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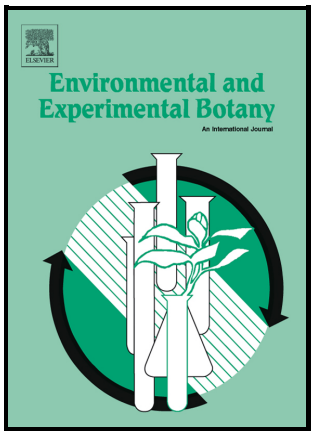

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Title:

The rice transcription factors OsHOX22 and OsHOX24 oppositely modulate the lamina joint inclination.

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Abstract

OsHOX22 and OsHOX24 are members of the homeodomain-leucine zipper I (HD-Zip I) subfamily of transcription factors (TFs) involved in ABA-mediated salt and drought tolerance. Phylogenetic analyses resolve these TFs into the same subgroup (γ-clade). We used CRISPR/Cas9 technology to obtain two *Japonica* CVs. Kitaake edited rice lines (*edOsHOX22* and *edOsHOX24*) by a non-homologous end-joining mechanism. These plants displayed opposite phenotypes in the lamina joint inclination of the second leaf of 9-day-old seedlings. The *edOsHOX22* genotype presented a larger leaf angle than the wild-type (WT), resembling plants with increased endogenous brassinosteroid (BR) levels. In addition, the BR treatment repressed *OsHOX22* expression in WT plants. Otherwise, the *edOsHOX24* plants exhibited the opposite phenotype, and BRs induced *OsHOX24* expression. This evidence suggested a mutual negative and positive regulation between OsHOX22 and OsHOX24 and the phytohormone, respectively. Besides, BR biosynthesis and signaling genes exhibited altered

levels in *edOsHOX22* and *edOsHOX24* plants compared with controls. Notably, under abiotic stress, they agonistically modified their expression, and the ABA signaling cascade masked the BR response, closing the leaf angle in both genotypes. Our findings indicated that OsHOX22 and OsHOX24 play differential roles in the lamina joint inclination event. This study contributes two new players to the complex mechanisms involved in the leaf angle opening under different environmental cues in rice.

Keywords:

OsHOX22; OsHOX24; lamina joint inclination; brassinosteroid; abscisic acid; abiotic stress.

1. Introduction

Rice (*Oryza sativa L.*) is one of the most critical crops because it feeds more than half of the world's population (D. Wang et al., 2017). Considering the increase in food demand and the current cycle of climatic changes, it is necessary to develop high-yielding stress-tolerant rice varieties to guarantee food security (Ansari et al., 2020; D. Wang et al., 2017). Therefore, rice breeding programs were devoted to improving agronomic traits such as enhanced yield, increased stress tolerance, and better nutritional quality (Ansari et al., 2020; Dong et al., 2021). Traditional breeding methods generated novel rice varieties (Tester and Langridge, 2010), albeit exhibiting limitations, such as randomness and low efficiency (Jun et al., 2019). On the other hand, genetically modified (GM) rice varieties showing beneficial traits have shown to be promising. However, they were associated with a negative public perception of environmental safety, which generated complex regulatory pipeline requests. Moreover, due to the high cost associated with the regulatory process, they did not represent a universal business for the case of abiotic stress tolerance, which led to their absence in the market (Ahmad and Mukhtar, 2017; Chan et al., 2020; Goberna et al., 2022). The development of new breeding techniques, such as genome-editing technology using the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)) system, provides a simple, flexible, efficient, and highly accurate tool for genetic engineering. CRISPR/Cas9 could contribute to crop improvement without the drawbacks mentioned for GM crops (Georges and Ray, 2017;

Romero and Gatica-Arias, 2019). However, these techniques have limitations as the choice of the endogenous targets and the potential off-target mutations, which makes knowledge of the whole genome sequence of the variety to be edited necessary (Aliaga-Franco et al., 2019). Transcription factors (TFs) are regulatory proteins that induce or repress entire signaling pathways (Ganie et al., 2019). TFs are abundant in plants, representing about 6% of the encoding genes. The alteration of their levels has been used to obtain varied plant species exhibiting beneficial traits, mainly by overexpression (Cabello et al., 2017; Jyoti et al., 2019; Ribichich et al., 2020; N. Wang et al., 2017). Plant TFs have been classified into families and subfamilies according to their DNA-binding domain. Among these families, the HD-Zip is unique to the plant kingdom because of the singular association between the homeodomain (HD) and the leucine zipper (Zip) dimerization motif. The HD-Zip members were divided into four groups (I–IV) according to their structure, size, and the presence of additional domains (Arce et al., 2011; Mukherjee et al., 2009). The HD-Zip I TFs were associated with developmental events related to abiotic stress (Ariel et al., 2007; Henriksson et al., 2005; Perotti et al., 2017). Thirty-three members of the HD-Zip family were identified in the rice genome, but only thirty were detected as expressed in different tissues and developmental stages (Agalou et al., 2008). Among members of subfamily I, OsHOX22 and OsHOX24 were resolved in the same clade as the well-characterized Arabidopsis paralog pair AtHB7 and AtHB12. The expression of *OsHOX22* and *OsHOX24* increased after drought, salt, and ABA treatments (Agalou et al., 2008; Bhattacharjee et al., 2016). Notably, given that most HD-Zip I TFs were described as positive regulators in front of abiotic stresses, the overexpression of these rice TFs increased the sensitivity to ABA, water deficit, and salinity (Bhattacharjee et al., 2017; Zhang et al., 2012). In agreement with a negative role in stress responses, the knockdown T-DNA insertion mutant *oshox22-1* showed decreased sensitivity to ABA and increased tolerance to water and salt stress without morphological differences compared with wild-type (WT) plants (Zhang et al., 2012).

In rice and other monocot species, the leaf angle between the leaf blade and the vertical stem constitutes a relevant architectural trait related to plant performance (Li et al., 2019). Plant

erection has been correlated with enhanced grain yield due to increased photosynthetic efficiency (Sakamoto et al., 2006). When flag leaves are erected, allowing sunlight penetration, lower leaves contribute to photosynthesis (Luo et al., 2016). Brassinosteroids (BRs) are the most relevant leaf-angle determinants; however, ABA also participates in this event, crosstalking with BRs to modulate the lamina joint inclination. Despite recent findings, the molecular mechanism of the interaction between ABA- and BR-signaling pathways in rice remains unknown (Li et al., 2021, 2019).

In this study, we show the obtaining of two novel rice varieties by genome editing *OsHOX22* and *OsHOX24* using CRISPR/Cas9 technology. These varieties exhibited an opposite phenotype related to the lamina joint inclination. Furthermore, our findings show that these TFs are involved in the BR regulatory pathway determining leaf angle opening.

2. Materials and Methods

2.1. Plant materials used and growth conditions.

The gene-edited plants in *OsHOX22* and *OsHOX24* were generated via *Agrobacterium tumefaciens*-mediated CRISPR/Cas9 technology (Main et al., 2015) using *Japonica* cv. Kitaake rice plants as WT background. Transformed embryogenic calluses resistant to 50 mg/L hygromycin were selected. After transformed plant regeneration, genome editing was verified by PCR using genomic DNA as a template and sequencing with specific oligonucleotides (Supplementary Table S1). A screening was performed to select the lines to be sequenced. Rice seeds were surface sterilized according to Main et al. (2015), germinated in flasks containing Murashige and Skoog (MS) medium with 300 mM NaCl and gentle shaking, and placed for 3 days in an environmentally controlled growth chamber with a photoperiod of 16/8 h light/dark regime at 28 °C, a light intensity of 180 μE m^{-2} s^{-1}, and 60% relative humidity. The DSDecode tool (http://skl.scau.edu.cn/dsdecode/) (Liu et al., 2015) was used to automatically decode biallelic, heterozygous, and homozygous mutations from sequencing chromatograms. Two homozygous independent lines were used for further analyses. The homozygous genome-edited lines were multiplied in a greenhouse.

2.2. Guide RNA design and genetic construction.

The guide RNA (gRNA) for genome editing in *OsHOX22* and *OsHOX24* was designed using CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) and obtained by annealing specific oligonucleotides (Supplementary Table S1). Each gRNA was cloned in a pRGEB32 vector (Addgene plasmid # 63142) (Xie et al., 2015) between *Bsa* I sites. The ability to modify the target genes of the genetic construction was evaluated by protoplast transformation, according to Lin et al. (2018). Supplementary Table S1 shows the specific oligonucleotides used for sequencing.

2.3. Lamina joint inclination assay and hormone treatments

The lamina joint assay was performed according to Li et al. (2019), with some modifications. Briefly, rice seeds were surface sterilized according to Main et al. (2015), germinated in flasks containing Murashige and Skoog (MS) medium with gentle shaking, and placed in an environmentally controlled growth chamber with a photoperiod of 16/8 h light/dark regime at 28 °C, a light intensity of 180 μE m^{-2} s^{-1}, and 60% relative humidity. After 96 hours, the seeds that germinated uniformly were transferred to a rubber net stretched over an 8.5 L plastic pot containing 0.5 x Hoagland solution for five days. First, leaf segments consisting of 1 cm of the second leaf blade, the lamina joint, and 1 cm of the leaf sheath were excised from the uniform seedlings. Next, leaf segments were floated on a Petri dish containing 10 μM ABA, 1 mM ABA, 200 nM BR (Brassinolide), or 50 μM BRZ (Brassinazole) and placed in a growth chamber under the same conditions for 2 or 24 h. Finally, ImageJ software measured the lamina joint angle formed by the lamina and leaf sheath. Twenty seedlings were used for each treatment, and all experiments were repeated three times.

