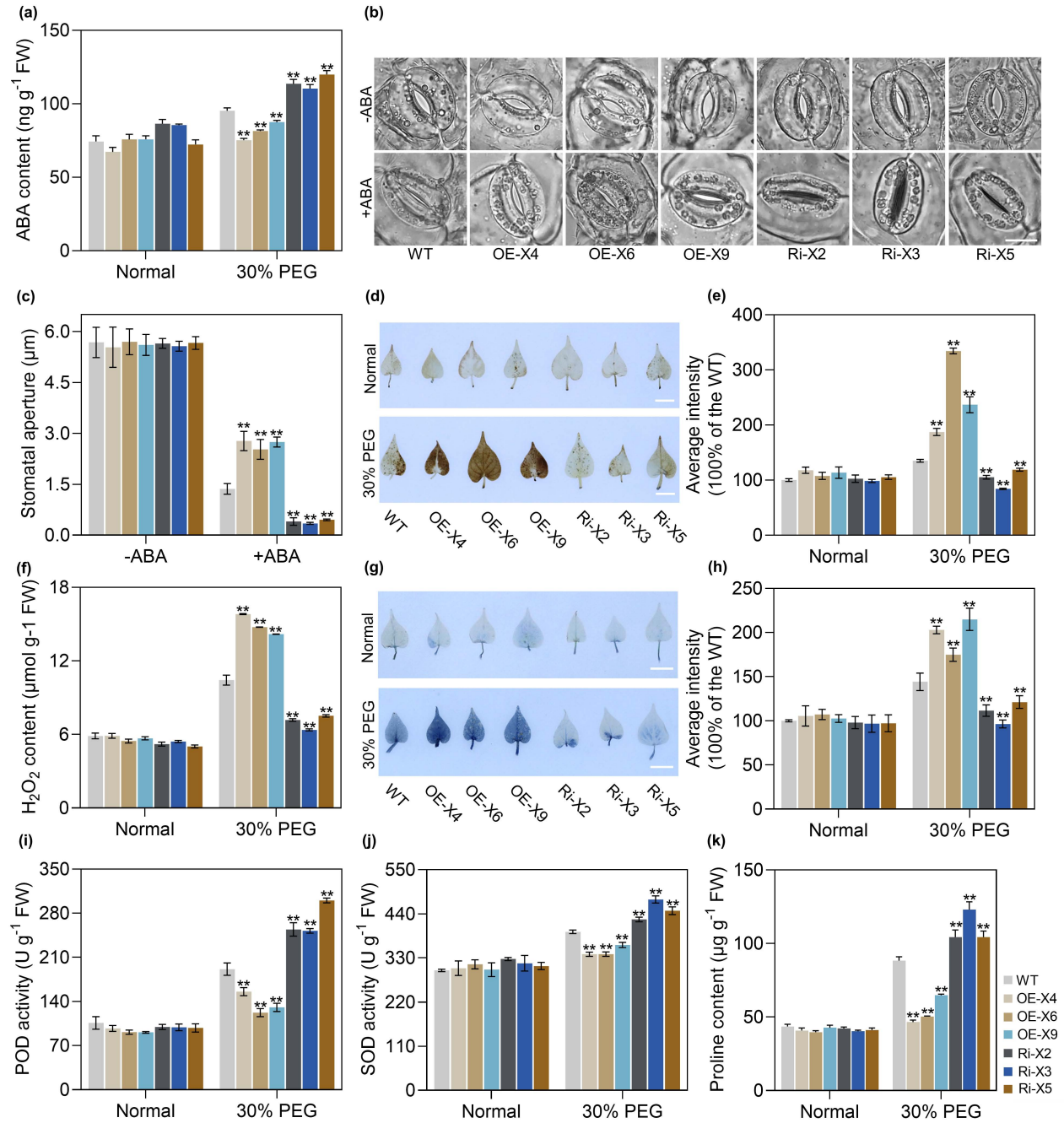
Fig. 9 The IbPYL8-IbbHLH66-IbbHLH118 complex targets the ABA-responsive gene *IbTIP1*. (a) Expression analysis of *IbTIP1* in 4-week-old *IbbHLH118* transgenic and WT plants under normal condition or subjected to 30% PEG. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test. (b) Expression analysis of *IbTIP1* in 5-week-old *IbbHLH66* transgenic and WT plants under normal condition or drought stress for 6 weeks. Data are presented as the means ± SD (n = 3). **, P < 0.01; Student's t-test. (c) Y1H assay showing that IbbHLH118 bound to the *IbTIP1* promoter. (d) Dual-LUC assays showing that IbbHLH118 suppressed the *IbTIP1* promoter, but the IbPYL8-IbbHLH66-IbbHLH118 complex interferes with IbbHLH118's repression of *IbTIP1*. Data are shown as mean ± SD (n = 3). Different letters indicate significant differences for each treatment at P < 0.05 based on Student's t-test. (e, f) ChIP-qPCR analysis using 35S:IbbHLH118-GFP, 35S:IbbHLH66-GFP, and 35S:GFP plants with anti-GFP antibody, which showed that IbbHLH118 could directly bind to the *IbTIP1* promoter, but IbbHLH66 could not. The *ACTIN* promoter was used as an internal reference for ChIP-qPCR. Data are shown as mean ± SD (n = 3). **, significant difference from 35S:GFP at P < 0.01 based on Student's t-test. (g) EMSA showing that IbbHLH118, but not IbbHLH66, could directly target *IbTIP1* by binding to the E-box in its promoter. The addition of IbbHLH66 inhibited the DNA binding activity of IbbHLH118. 50× indicates the usage of excess non-labeled probe as a competitor. (h-j) Responses, root length, and relative electrical conductivity of 2-month-old field-grown *IbbHLH66*-OE and WT sweet potato plants grown in transplanting boxes under normal condition or subjected to drought stress for 2 weeks. Bars = 5 cm. All data are presented as the means ± SD (n = 3). *, P < 0.05; **, P < 0.01; Student's t-test.

