Short title: Mechanism of miR2105-OsbZIP86 module in drought tolerance

miR2105 regulates ABA biosynthesis via OsbZIP86-*OsNCED3* module to contribute to drought tolerance in rice

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One-sentence summary: 'miR2105-OsbZIP86-*OsNCED3*' module plays crucial role in mediating ABA biosynthesis to contribute to drought tolerance with no penalty with respect to agronomic traits under normal conditions.

29 **Author contributions**

30 M. Zhang and K. Xia proposed the project. W. Gao, M. Li, S. Yang, Y. Su, C. Gao, Z. Jiao, W. Xu and
31 X. Zeng performed the experiments. W. Gao, M. Zhang and K. Xia drafted the manuscript. M. Zhang,
32 K. Xia and S. Yang revised the manuscript. All authors read and approved the final manuscript.

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38

Abstract

Induced abscisic acid (ABA) biosynthesis plays an important role in plant tolerance to abiotic stresses, including drought, cold and salinity. However, regulation pathway of the ABA biosynthesis in response to stresses is unclear. Here, we identified a rice miRNA, osa-miR2105 (miR2105), which plays a crucial role in ABA biosynthesis under drought stress. Analysis of expression, transgenic rice and cleavage site showed that *OsbZIP86* is a target gene of miR2105. Subcellular localization and luciferase activity assays showed that OsbZIP86 is a nuclear transcription factor. *In vivo* and *in vitro* analyses showed that OsbZIP86 directly binds to the promoter of *OsNCED3*, and interacts with OsSAPK10, resulting in enhanced-expression of *OsNCED3*. Transgenic rice plants with knock-down of miR2105 or overexpression of *OsbZIP86* showed higher ABA content, more tolerance to drought, a lower rate of water loss, more stomatal closure than wild type rice ZH11 under drought stress. These rice plants showed no penalty with respect to agronomic traits under normal conditions. By contrast, transgenic rice plants with miR2105 overexpression, *OsbZIP86* downregulation, or *OsbZIP86* knockout displayed less tolerance to drought stress and other phenotypes. Collectively, our results show that a regulatory network of 'miR2105-OsSAPK10/OsbZIP86-*OsNCED3*' control ABA biosynthesis in response to drought stress.

Keywords: abscisic acid, biosynthesis, drought, miRNA, rice, transcription factor.

Introduction

Rice (*Oryza sativa* L.) is one of the most important grain crops in the word, and both biotic and abiotic stresses are major threats to its yield. Therefore, breeding programs are directed towards increasing yield and improving tolerance to biotic and abiotic stresses (Kumar *et al.*, 2018). To cope with most stresses, plants synthesize abscisic acid (ABA), which activates ABA-mediated signaling pathways, including stomatal closure under drought stress, metabolic adjustment, growth regulation and regulation of defense-related genes (Finkelstein *et al.*, 2002; Joo *et al.*, 2020). Therefore, ABA is one of the most important phytohormones regulating plant growth, development, and stress response (Nambara & Marion-Poll, 2005; Chen *et al.*, 2020). The core components of ABA biosynthesis, catabolism, transport, and signaling have been identified (Chen *et al.*, 2020). ABA is synthesized in the plastids and the cytosol from zeaxanthin in a five-step biosynthetic process in *Arabidopsis*. The first

three steps of conversion from the precursor β-carotene to violaxanthin and neoxanthin, which are catalyzed by ABA DEFICIENT 1 (ABA1), ABA4, and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), take place in the plastids; violaxanthin and neoxanthin are then transported into the cytosol for the next two steps, in which ABA is produced by the catalysis of ABA2 and ABA3 in Arabidopsis (Nambara & Marion-Poll, 2005; Chen *et al.*, 2020).