2.4. RNA extraction and real-time qPCR analysis.

Leaf segments of 9-day-old rice seedlings were used for RNA extraction and gene expression analyses. According to the manufacturer's instructions, the total RNA was isolated from rice

tissues using Trizol® reagent (Invitrogen). RNA quality and quantity were evaluated using a NanoDrop 2000 (Thermo Scientific, MA, USA) system. One µg of RNA was reverse-transcribed using oligo(dT)18 and M-MLV reverse transcriptase (Promega®). Quantitative real-time PCR (qPCR) was performed using StepOne™ equipment (Applied Biosystems). Each reaction contained a final volume of 20 µl that included 2 µl of SyBr green (4×), 8 pmol of each primer, 2 mM $MgCl_2$, 10 µl of a 1/20 dilution of the RT reaction, and 0.1 µl of Taq Polymerase (Invitrogen). Thermocycler parameters were as follows: 94 ºC 12", 60 ºC 12", and 72 ºC 12". Fluorescence was measured over 40 cycles at 72 ºC. Specific primers for each gene are listed in Supplementary Table S1. Quantification of mRNA levels was performed by normalization with the elongation factor 1-alpha (*OsEF-1α*) transcript levels (Almas and Kamrodi, 2018) according to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

2.5. Stress treatments

Rice seeds were surface sterilized, germinated in flasks containing MS medium with gentle shaking, and placed in an environmentally controlled growth chamber, as mentioned above. After 96 hours, the seeds that germinated uniformly were transferred to a rubber net stretched over an 8.5 L plastic pot containing 0.5 x Hoagland solution for five days. 100, 150, and 200 mM NaCl was added to the Hoagland solution for salt stress treatment. After five days, for cold and drought stress treatments, the plastic pot containing plants was transferred to a cold chamber (4 °C) for 3, 6, and 10 hours or placed on blotting paper for 1, 1.5, and 2 hours, respectively. All assays were performed under the same photoperiod and humidity conditions. Lamina joint samples were photographed, and the angle was evaluated using ImageJ software. Leaf segments were excised from the uniform seedlings for RNA extraction and qPCR analysis. Twenty seedlings were used for each treatment, and all experiments were repeated three times.

2.6. Adaxial and abaxial sides lamina joint length evaluation

Lamina joint autofluorescence images were taken using a light microscope (Leica MZ10F). The lamina joint's adaxial and abaxial side lengths were measured and quantified using the ImageJ software.

2.7. Statistical analysis

The evaluations were performed using the Student's t-test. Significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) between means were denoted by asterisks. The numbers of biological replicates for each assessment are indicated in the corresponding figures. The evaluation in Table 1 was performed using a two-way analysis of variance (ANOVA), considering genotype and treatment as the main factors. When interaction terms were significant ($P < 0.01$), differences between means were analyzed using the Tukey comparison test and are indicated by different letters.

2.8. Accession Numbers

Sequence data from this article can be found in the MSU Rice Genome Annotation Project Database and Resource under the following accession numbers: LOC_Os04g45810 (*OsHOX22*); LOC_Os02g43330 (*OsHOX24*); LOC_Os03g08020 (*OsEF-1α*); LOC_Os07g39220 (*OsBZR1*); LOC_Os04g39430 (*OsD11*); LOC_Os03g40540 (*OsBRD1*); LOC_Os06g03710 (*OsDLT*).

3. Results

3.1. Generation of *OsHOX22* and *OsHOX24* gene-edited rice plants

OsHOX22 and *OsHOX24* participate in ABA signaling pathways related to drought and salinity stresses (Bhattacharjee et al., 2017; Zhang et al., 2012). Since *OsHOX22* and *OsHOX24* are putative paralogs resolved in the same clade, they may be functionally redundant. To further analyze their putative homology and redundancy, we performed a protein sequence alignment showing a 51% identity between them (Supplementary Figure S1). However, outside the HD-Zip domains and AHA motif (Aromatic, large Hydrophobic, Acidic context), OsHOX22 and

OsHOX24 exhibited differences impacting their structure and probably their function. Aiming to study the role of both TFs, we obtained *OsHOX22* and *OsHOX24* gene-edited plants in the *Japonica* cv. Kitaake background using the CRISPR/Cas9 system. The gRNAs were designed upstream of the HD domain, at 151 bp and 76 bp downstream of the start codons for *OsHOX22* and *OsHOX24*, respectively (Fig. 1A). Rice plants were transformed using *Agrobacterium tumefaciens*, and ten *OsHOX22* and fifteen *OsHOX24* putative genome-edited lines were obtained after the hygromycin selection. Lines were screened by germination in 300 mM NaCl since we determined that this NaCl concentration was lethal for Kitaake plants. After this, eight *edOsHOX22* (edited plants in *OsHOX22*) and four *edOsHOX24* (edited plants in *OsHOX24*) plant lines survived the treatment and were selected for DNA isolation and sequencing. The zygosity of each independent edited line was determined using the web-free tool DSDecode (http://skl.scau.edu.cn/dsdecode/) (Liu et al., 2015) (Supplementary Table S2).

To continue the study, we selected the homozygous gene-edited plants A8 and A9 (*edOsHOX22*) and A21 and A23 (*edOsHOX24*). Figure 1A shows the Cas9 activity's effect and cell-repairing machinery results. As expected, the modifications occurred 3-bp upstream from the PAM motif (Qi, 2019). From the non-homologous end joining (NHEJ) mechanism, we obtained two nucleotide deletions and one nucleotide insertion for *OsHOX22* and *OsHOX24*, respectively. The putative proteins generated in homozygous edited plants would be non-functional (Fig. 1B and Supplementary Fig. S2). Moreover, due to the modified open reading frame, no HD-Zip domains were structured and conformed because of premature stop codons (Supplementary Fig. S3).

3.2. OsHOX22 and OsHOX24 exhibit non-redundant functions in the lamina joint

Once we confirmed and selected the edited lines (*edOsHOX22* and *edOsHOX24*), we wondered how the lack of these genes affects the plant phenotype, aside from the differential germination in salinity conditions. To assess the characteristics of the newly obtained lines, we grew the plants in normal conditions. A visual inspection showed that the second leaf in *edOsHOX22* and *edOsHOX24* showed opposite leaf inclinations relative to the vertical stem nine days after

sowing (Fig. 2A, B). Compared with Kitaake, *edOsHOX22* plants showed a larger leaf angle determined by the increased adaxial side length of the lamina joint (Fig. 2C-F), whereas *edOsHOX24* ones exhibited the opposite phenotype with a decrease in the adaxial side length (Fig. 2C-F). These results strongly suggested that OsHOX22 and OsHOX24 play contrasting roles in the lamina joint in this developmental stage of rice plants. While OsHOX22, directly or indirectly, would harm leaf blade opening, OsHOX24 would have a positive role in this process.

3.3. OsHOX22 and OsHOX24 regulate each other in the lamina joint

We observed that *edOsHOX22* and *edOsHOX24* plants displayed contrasting phenotypes in the lamina joint. This phenomenon is similar to that shown by other HD-Zip I paralog pairs, such as AtHB7 and AtHB12 and AtHB13 and AtHB23, which exhibit opposite behavior in the same tissues or growth stages. We inspected whether these rice genes influence each other by measuring the transcript levels of *OsHOX22* and *OsHOX24* in Kitaake, *edOsHOX22*, and *edOsHOX24* 9-day-old seedlings. The results indicated that *OsHOX22* and *OsHOX24* have opposite regulation in the lamina joint; i.e., each gene is induced in the background of the other gene-edited plants (Fig. 3), supporting non-redundant functions for these genes. It is worth noting that the expression scenario resulted quite differently in distinct tissues of the same plants (Supplementary Fig. S4). In young leaves, *OsHOX24* and *OsHOX22* were repressed in *edOsHOX22* and *edOsHOX24*, respectively, whereas in distal leaf blades, induction was observed instead of repression. Notably, in proximal leaf blades, *OsHOX24* was repressed in the *edOsHOX22* genotype, whereas *OsHOX22* transcripts increased in *edOsHOX24* seedlings (Fig. S4).