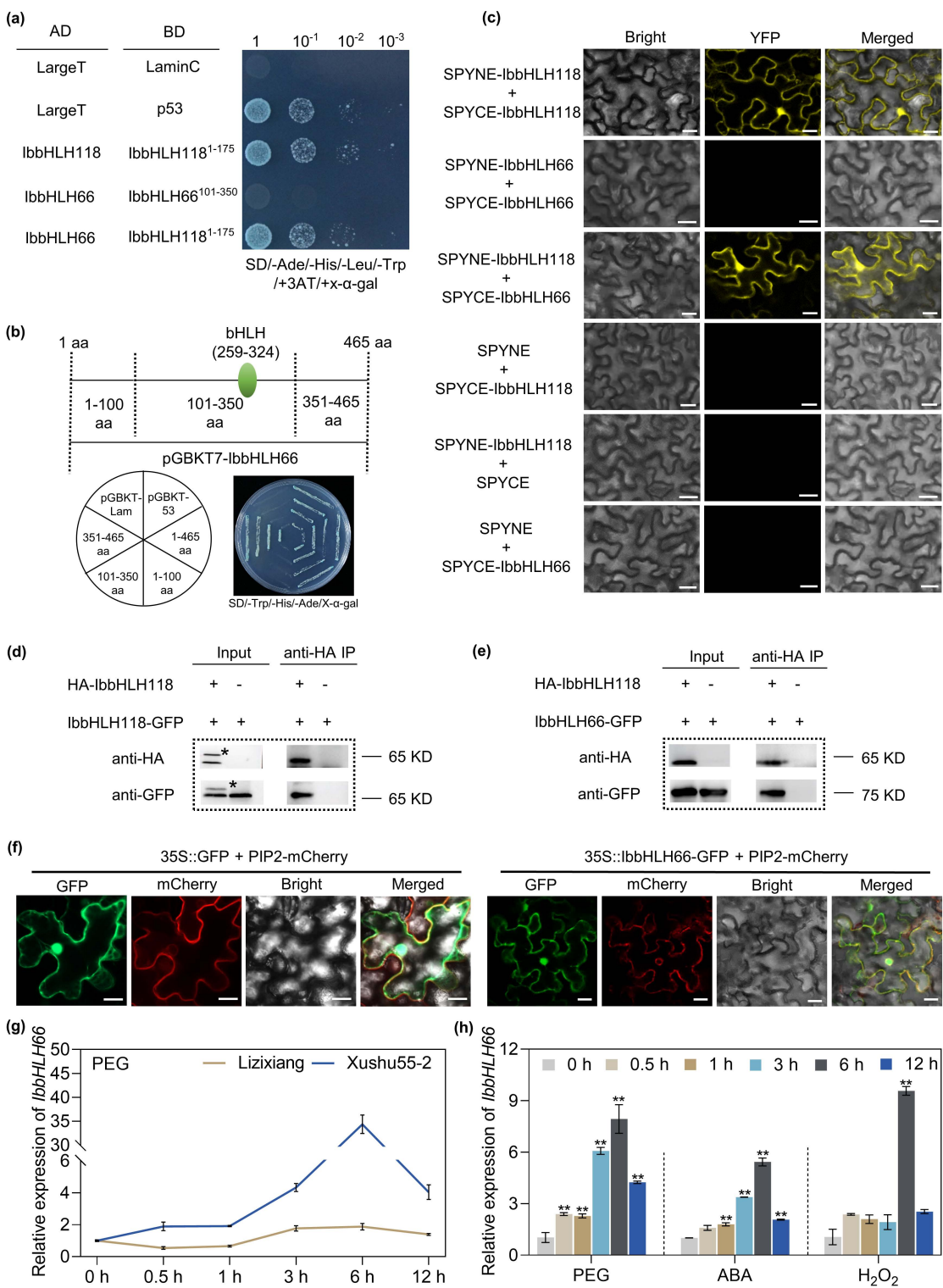
Fig. 10 Proposed working model of the IbPYL8-IbbHLH66-IbbHLH118 regulatory module in the ABA-dependent drought response of sweet potato. Under normal conditions, IbbHLH118 forms homodimers that bind to the promoters of *IbABI5*, *IbABF2*, and *IbTIP1*, inhibiting their expression. Under drought conditions, IbbHLH66 and IbPYL8 proteins are induced, but IbbHLH118 is repressed. Accumulated ABA is sensed by