NCEDs cleave violaxanthin and neoxanthin to produce xanthoxin in the ABA biosynthetic pathway; this is the rate-limiting step of ABA *de novo* biosynthesis (Nambara & Marion-Poll, 2005; Dong *et al.*, 2015). *NCED*s belong to a multigene family in plants, and their expression is tightly regulated in response to developmental or stress conditions. Rice has five *NCED*s. *OsNCED1* is most highly expressed in leaves as a housekeeping gene under normal conditions and is feedback-regulated by ABA, suppressed by water stress, and induced in cold-stressed anthers (Ye *et al.*, 2011). *OsNCED2* has roles in the seed germination (Zhu *et al.*, 2009) and grain ABA production (Nonhebel & Griffin, 2020). *OsNCED3*, *OsNCED4* and *OsNCED5* mediate ABA biosynthesis, and confer to different stress tolerance (Huang *et al.*, 2018; Hwang *et al.*, 2018; Huang *et al.*, 2019). *OsNCED3* is constitutively expressed in various tissues under normal conditions, and responses to multi-abiotic stresses in plant growth (Huang *et al.*, 2018). The expression of *OsNCED3* is rapidly induced by drought stress and quickly decreased after rehydration; thus, it is a major gene promoting ABA biosynthesis during drought stress in rice (Ye *et al.*, 2011; Mao *et al.*, 2017; Liu *et al.*, 2018).

The 20–22 nucleotide microRNAs (miRNAs) have critical roles in both plant development and biotic and abiotic stress responses, including ABA response (Nadarajah & Kumar, 2019). miRNAs can regulate gene expression at post-transcriptional levels through specific base-pairing to target mRNAs (Bartel, 2004). In rice, a serial of miRNAs, such as miR156, miR159, miR168, miR169, miR319, and miR395 are involved in various stress response (Zhou *et al.*, 2010). miR159 is induced by drought stress and provides tolerance against abiotic stresses such as drought in rice (Mohsenifard *et al.*, 2017). miR319 is also induced by salinity and drought stress and makes a positive contribution to the plant abiotic stress response (Koyama *et al.*, 2017). In Arabidopsis, miR165 and miR166 regulate expression of BG1, a glucosidase; BG1 hydrolyzes Glc-conjugated ABA, which in turn further modulates ABA homeostasis (Yan *et al.*, 2016). miR2105 has been isolated from developing rice seeds (Xue *et al.*, 2009), but its role has remained elusive.

The bZIP transcription factors (TFs) also have crucial regulatory roles in activating ABA-dependent

stress-responsive gene expression (Joo *et al.*, 2021). It is predicated that rice encodes 89 bZIP TFs, several of which have been found to be involved in rice stress responses (Joo *et al.*, 2021). For example, OsbZIP23 positively regulates the transcription of *OsNCED4* to mediate ABA biosynthesis (Xiang *et al.*, 2008; Zong *et al.*, 2016). OsbZIP71 directly binds to G-box sequences in the promoters of *OsNHX1* and *COR413-TM1* to contribute to drought and salt tolerance (Liu *et al.*, 2014). Rice OsbZIP86 (previously known as osZIP-1a) is a homolog of wheat G-box-binding factor EmBP-1; overexpression of *osZIP-1a* in rice protoplasts can enhance expression from the wheat *Em* gene promoter containing G-boxes only in the presence of ABA (Nantel & Quatrano, 1996). Moreover, phosphorylated OsbZIP72 directly binds to the G-box in the promoter of *AOC*, and activates *AOC* transcription (Wang *et al.*, 2020).

Members of the phosphorylation-activated sucrose nonfermenting 1–related protein kinase 2 (SnRK2) have also been reported to have crucial roles in phosphorylating AREB/ABF TFs, and subsequently activating downstream genes to respond to ABA signals (Banerjee & Roychoudhury, 2017). The rice SnRK2 protein family contains 10 members, denoted stress/ABA-activated protein kinase 1 (SAPK1) to SAPK10 (Kobayashi *et al.*, 2004). Among them, SAPK1, SAPK2, SAPK6, SAPK8, SAPK9, and SAPK10 have been reported to be functionally related to ABA signaling (Wang *et al.*, 2020; Fu *et al.*, 2021). SnRK2s usually function through their potential substrate proteins. A few TFs have been identified as SnRK2 substrates, including ABI5, OsbZIP23, OsbZIP46, OsbZIP62 and OsbZIP72 (Rehman *et al.*, 2021).