3.4. *OsHOX22* and *OsHOX24* expression is modulated by brassinosteroids impacting lamina joint inclination

Brassinosteroids (BRs) are the main hormones involved in the inclination grade of the leaf lamina joint (Luo et al., 2016). Given the differential and opposite phenotypes exhibited by *edOsHOX22* and *edOsHOX24* plants, we wondered whether BRs affected the expression of

these genes and the edited plant phenotypes. To answer this question, 9-day-old seedlings were treated with BR (200 mM brassinolide) for two hours, resulting in a marked repression and induction of *OsHOX22* and *edOsHOX24*, respectively, in the lamina joint (Fig. 4A). Then, we incubated a segment of the second leaf blade and leaf sheath of Kitaake and CRISPR-edited plants with 200 nM BR for 24 hours. As expected, Kitaake plants responded to the hormone treatment by opening their leaf angles (Fig. 4B) (Li et al., 2019), whereas *edOsHOX22* and *edOsHOX24* plants maintained their opposite leaf-angle phenotypes, which were emphasized by the BR treatment (Fig. 4B-D). BR treatment caused leaf angle opening in both edited genotypes, suggesting that HD-Zip FTs act upstream of the phytohormone. Alternatively, both pathways may be independent. When the edited plants were treated with 200 mM BR, the scenario significantly changed; *OsHOX22* expression continued to be repressed in the *edOsHOX24* genotype, but *OsHOX24*, induced by BR in the Kitaake plants (Fig. 4A), was repressed in the *edOsHOX22* one after the treatment (Fig. 4E). These observations suggested a negative feedback regulation between *OsHOX22* and the BR pathway (Fig. 4F).

In addition, after a 2-hour treatment with 50 μM BRZ, which inhibits the biosynthesis of BRs, no changes in the expression of *OsHOX22* and *OsHOX24* in the lamina joint of Kitaake were observed (Fig. 5A). Under the BRZ conditions assayed, *OsHOX24* expression decreased in *edOsHOX22* plants, and *OsHOX22* expression tended to increase in *edOsHOX24* plants (Fig. 5E, F). Furthermore, the incubation of a segment of the second leaf blade and leaf sheath of plants with 50 μM BRZ for 24 hours produced the closing of their leaf angles in Kitaake and *edOsHOX22* plants (Fig. 5B), as expected (Feng et al., 2016). Interestingly, the leaf inclination angle of *edOsHOX22* plants treated with BRZ resembled that of Kitaake plants in control conditions (Fig. 5C), and the percentage of leaf angle reductions due to the BRZ treatment was lower in *edOsHOX22* plants in comparison to Kitaake, indicating that their phenotype could be due to increased BR sensitivity or concentration in these edited plants. Likewise, this experiment allowed us to confirm that the leaf angle inclination of *edOsHOX24* plants resembles the phenotype of Kitaake plants treated with BRZ (Fig. 5D), suggesting that the phenotype of *edOsHOX24* plants could be due to decreased BR sensitivity or

content. Remarkably, *edOsHOX24* plants responded to this treatment by opening their leaf angles (Fig. 5D). So, the phenotypic response of the *edOsHOX24* plants to the BRZ treatment evidences the BR signaling complexity and that these conditions (*OsHOX24* editing and BRZ treatment) probably activated other BR pathways independent of the one proposed in this work involving HD-Zip FTs participation.

3.5. OsHOX22 and OsHOX24 modulate the expression of genes participating in BR biosynthesis and signaling

Given the crosstalk between the HD-Zip I TFs and BRs and to elucidate the relationship between these biomolecules, we evaluated the expression levels of genes participating in BR synthesis and signaling in the edited plants. *OsD11* (DWARF 11) encodes a cytochrome P450 (Tanabe et al., 2005) and *OsBRD1* (BR-DEFICIENT DWARF 1), an enzyme that catalyzes the C-6 oxidation step in BR synthesis (Hong et al., 2002); both are involved in BR biosynthesis. Neither the expression of *OsD11* nor that of *OsBRD1* changed in *edOsHOX22* plants (Fig. 6A), whereas the former was repressed and the latter induced in *edOsHOX24* plants (Fig. 6A), compared to Kitaake plants. Regarding BR signaling, *OsBZR1* (BRASSINAZOLE-RESISTANT 1) encodes a nuclear protein involved in the down-regulation of multiple BR-biosynthesis genes by a negative feedback mechanism, and *OsDLT* (DWARF AND LOW-TILLERING), the GRAS family protein that regulates BR responses (Kour et al., 2021; Tong et al., 2012), were assessed. The transcript levels of both genes were diminished in *edOsHOX22* plants and augmented in *edOsHOX24* ones (Fig. 6B). Altogether, these results suggested that only OsHOX24 could be involved in BR biosynthesis and both TFs in the BR signaling pathway.

3.6. ABA is involved in the lamina joint angle inclination in *edOsHOX22* and *edOsHOX24* plants

Considering that *OsHOX22* and *OsHOX24* are regulated by ABA (Bhattacharjee et al., 2017; Zhang et al., 2012), and ABA and BR interactions play crucial roles in the determination of the

leaf angle in rice (Li et al., 2021, 2019), we wondered about the role of both HD-Zip TFs in this crosstalk. *OsHOX22* and *OsHOX24* transcripts were evaluated after treatments with different ABA concentrations in the lamina joint of Kitaake plants. Lower ABA concentration (10 μM) induced the expression of *OsHOX24*, and the higher ones (1 mM) positively affected both genes (Fig. 7A). Then, we incubated a segment of the second leaf blade and leaf sheath of plants in 10 μM and 1 mM ABA for 24 hours (Fig. 7B). The lowest ABA concentration (10 μM) increased the leaf angle of Kitaake plants as expected (Li et al., 2021) and also of both CRISPR-edited plants (Fig. 7B-D). However, the higher ABA concentration (1 mM) triggered the closure of the lamina joint angle in all the genotypes (Fig. 7B). This scenario supported the idea that in the presence of high ABA concentrations, the leaf inclination phenotype becomes independent of the BR-pathway response, masking the roles of OsHOX22 and OsHOX24 in this process.

3.7. Abiotic stress suppresses the effect of OsHOX22 and OsHOX24 on the lamina joint inclination

To test the previous idea that the high ABA concentration masked the roles of OsHOX22 and OsHOX24 in the leaf inclination angle mechanism, we evaluated the leaf angle responses of Kitaake and CRISPR-edited plants under abiotic stress as an endogenous source of ABA. We exposed the plants to different salinity, cold, and drought conditions while the plants remained viable. The treatments applied were incubation at 4°C for 3, 6, and 10 hours, exposition to different salt concentrations for 5 days (100, 150, and 200 mM NaCl), and drought-induced treatment for 1, 1.5, and 2 hours. Thus, all genotypes closed their leaf angles (Table 1 and Fig. 8B) similarly to the Kitaake plants treated with the higher ABA concentration (Figs. 7B-D). *OsHOX22* and *OsHOX24* expression increased in Kitaake and CRISPR-edited plants, evidencing higher ABA endogenous levels (Figs. 8A-B, D-E). On the one hand, these findings supported the previous reports that both HD-Zip TFs agonistically contribute to the ABA response under abiotic stress (Bhattacharjee et al., 2016; Zhang et al., 2012). On the other hand, as the ABA pathway masks the BR response under these conditions, OsHOX22 and OsHOX24 functions do not impact the lamina joint inclination mechanism.

4. Discussion

In this study, we analyzed the phenotype of two rice varieties obtained by genome editing of *OsHOX22* and *OsHOX24* genes using CRISPR/Cas9 technology. The mutations obtained by the Cas9 activity and the NHEJ mechanism were consistent with the rice CRISPR/Cas9 system predictions, pointing out that the most common editions are 1-bp insertions or short deletions (Fig. 1). Mainly A (44.8%) or T (43.4%) (H. Zhang et al., 2014) were predicted as insertions. *OsHOX22* and *OsHOX24* are paralog members of the HD-Zip I family, which is unique to the plant kingdom (Agalou et al., 2008). Previous studies reported that both TFs were involved in the ABA response to salinity and drought stresses, and their overexpression produced similar phenotypes (Bhattacharjee et al., 2017, 2016; Zhang et al., 2012). However, our results evidenced different activities in the lamina joint inclination: a negative regulatory function for *OsHOX22* and a positive role for *OsHOX24*, as shown by the opposite phenotype of the CRISPR-edited plants (Fig. 2), and the opposite regulation of their expression levels in *edOsHOX22* and *edOsHOX24* lamina joints (Fig. 3). In agreement, the *Arabidopsis thaliana* paralog pair of the same clade as *OsHOX22* and *OsHOX24*, described by Agalou et al., (2008), also exhibited the same crosstalk regulation (Ré et al., 2014). The TFs involved in the crossed regulation between OsHOX22 and OsHOX24 still need to be experimentally determined since their promoter regions lack the pseudo-palindrome CAAT(A/T)ATTG, which was previously reported as a target sequence of HD-Zip I proteins (Palena et al., 1999). Moreover, AtHB13 and AtHB23, two other HD-Zip I paralogs (α-clade), were found to be non-redundant in several development events and cooperatively acting in others (Ribone et al., 2015), indicating that the expression of the HD-Zip I family duplicated members during development in different tissues is finely regulated on a case-by-case basis, according to experimental evidence.