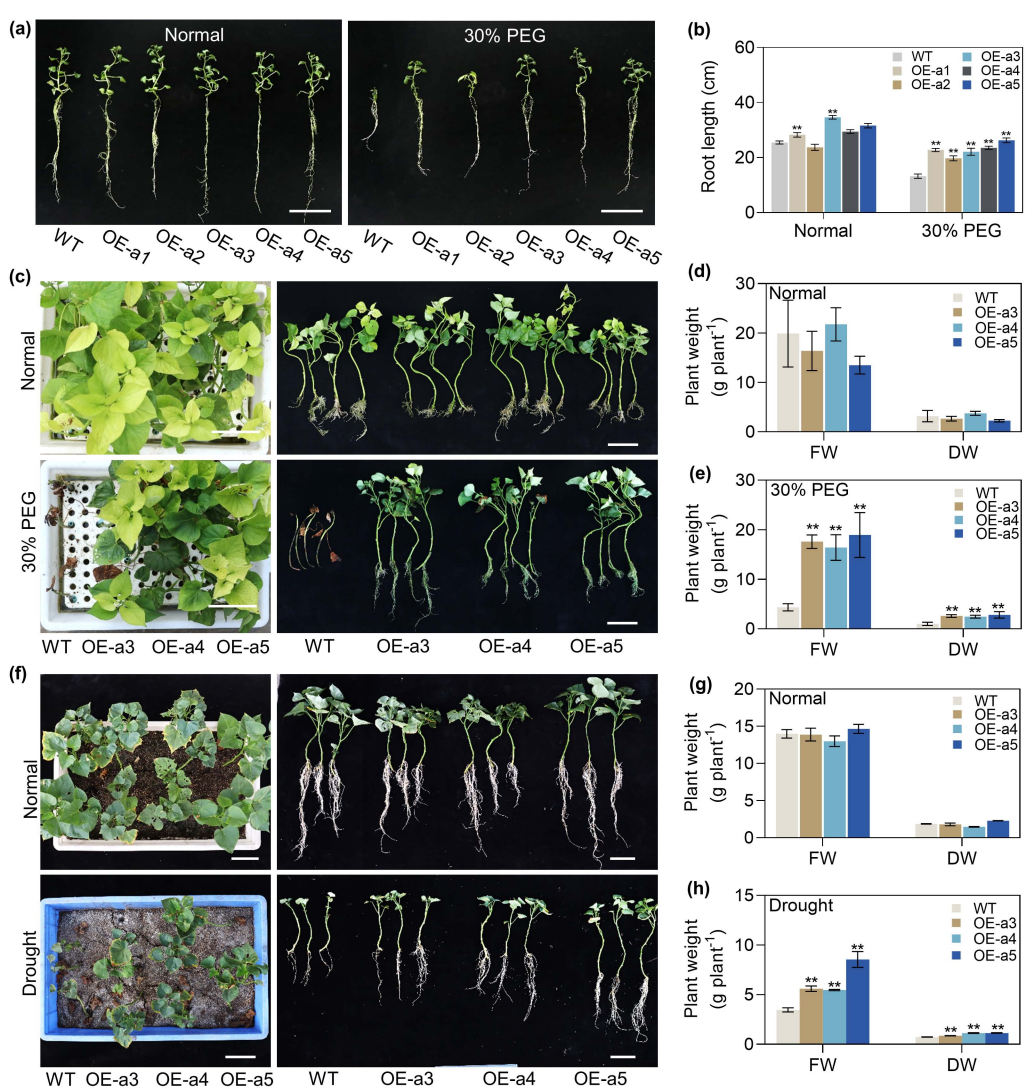
IbPYL8 and promotes the formation of the IbPYL8-IbbHLH66-IbbHLH118 complex, which relieves IbbHLH118's repression of ABA-responsive genes, such as *IbABI5*, *IbABF2*, and *IbTIP1*, thereby promoting ABA signaling and drought tolerance. Orange circle, ABA; yellow circle, IbbHLH118; blue circle, IbbHLH66; green circle, IbPYL8. Blunt-ended black arrow, promote gene expression; pointed green arrow, suppression; pointed red arrow, activation.