Previous efforts have provided evidence that miRNAs and bZIP TFs contribute to ABA biosynthesis in regulating drought resistance (Nadarajah & Kumar, 2019). However, regulation pathway of ABA biosynthesis and the cross talk between miRNAs and target genes are not well understood in rice. Here, we report that *OsbZIP86*, the target gene of miR2105, regulates drought-induced ABA biosynthesis through directly increasing the expression of *OsNCED3* to modulate drought tolerance, without penalty with respect to main agronomic traits under normal conditions. OsSAPK10 can interact with OsbZIP86, and increase *OsNCED3* promoter activity. Taken together, our results demonstrate the crucial importance of the 'miR2105-OsSAK10/OsbZIP86-*OsNCED3*' module in mediating ABA biosynthesis under drought stress.

Results

OsbZIP86 is a target gene of miR2105

Previously, we identified a drought-repressed miRNA osa-miR2105 (miR2105) from rice seedlings by miRNA sequencing; this miRNA was also isolated from developing rice seeds (Xue *et al.*, 2009; Yi *et al.*, 2013). In order to investigate the functions of miR2105 in stress responses in rice, we detected its expression under ABA, drought, and salt treatments by real-time qRT-PCR. The expression of miR2105 was repressed under ABA treatment in wild-type rice ZH11 seedlings (Figure **1A**). miR2105 was also downregulated after 0.5–4 h of water deficiency treatment and returned to relatively high expression levels after 1 h of re-watering (Figure **1B**). Expression of miR2105 was also downregulated by NaCl treatment (Figure **1C**). These results suggest that miR2105 is an ABA-, drought-, and salt-repressed miRNA.

It is well known that miRNAs exert their functions by inhibiting the expression of their target genes (Bartel, 2004). To identify the target gene of miR2105, we used two methods to identify its downstream targets. A total of 13 genes were predicted to be targets of miR2105 using psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Supplemental Table **S1**). To further validate the targets, we generated miR2105-overexpressing (miR2105-ox) and miR2105-downregulation lines (STTM2105) (Supplemental Figure **S1A,B**). Among the predicted 13 target genes, *OsbZIP86* (LOC_Os12g13170) (Nijhawan *et al.*, 2008) was downregulated in the T_2–T_4 generations of miR2105-ox and upregulated in the T_2–T_4 generations of STTM2105 (Figure **1D** and Supplemental Figure **S2A**). These results show that the expression change of *OsbZIP86* occurred in the opposite direction to that of miR2105 in miR2105-ox and STTM2105.

To further confirm *OsbZIP86* as a target gene of miR2105, a 5′ RNA ligase-mediated rapid amplification of cDNA ends (5′-RLM-RACE) assay was performed *in vivo* using ZH11 seedlings grown under normal condition. Sequencing of the 5′-RLM-RACE clones revealed that *OsbZIP86* mRNA was cleaved at the miR2105/*OsOsbZIP86* mRNA complementary site (Figure **1E**). We also searched the *OsbZIP86* expressed sequence tags (ESTs) in the NCBI database and found that an EST (CF324346) had its first base pair located within the complementary site. Collectively, these data demonstrated that miR2105 could direct cleavage of *OsbZIP86* mRNA to regulate transcript levels of *OsbZIP86*.

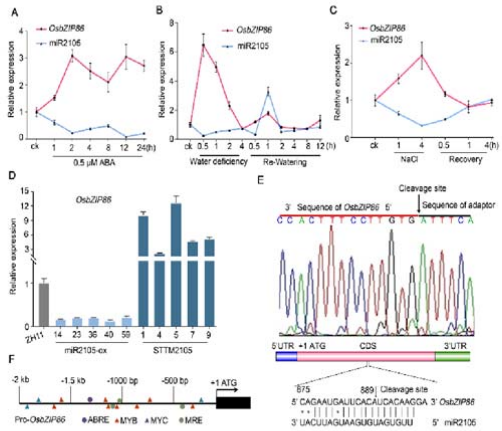

Figure 1. miR2105 regulates expression of *OsbZIP86* under ABA, drought, and salt treatments.