The leaf angle is an important agronomic trait contributing to crop yield determination since it influences the light capture for photosynthesis, grain filling, and plant density (Sakamoto et al., 2006). The current knowledge indicates that most phytohormones such as auxin, gibberellin (GA), methyl jasmonate (MeJA), and ethylene regulate the leaf angle through BRs signaling

pathways (Luo et al., 2016). Many mutants involved in BR biosynthesis or signaling showed differential lamina joint inclination angles, correlating with the *in vivo* BR level changes (Luo et al., 2016; Zhang et al., 2014). Although BRs are the main hormones controlling leaf angle opening (Luo et al., 2016; Sakamoto et al., 2006), ABA has been recently involved in this event (Li et al., 2021, 2019). The *OsHOX22* and *OsHOX24* gene expression patterns in Kitaake and CRISPR-edited plants under BR treatment (Fig. 4) suggested their opposite participation in one of the multiple feedback mechanisms regulating BR levels (Tong et al., 2012). In addition, it was observed that the *edOsHOX22* plants exhibited a phenotype that was similar to Kitaake plants treated with BRs. Furthermore, *edOsHOX24* plants treated with BRZ resembled the Kitaake plants, which had a contrasting sensitivity to BR levels (as seen in Fig. 5). It was also noted that the *OsHOX24* expression significantly reduced after BR and BRZ treatments in *edOsHOX22* (Figs. 4 and 5). Notably, the BRZ treatment in *edOsHOX24* plants generated the opposite of the expected phenotype. These observations suggest that the balance between both HD-Zip I TFs plays a crucial role in regulating BR levels under normal conditions. However, when the BR content is exogenously modified, the CRISPR-edited plants evidence the complexity of the BR pathway.

Although OsHOX22 and OsHOX24 are involved in ABA-related abiotic stress (Bhattacharjee et al., 2016; Zhang et al., 2012), our results suggest they also participate in the leaf angle opening through the BR response during normal growth conditions. Under abiotic stress or high ABA concentration treatment, the expression of both HD-Zip TFs increased. At the same time, the leaf angle of all genotypes was closed, indicating that ABA signaling masked the BR-response impact on the leaf blade inclination. This evidence strongly suggests that the OsHOX22 and OsHOX24 roles in the leaf inclination angle mechanism are irrelevant at high ABA concentrations (Figs. 7 and 8 and Table 1). The scheme presented in Figure 9 summarizes a proposed model for OsHOX22 and OsHOX24 functions in the feedback mechanism of the BR pathway in the lamina joint under normal and stress conditions. Accordingly, the differential gene expression related to BR biosynthesis or signaling also evidenced their antagonistic role in the BR response. The *edOsHOX24* plants showed a decrease in *OsD11* and an increase in

OsBRD1 transcript levels (Fig. 6A) compared with Kitaake. In agreement, a previous study reported a similar expression pattern of BR-biosynthesis genes under high-ABA concentration treatment (Li et al., 2019), which resembled the phenotype of *edOsHOX24* plants. As BR signaling could inhibit both BR biosynthesis and signaling through multiple feedback mechanisms (Tong et al., 2014, 2012), BR perception suppressed the *OsBZR1* expression that regulates cell elongation and, consequently, the lamina joint inclination (Gruszka, 2020). Both OsBZR1 and OsDLT TFs are regulators of BR signaling in rice. According to the hypothesis of a differential BR perception, *OsBZR1* and *OsDLT* were repressed in *edOsHOX22* plants and increased in *edOsHOX24* plants (Fig. 6B). *OsDLT* is a member of a plant-specific GRAS family of TFs (Gruszka, 2020). Based on a feedback mechanism, BRs also repressed *OsDLT* gene expression at the transcriptional level via OsBZR1 in rice seedlings (Tong et al., 2014, 2012). Although OsBZR1 binds to the BR-response element CGTG(T/C)G of BR-repressed genes (He et al., 2005), 1 µM BR reduces *OsBZR1* and *OsDLT* transcript accumulation, indicating that this hormone regulates both genes in different ways (Yang et al., 2016). Interestingly, *OsHOX22* and *OsHOX24* contain BR-response elements in their promoter regions (Table S3), which reinforces the idea of their participation in the BR regulation levels. On the other hand, OsDLT participates in the feedback inhibition of the BR biosynthetic genes (Tong et al., 2009), as shown by the repression of *OsD11* transcripts in *edOsHOX24* plants (Fig. 6A), suggesting the participation of OsHOX24 in this pathway.

Our study has shed light on the intricate hormonal regulatory mechanisms involved in the BR pathway's determination of leaf angle opening. With rice being a significant crop and BR having immense potential in biotechnology (Divi and Krishna, 2009), the distinct contributions of OsHOX22 and OsHOX24 underscore the complexity of the BR response mechanism under various environmental conditions. Our findings add to the knowledge of the molecular factors that regulate leaf angle opening in the BR pathway.

5. Conclusion

OsHOX22 and OsHOX24 differentially influence the leaf angle opening in the lamina joint, constituting an important agronomic trait. Moreover, these two TFs actively participate in a feedback mechanism that modulates BR levels in this tissue. Future studies will be necessary to elucidate this intricate hormonal and molecular network.

Author contributions

CVA conceived and supervised the whole study. VT and MC performed all the experiments. VT, EW, RLC, and CVA analyzed the data. EW, RLC, and CVA wrote the manuscript.

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Conflict of interest

The authors declare no competing interests.

Supplementary material

Figure S1: Alignment of OsHOX22 and OsHOX24 protein sequences. Protein sequences were aligned using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).
In gray is shown the homeodomain, and in orange are the typical leucine (L) or isoleucine (I) residues of the zipper domain. Asterisks indicate conservation, ".." similarity, and ":" divergence.

Figure S2: Templates used to build the models for the different protein products obtained in each CRISPR-edited plant. The Swiss-Model tool (https://swissmodel.expasy.org/) was used.

Figure S3: Protein sequence alignment of OsHOX22 and OsHOX24 with the respective protein products of homozygous CRISPR-edited plants using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). In gray is shown the homeodomain, and in orange are the typical leucine (L) or isoleucine (I) residues of the zipper domain. Asterisks indicate conservation, ".." similarity, and ":" divergence.

Figure S4: *OsHOX24* and *OsHOX22* exhibit different expression patterns depending on the tissue. The upper panel shows *OsHOX24* levels in young leaves **(A)**, the proximal leaf blade **(B)**, and the distal leaf blade of *edOsHOX22* plants **(C)**. The lower panel shows *OsHOX22* levels in young leaves **(D)**, the proximal leaf blade **(E)**, and the distal leaf blade **(F)** in *edOsHOX24* plants. **(G)** Schematic representation of the proposed expression effects of each protein on the other (positive effect: →; negative effect: —ı). **(H)**: different leaf sections used for the analysis: young leaves; (2): proximal leaf blade; and (3): distal leaf blade. Transcript levels were normalized with the one obtained in the Kitaake genotype and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 seedlings per homozygous line) + SD. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are denoted by asterisks, according to the Student's t-test.

Table S1: Oligonucleotides used for gRNA preparation, gene expression, and editing analysis.

Table S2: Genome edition results for T_0 plants based on the web tool DSDecode.

Table S3: PlantPAN 3.0-based BR-response elements CGTG(T/C)G in the promoter sequences of BR-repressed genes (http://plantpan.itps.ncku.edu.tw/search.php#species) (Chow et al., 2019).

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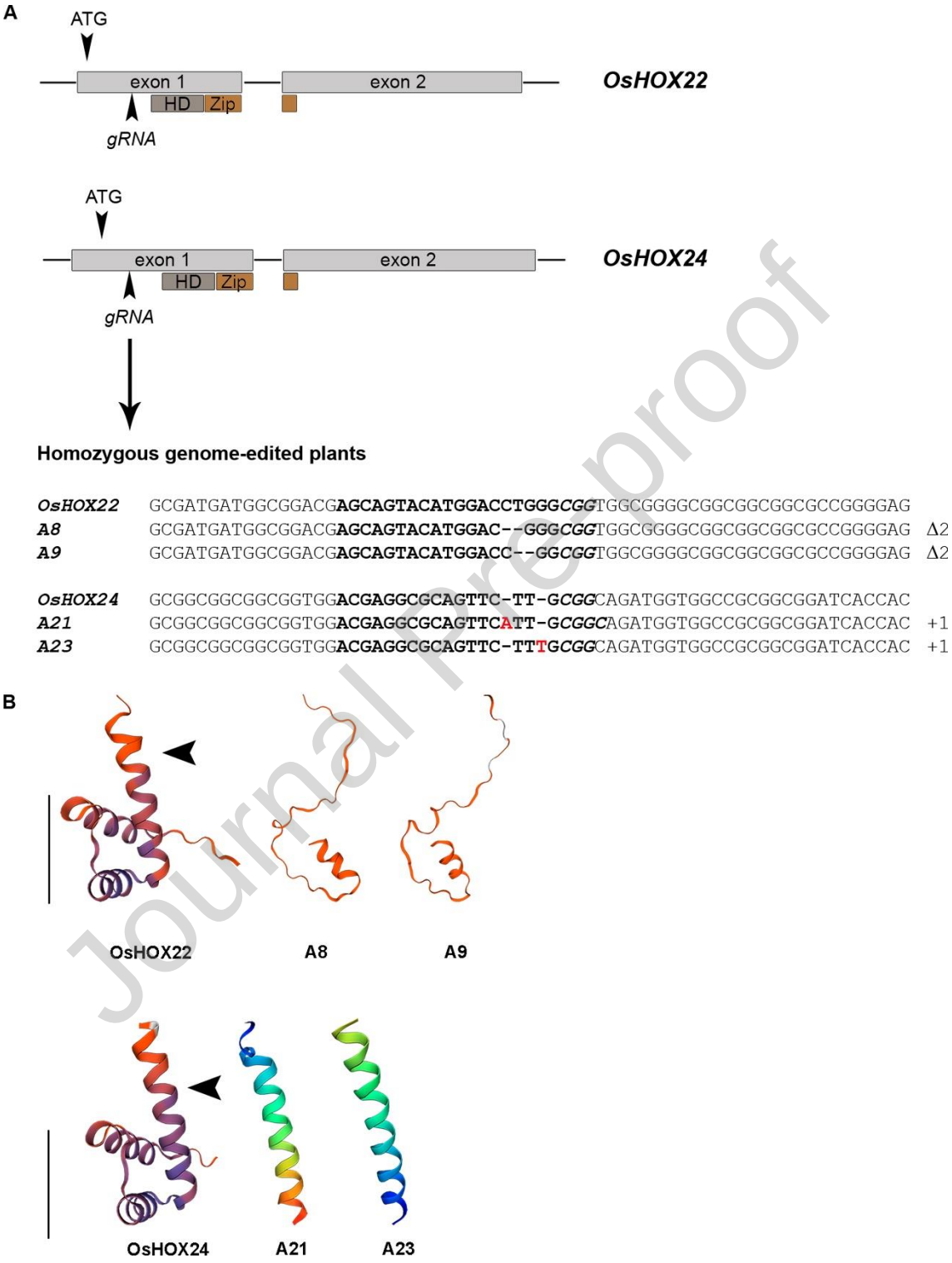