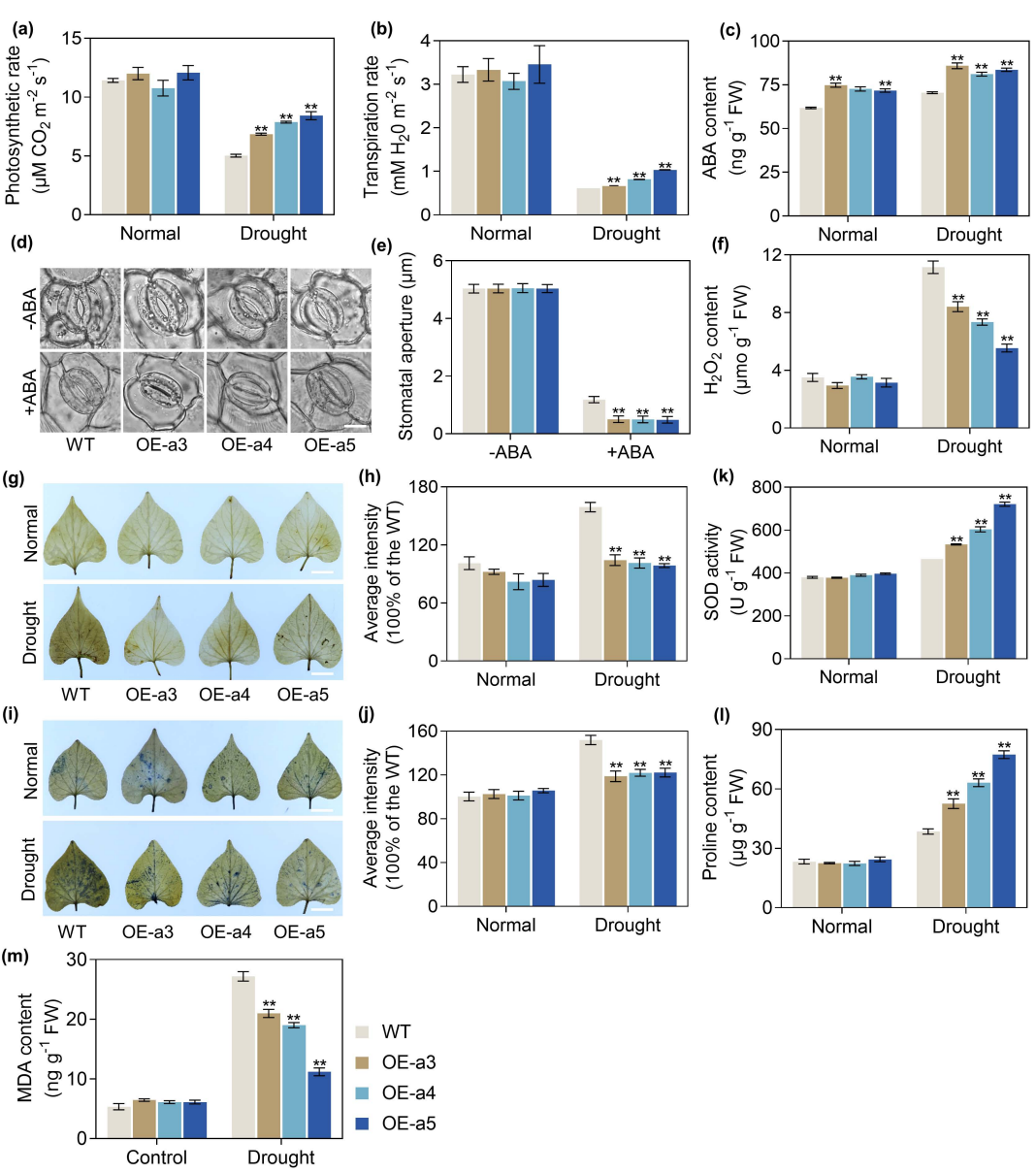

(a)
PEG
Lizixiang
Xushu55-2
Relative expression
0 h 0.5 h 1 h 3 h 6 h 12 h
(b)
ABA
Lizixiang
Xushu55-2
Relative expression
0 h 0.5 h 1 h 3 h 6 h 12 h
(c)
H2O2
Lizixiang
Xushu55-2
Relative expression
0 h 0.5 h 1 h 3 h 6 h 12 h
(d)
bHLH
IbbHLH118
AtbHLH118
NtbHLH118
NsbHLH118
NabHLH36
SpbHLH36
StbHLH118
Consensus
(e)
IbbHLH118
AtbHLH118
NtbHLH118
NsbHLH118
NabHLH36
SpbHLH36
StbHLH118
98
99
100
100
0.10
(f)
IbbHLH118
AtbHLH118
NtbHLH118
NsbHLH118
NabHLH36
SpbHLH36
StbHLH118
Exon
Intron
5'
3'
0 bp
500 bp
1000 bp
1500 bp
2000 bp
(g)
GFP
mCherry
Bright
Merged
35S::GFP + PIP2-mCherry
35S::IbbHLH118-GFP + PIP2-mCherry

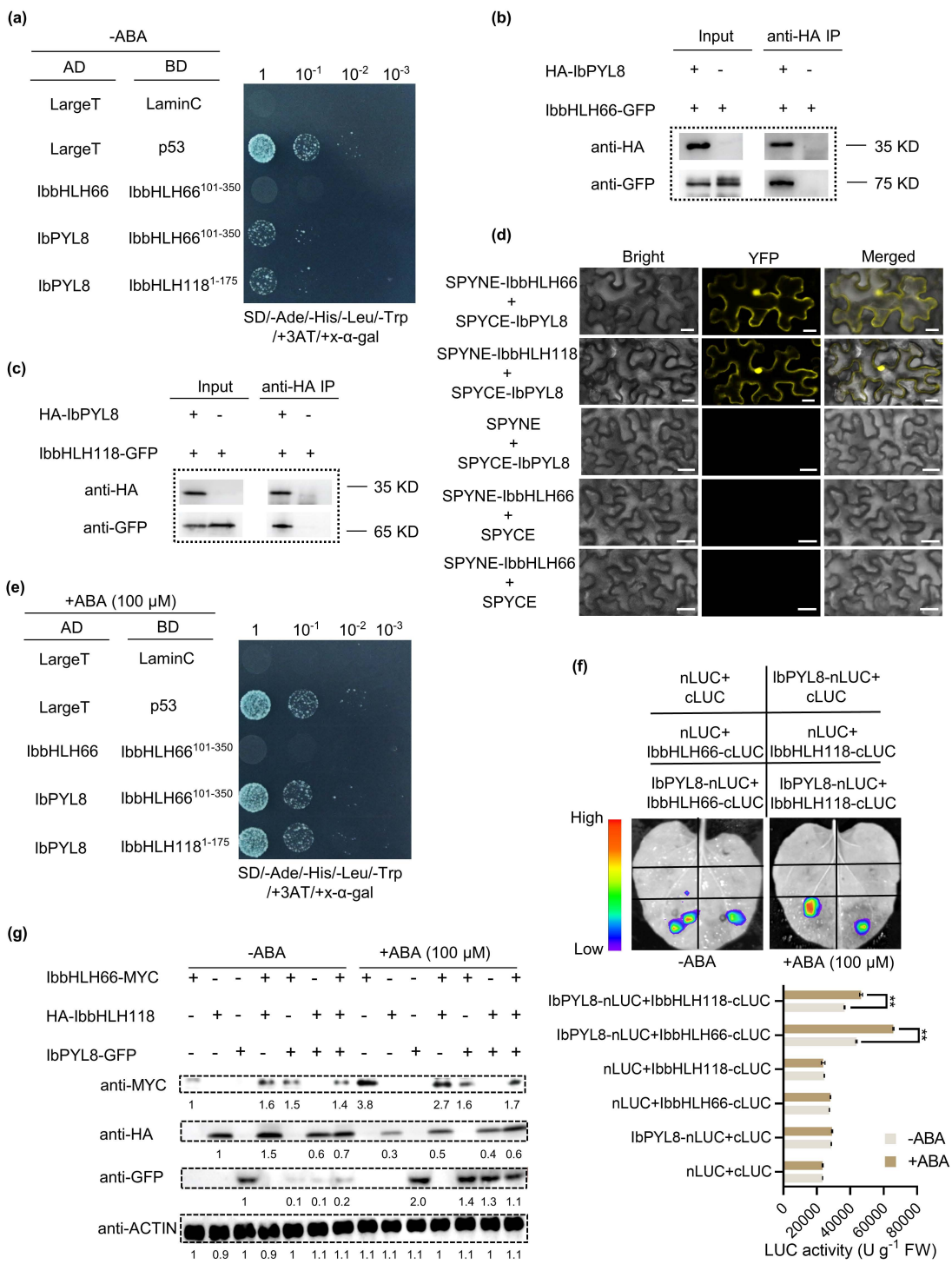

(a)
Normal
30% PEG
WT OE-X4 OE-X6 OE-X9 Ri-X2 Ri-X3 Ri-X5
(b)
Normal
30% PEG
FW
DW
Plant weight (g plant-1)
(c)
Normal
Drought
WT OE-X4 OE-X6 OE-X9
(d)
Normal
WT
OE-X4
OE-X6
OE-X9
Plant weight (g plant-1)
FW
DW
(e)
Drought
(f)
WT Ri-X2 Ri-X3 Ri-X5
(g)
Ri-X2
Ri-X3
Ri-X5
(h)