(A–C) Expression changes of *OsbZIP86* and miR2105 under ABA, drought, and salt treatments. RNA was isolated from 2-week-old ZH11 rice seedlings grown in Yoshida solution supplied with 0.5 μM ABA at the indicated time (A). For drought and salt treatments, 2-week-old seedlings grown in Yoshida solution were exposed to air (B) or treated with 150 mM NaCl (C) for 4 h, and then the seedlings were transferred to Yoshida solution again for recovery. (D) Expression changes of *OsbZIP86* in transgenic rice overexpressing osa-miR2105 (miR2105-ox) or with downregulation of osa-miR2105 (STTM2105) under normal growth conditions. (E) The cleavage site targeted by miR2105 in the *OsbZIP86* mRNA. The arrow on the miRNA:mRNA alignment indicates the cleavage site from 10 sequencing clones identified in ZH11 seedlings by 5′ -RLM-RACE. *U6* and *e-EF-1a* were used as miRNA and mRNA reference genes, respectively, and mean ± SD (n = 3) values are shown in (A–D). All qRT-PCR analyses for gene expression were performed in three biological replicates with similar results. (F) Main stress-related *cis*-acting elements in the 2-kb *OsbZIP86* promoter region. The *cis* elements are indicated.

159 **Drought, salt and ABA induce expression of *OsbZIP86* and repress that of miR2105**

OsbZIP86 (*osZIP-1a*) belongs to the OsbZIP TF family (Nantel & Quatrano, 1996; Nijhawan *et al*., 2008), and its promoter contains putative stress response-related *cis*-elements (Figure **1F**). Through searching the Rice eFP database (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi), we found that drought stress could significantly induce *OsbZIP86* expression, and *OsbZIP86* also responded to salt stress (Supplemental Figure **S2C,D**). Our qRT-PCR results also showed that expression of *OsbZIP86* was induced, whereas miR2105 expression was repressed after ABA and salt treatments in ZH11 seedlings (Figure **1A,C**). *OsbZIP86* and miR2105 were up- and downregulated, respectively after 0.5–2 h of water-pouring-out treatment (Figure **1B)**. miR2105 expression rapidly increased within 0.5–1 h recovery; by contrast, *OsbZIP86* expression returned to its normal level after 2–12 h of re-watering (Figure **1B**). Thus, *OsbZIP86* showed the opposite trend of expression change to that of miR2105 in ZH11 seedlings under ABA, salt, and drought treatments. To further evaluate the expression pattern of *OsbZIP86* in different tissues, qRT-PCR was performed. The results showed that *OsbZIP86* was mainly expressed in the stem, followed by the leaf sheath and young leaf (Supplemental Figure **S2B**), similar to the results from the Rice eFP database (Supplemental Figure **S2E,F**). GUS staining of *OsbZIP86pro:GUS* rice also showed that *OsbZIP86* was highly expressed in young leaf, leaf sheath, stem, and seed, with lower expression in the panicle (Supplemental Figure **S2G**), consistent with the qRT-PCR data (Supplemental Figure **S2B**). The temporal and spatial expression patterns of miR2105 and *OsbZIP86* in ZH11 also showed highly negative correlations at the same growth phases (Supplemental Figure **S2B**). Therefore, we concluded that ABA, salt, and drought repressed expression of miR2105, resulting in increased expression of *OsbZIP86*.

To test the subcellular localization of OsbZIP86, its the coding sequence (CDS) was fused with *GFP* and introduced into rice protoplasts using PEG (Supplemental Figure **S3A**). The recombinant OsbZIP86-GFP was found to exclusively co-localize with a nuclear marker, NSL-rk-mCherry (Supplemental Figure **S3A**). The stable *Ubi: OsbZIP86-GFP* transgenic rice also showed that the green fluorescence signal of the OsbZIP86-GFP fusion protein was located in the nuclei of root cells (Supplemental Figure **S3B**). These data showed that OsbZIP86 is a nuclear-localized protein.

miR2105 and *OsbZIP86* had opposite effects on the tolerance of rice to drought and salt stresses

To determine the detailed functions of *OsbZIP86* and miR2105 in response to different stresses in rice, they were used to generate transgenic rice (Supplemental Figure **S1**), which were subsequently tested