Figure 1: Genome editing results for *OsHOX22* and *OsHOX24*. (A) Gene structure of *OsHOX22* and *OsHOX24*. The Homeodomain (HD) is shown in light gray, while the Leucine zipper (Zip) is denoted in brown. Arrows indicate the translation start codon (ATG) and the gRNA

recognition site. The gRNA recognition sequence is shown in bold letters at the bottom. The deletions obtained for homozygous lines of CRISPR-edited plants to *OsHOX22* (A8 and A9) are shown as "-", whereas the insertions for CRISPR-edited plants to *OsHOX24* (A21 and A23) are signaled in red. The PAM sequences are shown in italics. **(B)** Protein structure prediction of OsHOX22, OsHOX24, and the probable protein products in the CRISPR-edited plants (A8, A9, A21, and A23) using the Swiss-Model tool (https://swissmodel.expasy.org/). The HD is shown with vertical lines and the Zip with arrows.

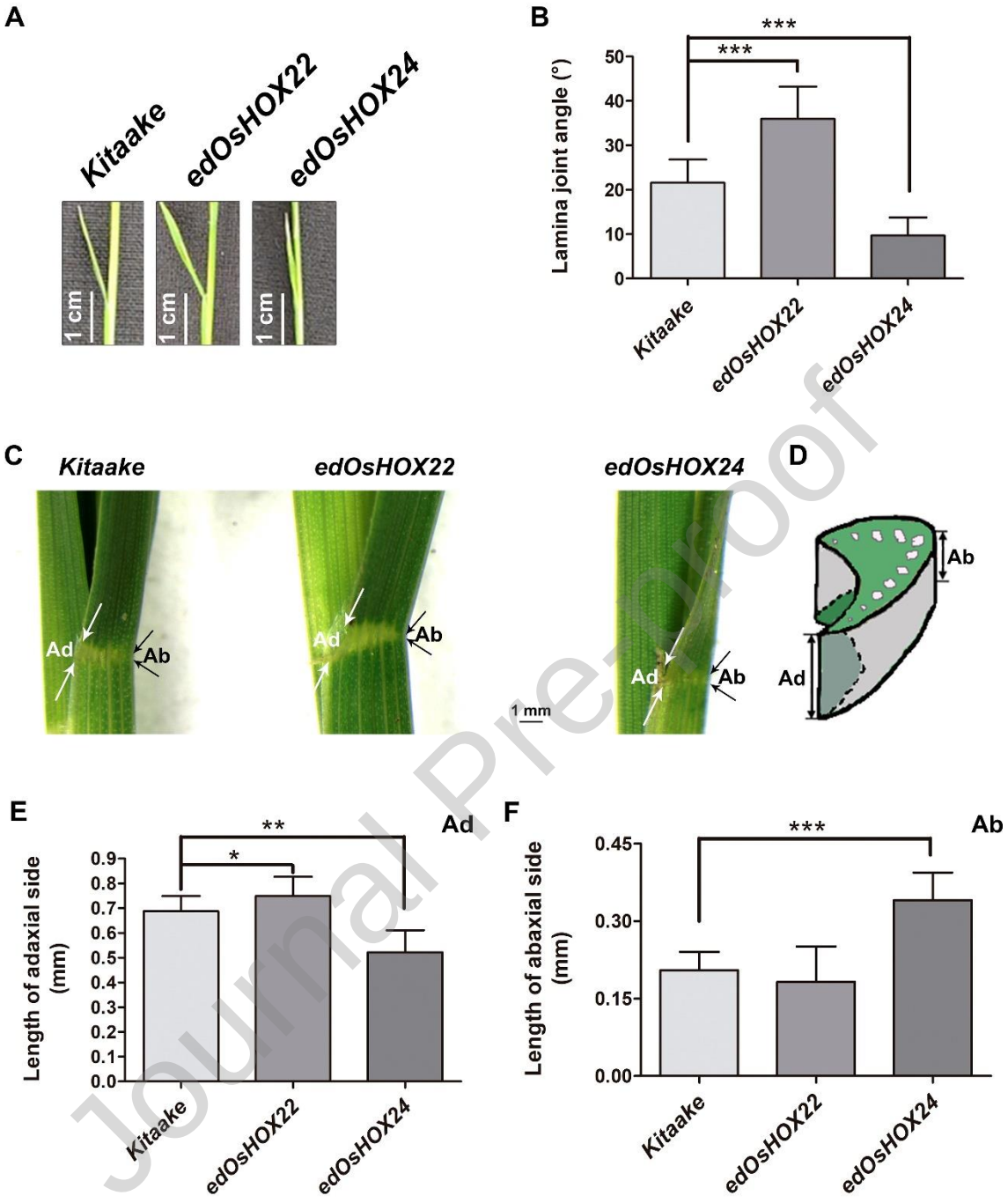

Figure 2: *edOsHOX22* and edOsHOX24 edited plants exhibit opposite lamina joint inclination phenotypes. (A) Illustrative image of the second-leaf lamina joint, showing the differential inclination between wild-type Kitaake (*Oryza sativa* ssp. japonica) and CRISPR-edited in *OsHOX22* (*edOsHOX22*) and *OsHOX24* (*edOsHOX24*) 9-day-old seedlings. **(B)** Data evaluation of the images showed in (A). **(C)** Amplification of the images shown in (A), indicating the abaxial (Ab) and adaxial (Ad) sides. **(D)** Schematic representation of the evaluated lengths corresponding to the adaxial and abaxial sides of the lamina joint. **(E)** and (**F**)

Quantitative assessment of the adaxial (E) and abaxial (F) side lengths shown in (C). Bars represent the mean of 20 seedlings (10 per homozygous line) + SD. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) are denoted by asterisks, according to the Student's t-test.

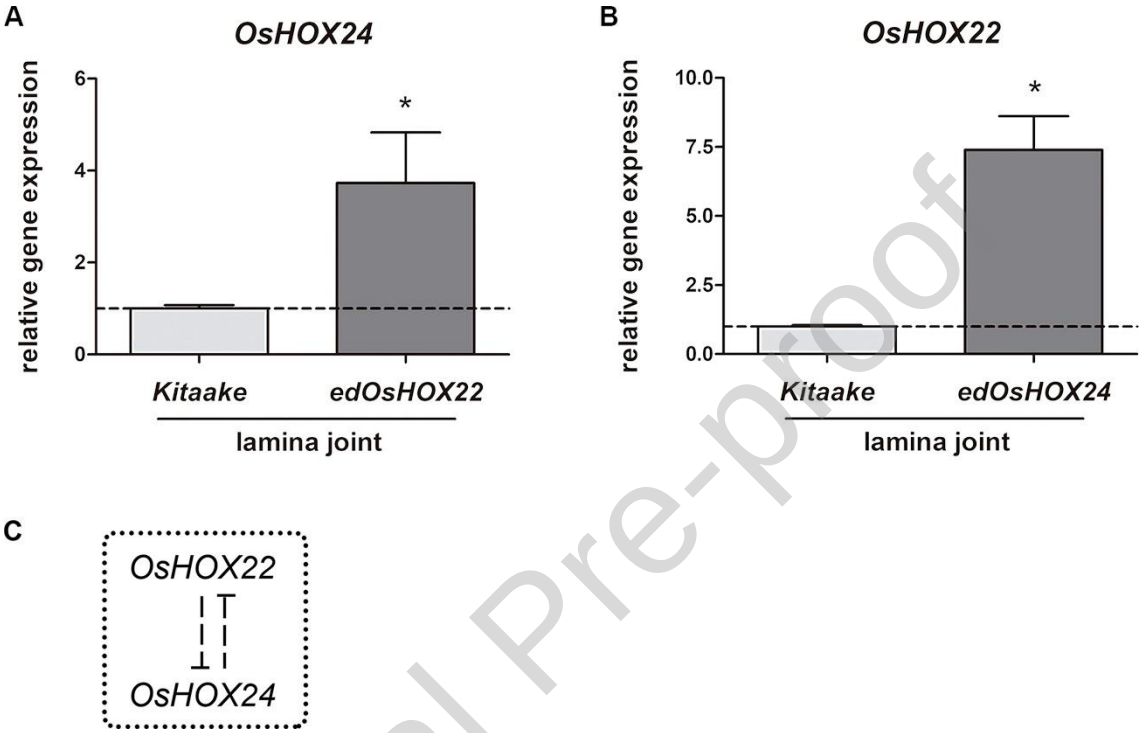

Figure 3: *OsHOX22* and *OsHOX24* modulate each other expression in CRISPR-edited plants. Transcriptlevels of *OsHOX24* **(A)** and *OsHOX22* **(B)** in the second-leaf lamina joint of 9-day-old Kitaake and CRISPR-edited plants. **(C)** Schematic representation of the proposed effect for each TF on the other (negative effect: ⊣). The values were normalized with the one obtained in the Kitaake genotype, arbirtrary assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 per homozygous line) + SD. Statistically significant differences (* $p < 0.05$) are denoted by asterisks, according to the Student's t-test.