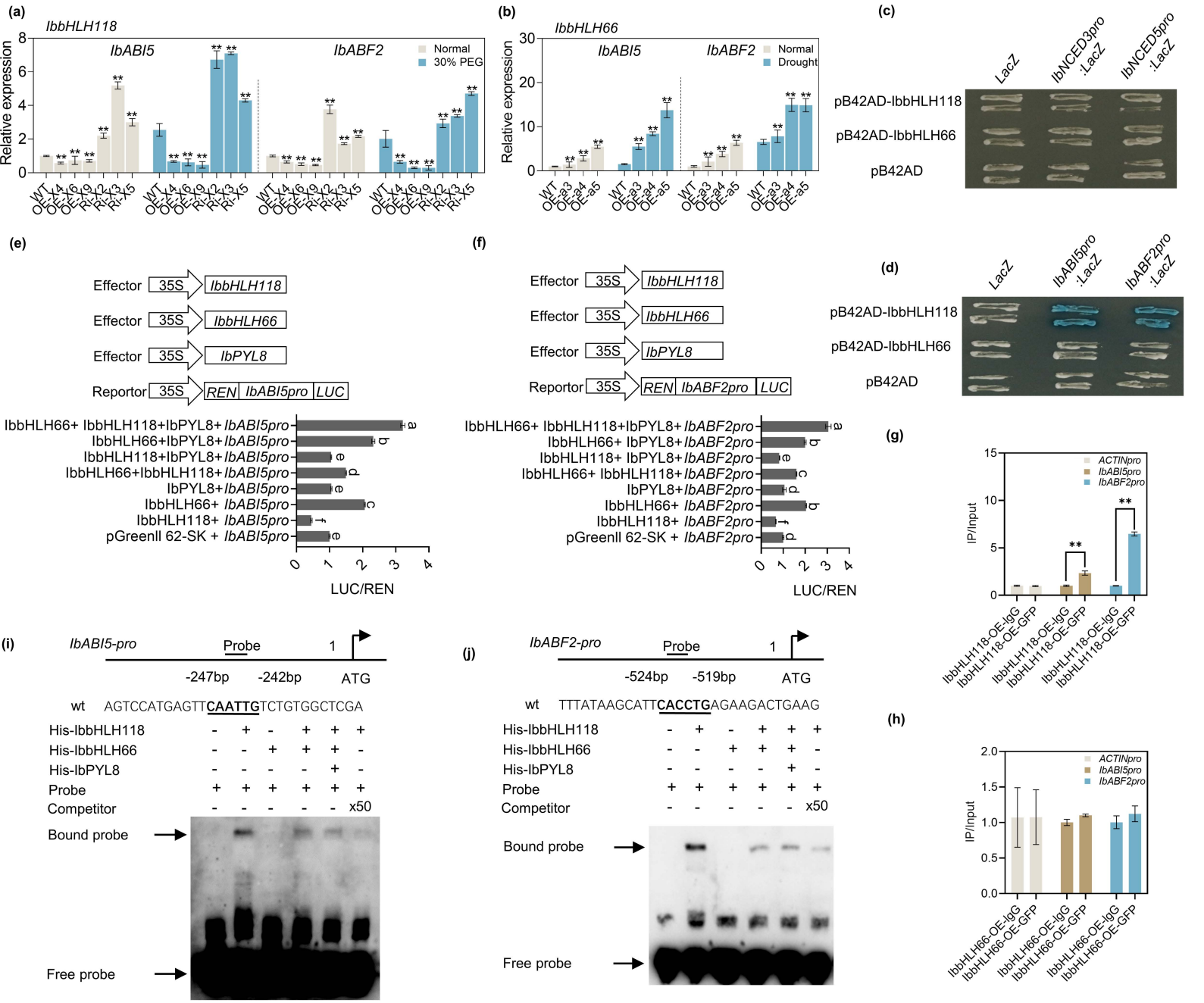

(a)
ABA content (ng g-1 FW)
150
100
50
0
Normal
30% PEG
(b)
-ABA
+ABA
WT
OE-X4
OE-X6
OE-X9
Ri-X2
Ri-X3
Ri-X5
(c)
Stomatal aperture (μm)
6.0
4.5
3.0
1.5
0.0
-ABA
+ABA
(d)
Normal
30% PEG
WT
OE-X4
OE-X6
OE-X9
Ri-X2
Ri-X3
Ri-X5
(e)
Average intensity (100% of the WT)
400
300
200
100
0
Normal
30% PEG
(f)
H2O2 content (μmol g-1 FW)
18
12
6
0
Normal
30% PEG
(g)
Normal
30% PEG
WT
OE-X4
OE-X6
OE-X9
Ri-X2
Ri-X3
Ri-X5
(h)
Average intensity (100% of the WT)
250
200
150
100
50
0
Normal
30% PEG
(i)
POD activity (U g-1 FW)
350
280
210
140
70
0
Normal
30% PEG
(j)
SOD activity (U g-1 FW)
550
440
330
220
110
0
Normal
30% PEG
(k)
Proline content (μg g-1 FW)
150
100
50
0
Normal
30% PEG
WT
OE-X4
OE-X6
OE-X9
Ri-X2
Ri-X3
Ri-X5

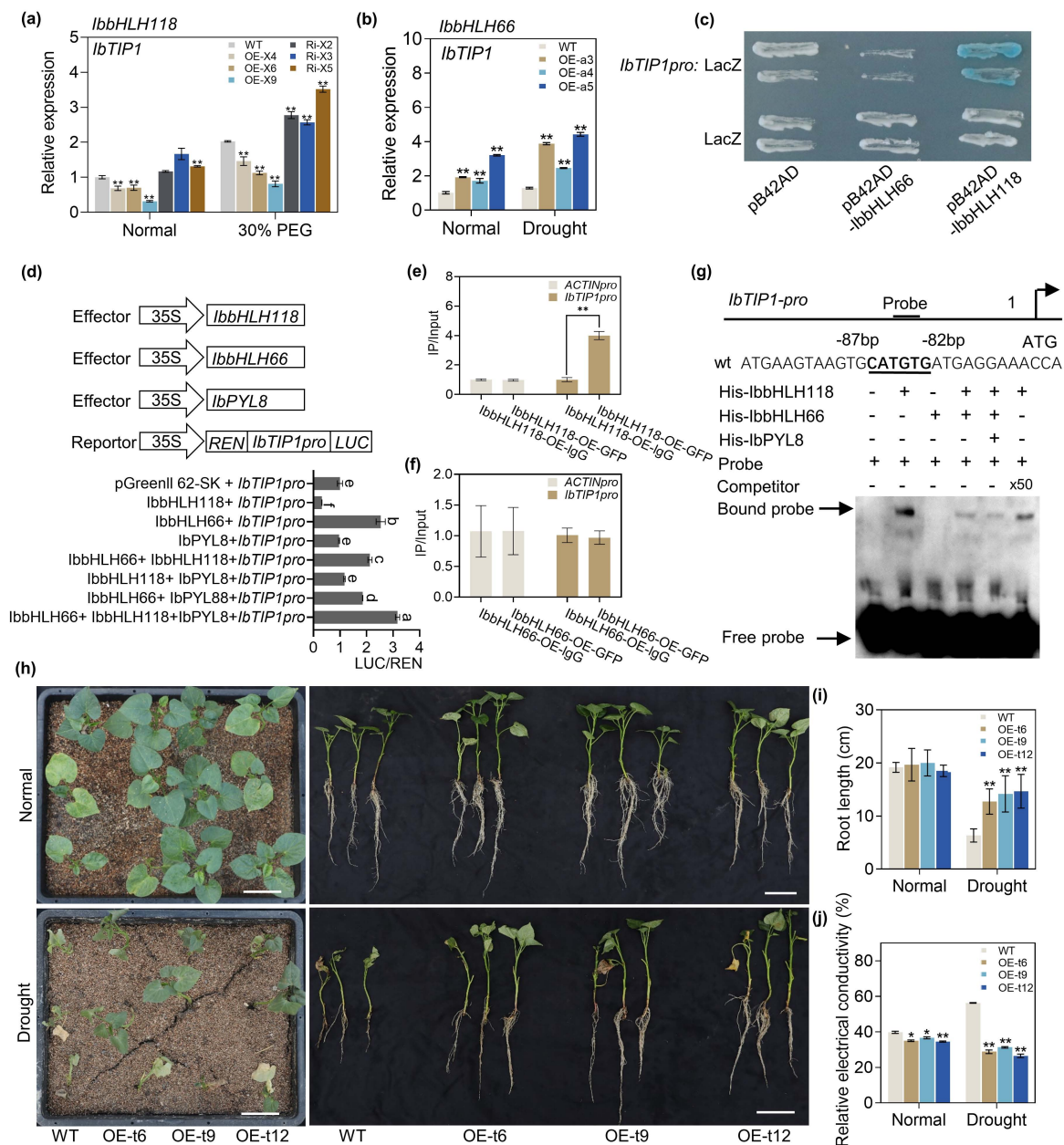

(a)
AD
BD
LargeT
LaminC
LargeT
p53
IbbHLH118
IbbHLH118 1-175
IbbHLH66
IbbHLH66 101-350
IbbHLH66
IbbHLH118 1-175
1
10-1
10-2
10-3
SD/-Ade/-His/-Leu/-Trp
/+3AT/+x-α-gal
(b)
1 aa
bHLH
(259-324)
465 aa
1-100
aa
101-350
aa
351-465
aa
pGBKT7-IbbHLH66
pGBKT-
Lam
pGBKT-
53
351-465
aa
1-465
aa
101-350
aa
1-100
aa
SD/-Trp/-His/-Ade/X-α-gal
(c)
Bright
YFP
Merged
SPYNE-IbbHLH118
+
SPYCE-IbbHLH118
SPYNE-IbbHLH66
+
SPYCE-IbbHLH66
SPYNE-IbbHLH118
+
SPYCE-IbbHLH66
SPYNE
+
SPYCE-IbbHLH118
SPYNE-IbbHLH118
+
SPYCE
SPYNE
+
SPYCE-IbbHLH66
(d)
Input
anti-HA IP
HA-IbbHLH118
+ - + -
IbbHLH118-GFP
+ + + +
anti-HA
anti-GFP
65 KD
65 KD
(e)
Input
anti-HA IP
HA-IbbHLH118
+ - + -
IbbHLH66-GFP
+ + + +
anti-HA
anti-GFP
65 KD
75 KD
(f)
35S::GFP + PIP2-mCherry
GFP
mCherry
Bright
Merged
35S::IbbHLH66-GFP + PIP2-mCherry
GFP
mCherry
Bright
Merged
(g)
Relative expression of IbbHLH66
PEG
Lizixiang
Xushu55-2
0 h
0.5 h
1 h
3 h
6 h
12 h
(h)
Relative expression of IbbHLH66
0 h
0.5 h
1 h
3 h
6 h
12 h
PEG
ABA
H2O2