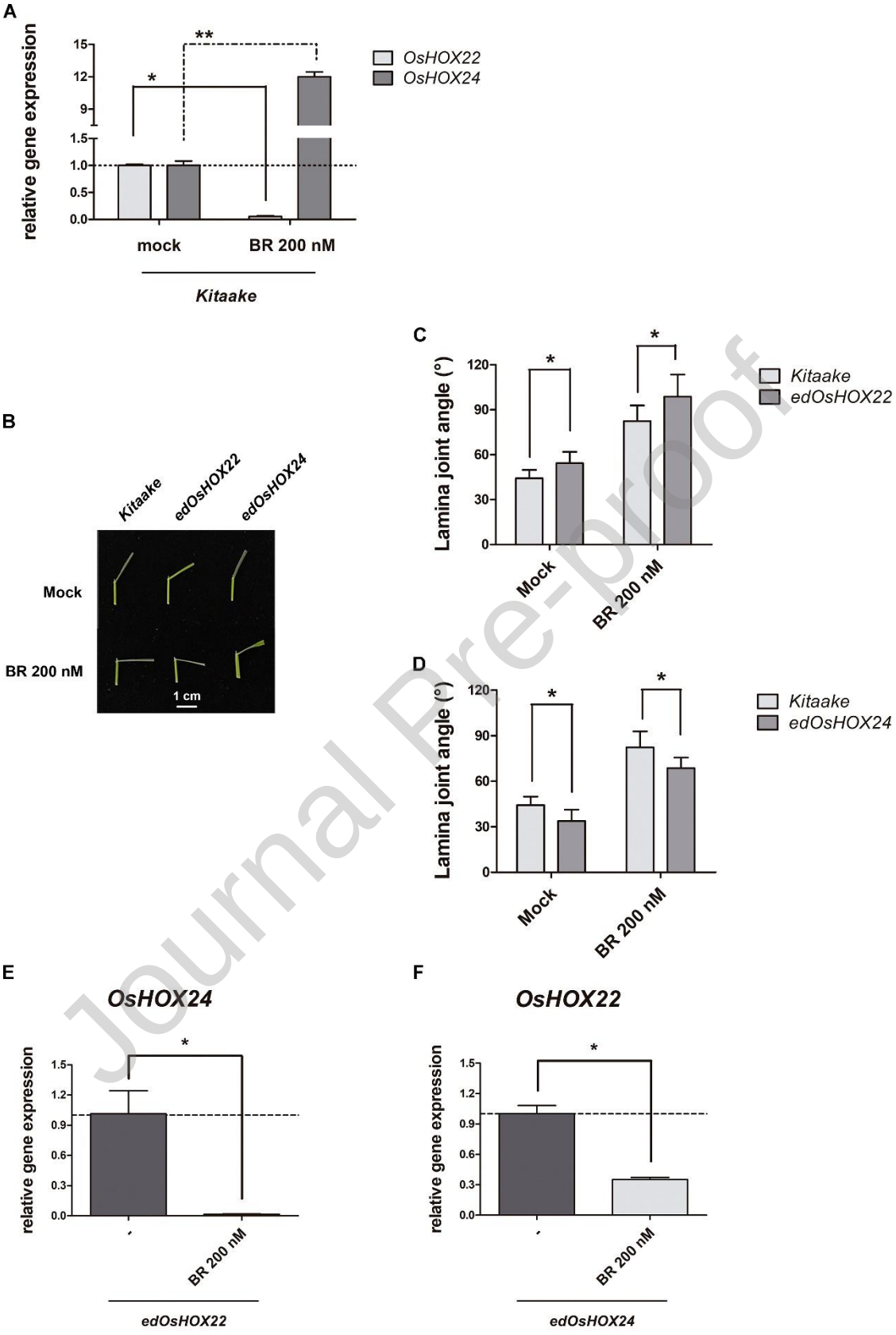

Figure 4: Brassinosteroids affect *OsHOX22* and *OsHOX24* expression, impacting the lamina joint inclination phenotype. (A) Expression levels of *OsHOX22* and *OsHOX24* in 9-

day-old Kitaake plants treated for 2 hours with 200 nM BR. **(B)** Illustrative pictures of the lamina joint phenotype of Kitaake, *edOsHOX22,* and ed*OsHOX24* plants treated for 24 hours with 200 nM BR. Quantitative evaluation of the lamina joint angle of *edOsHOX22* **(C)** and *edOsHOX24* **(D)** plants. Transcript levels of *OsHOX24* **(E)** and *OsHOX22* **(F)** in 9-day-old *edOsHOX22* and *edOsHOX24* plants treated for 2 hours with 200 nM BR. Transcript levels were normalized with the one obtained in the Kitaake or CRISPR-edited genotype, and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 seedlings per homozygous line) + SD. Asterisks denote statistically significant differences (* $p < 0.05$, and ** $p < 0.01$) according to the Student's t-test.

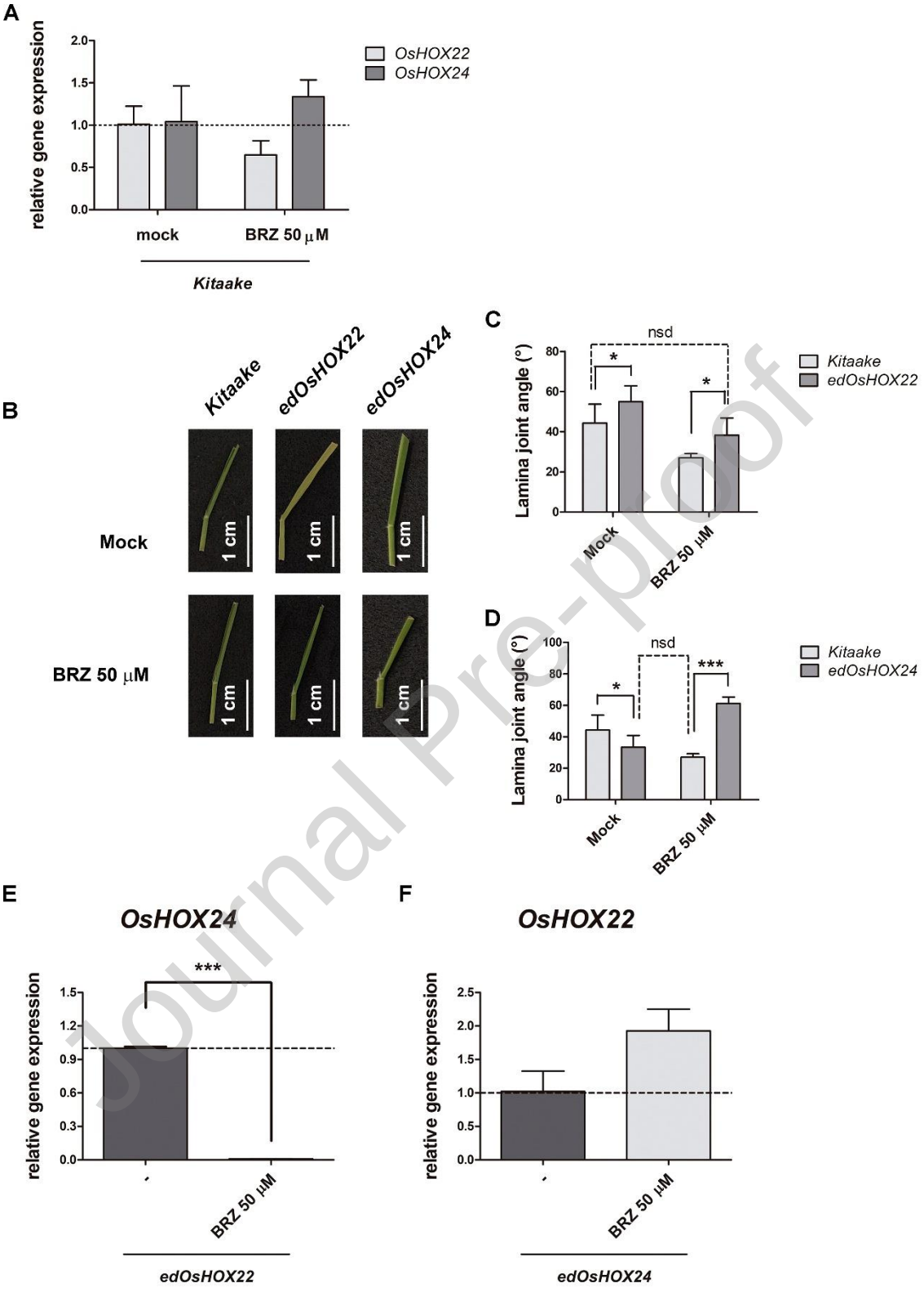

Figure 5: Brassinazole impacts the lamina joint inclination phenotype. (A) Expression levels of *OsHOX22* and *OsHOX24* in 9-day-old Kitaake plants treated for 2 hours with 50 µM BRZ. **(B)** Illustrative pictures of the lamina joint phenotype of Kitaake, *edOsHOX22*, and ed*OsHOX24* plants treated for 24 hours with 50 µM BRZ. Quantitative evaluation of the lamina