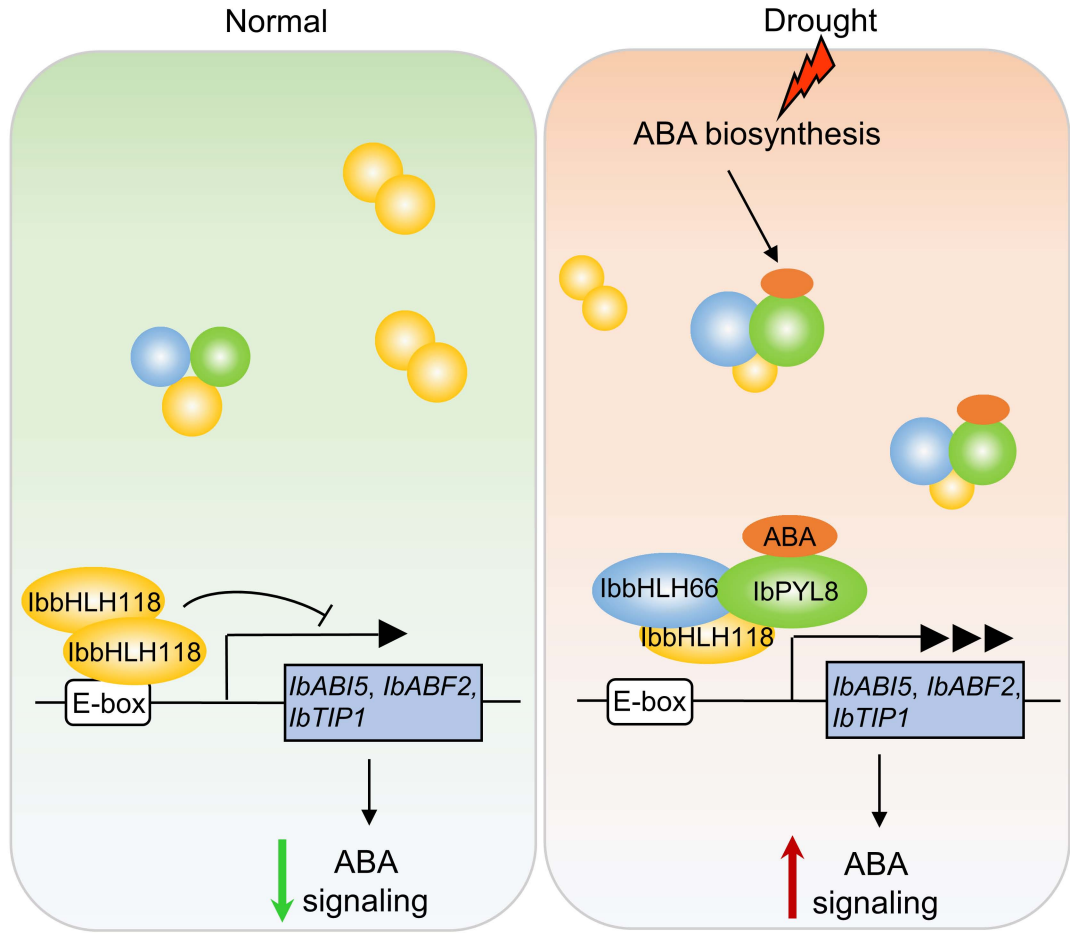

(a)
Normal
30% PEG
WT
OE-a1
OE-a2
OE-a3
OE-a4
OE-a5
(b)
Root length (cm)
WT
OE-a1
OE-a2
OE-a3
OE-a4
OE-a5
Normal
30% PEG
(c)
Normal
30% PEG
WT
OE-a3
OE-a4
OE-a5
(d)
Normal
Plant weight (g plant-1)
FW
DW
(e)
30% PEG
(f)
Drought
(g)
(h)


(a)
Photosynthetic rate (μM CO2 m-2 s-1)
Normal
Drought
(b)
Transpiration rate (mM H20 m-2 s-1)
(c)
ABA content (ng g-1 FW)
(d)
-ABA
+ABA
WT
OE-a3
OE-a4
OE-a5
(e)
Stomatal aperture (μm)
(f)
H2O2 content (μmo g-1 FW)
(g)
Normal
Drought
(h)
Average intensity (100% of the WT)
(k)
SOD activity (U g-1 FW)
(i)
(j)
(l)
Proline content (μg g-1 FW)
(m)
MDA content (ng g-1 FW)
Control
WT
OE-a3
OE-a4
OE-a5


(a)
-ABA
AD
BD
LargeT
LaminC
LargeT
p53
IbbHLH66
IbbHLH66101-350
IbPYL8
IbbHLH66101-350
IbPYL8
IbbHLH1181-175
1
10-1
10-2
10-3
SD/-Ade/-His/-Leu/-Trp
/+3AT/+x-α-gal
(b)
Input
anti-HA IP
HA-IbPYL8
IbbHLH66-GFP
anti-HA
anti-GFP
35 KD
75 KD
(c)
Input
anti-HA IP
HA-IbPYL8
IbbHLH118-GFP
anti-HA
anti-GFP
35 KD
65 KD
(d)
Bright
YFP
Merged
SPYNE-IbbHLH66
+
SPYCE-IbPYL8
SPYNE-IbbHLH118
+
SPYCE-IbPYL8
SPYNE
+
SPYCE-IbPYL8
SPYNE-IbbHLH66
+
SPYCE
SPYNE-IbbHLH66
+
SPYCE
(e)
+ABA (100 μM)
AD
BD
LargeT
LaminC
LargeT
p53
IbbHLH66
IbbHLH66101-350
IbPYL8
IbbHLH66101-350
IbPYL8
IbbHLH1181-175
1
10-1
10-2
10-3
SD/-Ade/-His/-Leu/-Trp
/+3AT/+x-α-gal
(f)
nLUC+
cLUC
IbPYL8-nLUC+
cLUC
nLUC+
IbbHLH66-cLUC
nLUC+
IbbHLH118-cLUC
IbPYL8-nLUC+
IbbHLH66-cLUC
IbPYL8-nLUC+
IbbHLH118-cLUC
High
Low
-ABA
+ABA (100 μM)
IbPYL8-nLUC+IbbHLH118-cLUC
IbPYL8-nLUC+IbbHLH66-cLUC
nLUC+IbbHLH118-cLUC
nLUC+IbbHLH66-cLUC
IbPYL8-nLUC+cLUC
nLUC+cLUC
-ABA
+ABA
0
20000
40000
60000
80000
LUC activity (U g-1 FW)
(g)
-ABA
+ABA (100 μM)
IbbHLH66-MYC
HA-IbbHLH118
IbPYL8-GFP
anti-MYC
1 1.6 1.5 1.4 3.8 2.7 1.6 1.7
anti-HA
1 1.5 0.6 0.7 0.3 0.5 0.4 0.6
anti-GFP
1 0.1 0.1 0.2 2.0 1.4 1.3 1.1
anti-ACTIN
1 0.9 1 0.9 1 1.1 1.1 1.1 1.1 1.1 1 1.1 1 1.1