joint angle of *edOsHOX22* **(C)** and *edOsHOX24* **(D)** plants. Transcript levels of *OsHOX24* **(E)** and *OsHOX22* **(F)** in 9-day-old *edOsHOX22* and *edOsHOX24* plants treated for 2 hours with 50 µM BRZ. Transcript levels were normalized with the one obtained in the Kitaake or CRISPR-edited genotype, and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 seedlings per homozygous line) + SD. Asterisks denote statistically significant differences (* $p < 0.05$, and *** $p < 0.001$) according to the Student's t-test.

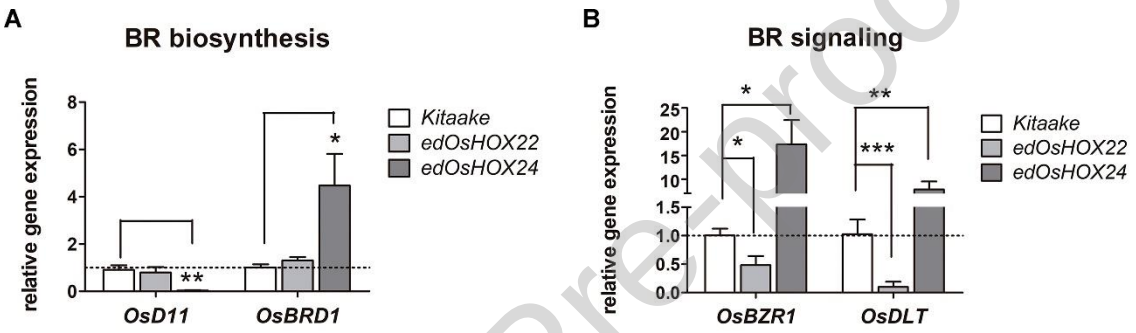

Figure 6: *OsHOX22* and *OsHOX24* modulate genes involved in brassinosteroid biosynthesis and signaling. (A) Transcript levels of *OsD11* and *OsBDR1*, which are involved in BR synthesis. **(B)** Transcript levels of *OsBZR1* and *OsDLT*, which are involved in BR signaling. Transcript levels were normalized with the one obtained in the Kitaake genotype, and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 per homozygous line) + SD. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are denoted by asterisks, according to the Student's t-test.

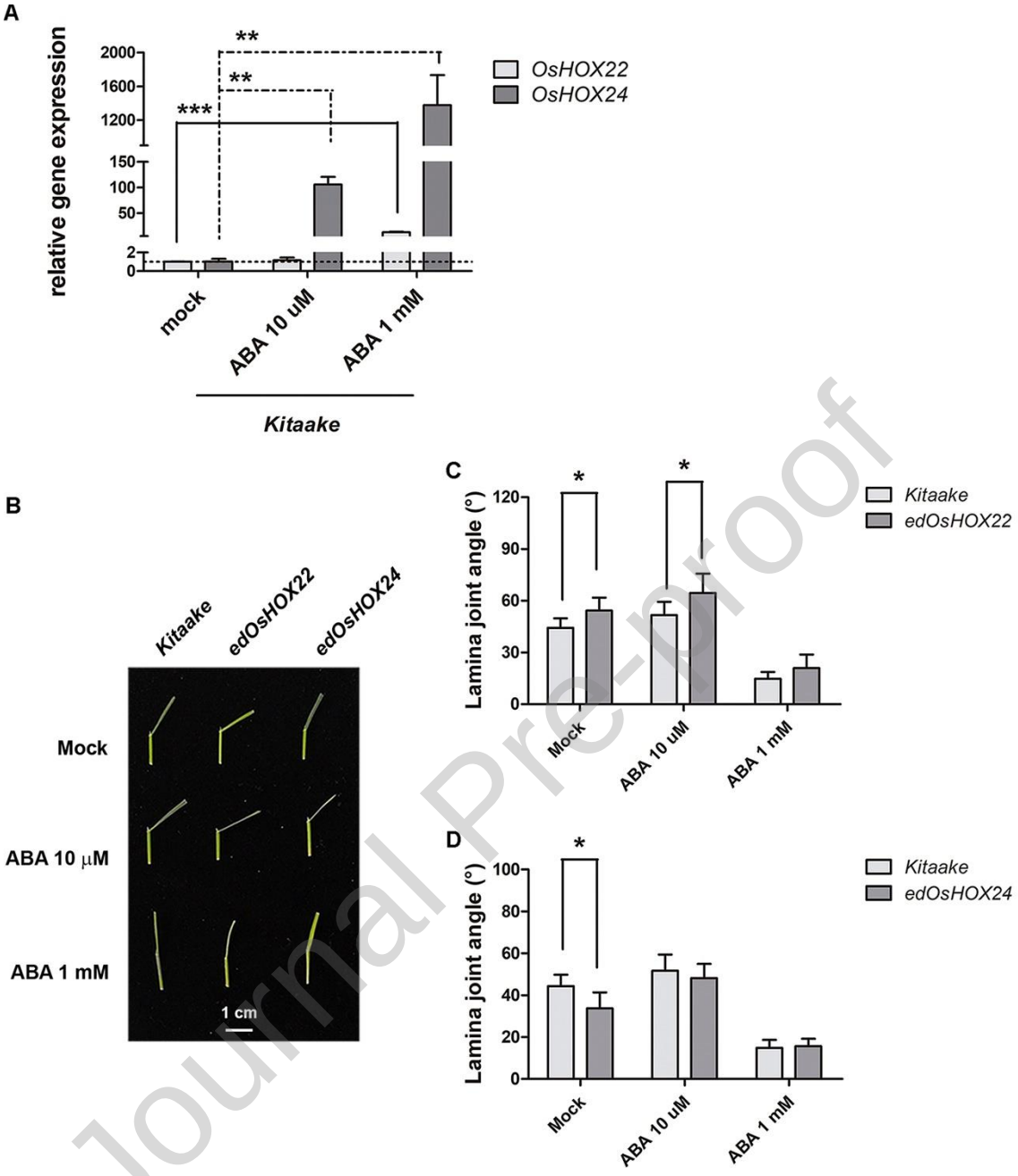

Figure 7: ABA treatment affects the lamina joint inclination of *edOsHOX22* and *edOsHOX24* plants. (A) *OsHOX22* and *OsHOX24* expression in 9-day-old Kitaake plants treated for 2 hours with 10 μM ABA and 1 mM ABA. **(B)** Illustrative picture of the lamina joint phenotype of Kitaake, *edOsHOX22*, and *OsHOX24* 9-day-old plants treated for 24 hours with 10 μM ABA and 1 mM ABA. Quantitative evaluation of the parameters shown in (B) in *edOsHOX22* **(C)** and *edOsHOX24* **(D)** plants. Transcript levels were normalized with the one obtained in the Kitaake genotype, and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 per homozygous line) + SD.

Statistically significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are denoted by asterisks, according to the Student's t-test.