(a)
IbbHLH118
IbABI5
IbABF2
Normal
30% PEG
Relative expression
WT
OE-14
OE-16
OE-19
RI-X2
RI-X3
RI-X5
(b)
IbbHLH66
IbABI5
IbABF2
Normal
Drought
Relative expression
WT
OE-a3
OE-a4
OE-a5
(c)
LacZ
IbNCED3pro :LacZ
IbNCED5pro :LacZ
pB42AD-IbbHLH118
pB42AD-IbbHLH66
pB42AD
(d)
LacZ
IbABI5pro :LacZ
IbABF2pro :LacZ
pB42AD-IbbHLH118
pB42AD-IbbHLH66
pB42AD
(e)
Effector 35S IbbHLH118
Effector 35S IbbHLH66
Effector 35S IbPYL8
Reportor 35S REN IbABI5pro LUC
IbbHLH66+ IbbHLH118+IbPYL8+ IbABI5pro
IbbHLH66+IbPYL8+ IbABI5pro
IbbHLH118+IbPYL8+ IbABI5pro
IbbHLH66+IbbHLH118+ IbABI5pro
IbPYL8+ IbABI5pro
IbbHLH66+ IbABI5pro
IbbHLH118+ IbABI5pro
pGreenII 62-SK + IbABI5pro
LUC/REN
(f)
Effector 35S IbbHLH118
Effector 35S IbbHLH66
Effector 35S IbPYL8
Reportor 35S REN IbABF2pro LUC
IbbHLH66+ IbbHLH118+IbPYL8+ IbABF2pro
IbbHLH66+ IbPYL8+ IbABF2pro
IbbHLH118+ IbPYL8+ IbABF2pro
IbbHLH66+ IbbHLH118+ IbABF2pro
IbPYL8+ IbABF2pro
IbbHLH66+ IbABF2pro
IbbHLH118+ IbABF2pro
pGreenII 62-SK + IbABF2pro
LUC/REN
(g)
ACTINpro
IbABI5pro
IbABF2pro
IP/Input
IbbHLH118-OE-IgG
IbbHLH118-OE-GFP
(h)
ACTINpro
IbABI5pro
IbABF2pro
IP/Input
IbbHLH66-OE-IgG
IbbHLH66-OE-GFP
(i)
IbABI5-pro
Probe
-247bp
-242bp
ATG
wt AGTCCATGAGTTCAATTGTCTGTGGCTCGA
His-IbbHLH118
His-IbbHLH66
His-IbPYL8
Probe
Competitor
x50
Bound probe
Free probe
(j)
IbABF2-pro
Probe
-524bp
-519bp
ATG
wt TTTATAAGCATTCACCTGAGAAGACTGAAG
His-IbbHLH118
His-IbbHLH66
His-IbPYL8
Probe
Competitor
x50
Bound probe
Free probe


(a)
IbbHLH118
IbTIP1
Relative expression
WT
OE-X4
OE-X6
OE-X9
Ri-X2
Ri-X3
Ri-X5
Normal
30% PEG
(b)
IbbHLH66
IbTIP1
Relative expression
WT
OE-a3
OE-a4
OE-a5
Normal
Drought
(c)
IbTIP1pro: LacZ
LacZ
pB42AD
pB42AD -IbbHLH66
pB42AD -IbbHLH118
(d)
Effector 35S IbbHLH118
Effector 35S IbbHLH66
Effector 35S IbPYL8
Reportor 35S REN IbTIP1pro LUC
pGreenII 62-SK + IbTIP1pro
IbbHLH118+ IbTIP1pro
IbbHLH66+ IbTIP1pro
IbPYL8+IbTIP1pro
IbbHLH66+ IbbHLH118+IbTIP1pro
IbbHLH118+ IbPYL8+IbTIP1pro
IbbHLH66+ IbPYL88+IbTIP1pro
IbbHLH66+ IbbHLH118+IbPYL8+IbTIP1pro
LUC/REN
(e)
ACTINpro
IbTIP1pro
IP/Input
IbbHLH118-OE-IgG
IbbHLH118-OE-GFP
IbbHLH118-OE-IgG
IbbHLH118-OE-GFP
(f)
ACTINpro
IbTIP1pro
IP/Input
IbbHLH66-OE-IgG
IbbHLH66-OE-GFP
IbbHLH66-OE-IgG
IbbHLH66-OE-GFP
(g)
IbTIP1-pro
Probe
1
-87bp
-82bp
ATG
wt ATGAAGTAAGTGCATGTGATGAGGAAACCA
His-IbbHLH118
His-IbbHLH66
His-IbPYL8
Probe
Competitor
x50
Bound probe
Free probe
(h)
Normal
Drought
WT
OE-t6
OE-t9
OE-t12
(i)
Root length (cm)
Normal
Drought
(j)
Relative electrical conductivity (%)
Normal
Drought


Normal
Drought
ABA biosynthesis
IbbHLH118
IbbHLH118
E-box
IbABI5, IbABF2, IbTIP1
ABA signaling
ABA
IbbHLH66
IbPYL8
IbbHLH118
E-box
IbABI5, IbABF2, IbTIP1
ABA signaling