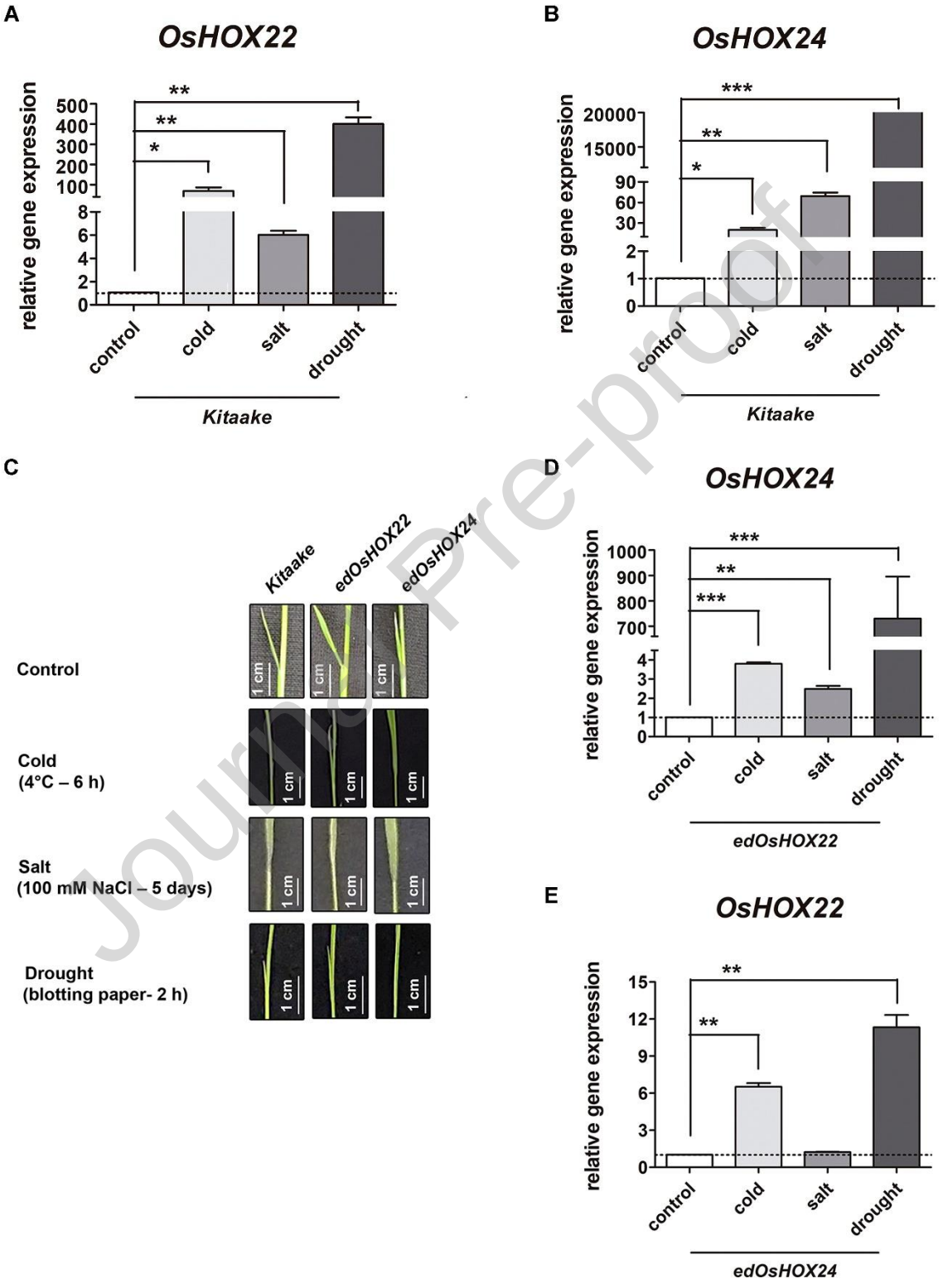

Figure 8. Abiotic stress factors affect *OsHOX22* and *OsHOX24* expression, impacting the lamina joint angle. *OsHOX22* **(A)** and *OsHOX24* **(B)** gene expression in the lamina joint of 9-day-old Kitaake plants, subjected to cold stress of 4°C for 6 hours, exposed under salt stress conditions of 100 mM NaCl for 5 days, or under drought stress conditions for 2 hours on blotting paper. **(C)** Illustrative pictures of the lamina joint phenotypes of 9-day-old plants exposed to 4 °C for 6 hours, 100 mM NaCl for five days, and water deficit for 2 hours on blotting paper. Relative transcript levels of *OsHOX24* **(D)** and *OsHOX22* **(E)** in 9-day-old *edOsHOX22* and *edOsHOX24* plants exposed to the conditions described in (B). Transcript levels were normalized with the one obtained in the Kitaake genotype, and arbitrarily assigned a value of 1 (one), using *OsEF-1α* as housekeeping. Bars represent the mean of 20 seedlings (10 seedlings per homozygous line) + SD. Statistically significant differences (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) are denoted by asterisks, according to the Student's t-test.

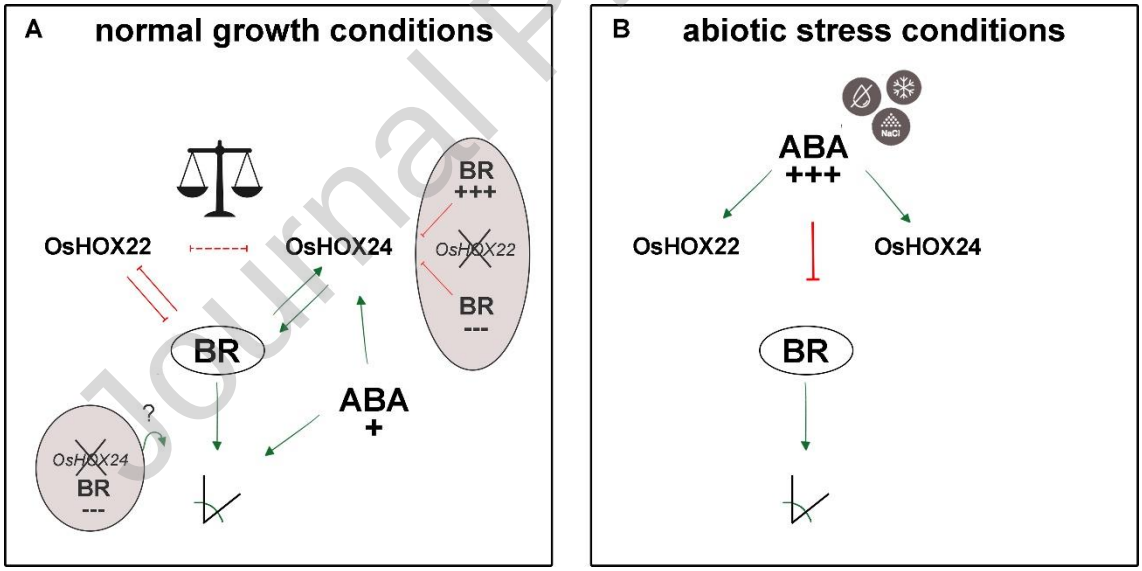

Figure 9: Proposed model of OsHOX22 and OsHOX24 functions related to lamina joint angle. Schematic representation of the proposed mechanisms of action of both TFs in normal growth conditions **(A)** and under abiotic stress or increased ABA concentration treatment **(B)**. BR+++ denotes the exogenous BR treatment; BR---, the BRZ treatment; ABA+, the 10 μM ABA treatment; and ABA+++, the 1 mM ABA treatment. In normal growth conditions (A), the gray

circles represent the differential responses shown for *edOsHOX22* plants (at right) and *edOsHOX24* plants (at left).

Table 1: Quantitative data for analysis of the lamina joint angle under abiotic stress conditions.

Genotype	Lamina joint angle (°)	Treatment	*
Kitaake	16.1 ± 5.7	Control	A
edOsHOX22	24.5 ± 7.7		B
edOsHOX24	10.6 ± 4.8		C
Kitaake	6.3 ± 2.0	Cold (4°C – 3 h)	C
edOsHOX22	13.2 ± 4.7		AC
edOsHOX24	6.7 ± 2.2		C
Kitaake	6.0 ± 1.6	Cold (4°C – 6 h)	C
edOsHOX22	7.1 ± 1.2		C
edOsHOX24	5.8 ± 1.9		C
Kitaake	5.7 ± 1.9	Cold (4°C – 10 h)	C
edOsHOX22	6.6 ± 1.4		C
edOsHOX24	5.4 ± 1.5		C
Kitaake	9.3 ± 3.4	Salt (100 mM NaCl – 5 days)	C
edOsHOX22	8.1 ± 3.6		C
edOsHOX24	8.4 ± 1.6		C
Kitaake	6.7 ± 1.4	Salt (150 mM NaCl – 5 days)	C
edOsHOX22	7.0 ±1.9		C
edOsHOX24	6.6 ± 2.0		C
Kitaake	9.5 ± 2.8	Salt (200 mM NaCl – 5 days)	C
edOsHOX22	7.5 ± 1.8		C
edOsHOX24	6.0 ± 2.0		C
Kitaake	13.3 ± 6.0	Drought (blotting paper- 1 h)	AC
edOsHOX22	15.7 ± 6.0		AC
edOsHOX24	12.7 ± 4.3		AC
Kitaake	6.8 ± 2.1	Drought (blotting paper- 1:30 h)	C
edOsHOX22	7.7 ± 0.5		C
edOsHOX24	7.1 ± 1.8		C
Kitaake	6.7 ± 2.4	Drought (blotting paper- 2 h)	C

edOsHOX22	8.4 ± 2.1	C
edOsHOX24	5.1 ± 2.3	C

* Significant differences ($p < 0.01$) as determined by two-way ANOVA followed by Tukey's test are indicated by distinct letters.

Author contributions

CVA conceived and supervised the whole study. VT and MC performed all the experiments.

VT, EW, RLC, and CVA analyzed the data. EW, RLC, and CVA wrote the manuscript.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Carolina V. Attallah reports financial support was provided by National University of the Littoral. Raquel L. Chan reports financial support was provided by National Scientific and Technical Research Council. Raquel L. Chan reports financial support was provided by Santa Fe Agency of Science Technology and Innovation.

Highlights:

- Edited plants in the HD-Zip I encoding genes OsHOX22 and OsHOX24 were obtained.
- The analysis of edited plants indicates a mutual negative regulation between these proteins.
- OsHOX22 and OsHOX24 modulate BR signaling, impacting lamina joint angle inclination.
- OsHOX22 and OsHOX24 exhibit opposite functions related to lamina joint architecture.
- Drought, cold, and salinity stresses do not differentially affect the phenotype.