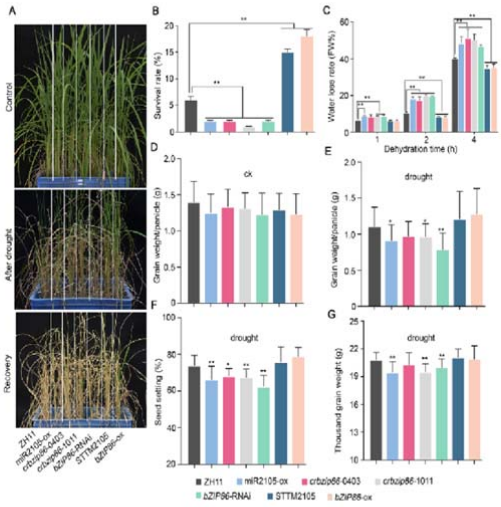

Figure 2. miR2105 and *OsbZIP86* mediate drought-resistance and grain yield of rice under drought condition.

(A–B) Phenotypes (A) and survival rates (B) of transgenic rice seedlings under drought treatment. Two-week-old rice seedlings were grown in boxes with sandy soil, water was poured out, and irrigation was stopped for 2 weeks until the leaves wilted for 3 d (middle); then, irrigation was resumed for 1 week (bottom), and the seedlings were watered as the control (top). (C) Water loss rate in detached leaves of the transgenic rice seedlings. Values are means ± SD of 30 independent plants. (D–E) Grain weights per panicle under normal (D) and drought conditions (E). (F–G) Seed setting rate (F) and thousand grain weight (G) under drought treatment. All plants were grown in boxes filled with sandy soil. For drought treatment (F–G), all plants were grown under normal conditions until flowering, then all the water in the boxes was poured out and watering was stopped for 1 week, before plants were recovered with water for 3 d. Lacunar drought treatment was carried out from flowering to mature grain. The experiments were performed in three replicates with similar results, and two independent lines of each transgenic construction were tested. Each repeat was measured in at least 30 seedlings in (A–B) and in 20 independent plants in (D–G). Values are means ± SD; $*p < 0.05$, $**p < 0.01$ according to student's *t*-test in (B–G). miR2105-ox, miR2105 overexpression; *cribzip86-0403*/-1011, *OsbZIP86*-CRISPR; *bZIP86*-RNAi, *OsbZIP86* RNAi; STTM2105, miR2105 downregulation; *bZIP8686*-ox, *OsbZIP86* overexpression.

190 with respect to their response to drought and NaCl treatments. Under normal growth conditions (ck),

191 miR2105 and *OsbZIP86* transgenic rice did not show phenotypic difference compared with ZH11,
192 including with respect to plant growth, stomatal area, seed germination, and the main agronomic traits
193 (Figure **2A,D, 3, 6,** and Supplemental Figure **S4**). However, when these transgenic rice seedlings were
194 recovered after water deficiency stress, the seedling survival rate of ZH11 was about 7%, whereas
195 those of STTM2105 and *bZIP86*-ox seedlings ranged from 15% to 18%, and those of miR2105-ox,
196 *crbzip86*, and *bZIP86*-RNAi seedlings ranged from 0% to 2% (Figure **2A,B**). The leaf water loss rates
197 of STTM2105 and *bZIP86*-ox seedlings were slower whereas those of miR2105-ox, *crbzip86*, and
198 *bZIP86*-RNAi were faster than those of ZH11 under dehydration conditions (Figure **2C**). To test the
199 effects of *OsbZIP86* and miR2105 on agronomic traits under drought conditions, the transgenic rice
200 were grown in boxes filled with sandy with lacunar drought. We found that miR2105-ox, *crbzip86*, and
201 *bZIP86*-RNAi plants showed lower grain weight per panicle, seed setting, and thousand grain weight
202 than ZH11 plants, whereas STTM2105 and *bZIP86*-ox did not show any significant difference
203 compared with ZH11 (Figure **2E–G**). STTM2105 and *OsbZIP86*-ox also enhanced the salt stress
204 tolerance of rice; by contrast, miR2105-ox, *crbzip86*, and *bZIP86*-RNAi decreased salt stress tolerance
205 during the seed germination and seedling stages (Supplemental Figure **S4**). These results indicated that
206 decreased miR2105 expression and increased *OsbZIP86* expression improved the drought and salt
207 tolerance of rice, whereas increased miR2105 expression and decreased *OsbZIP86* expression reduced
208 the drought and salt tolerance of rice.

209 When the transgenic rice were grown under water deficiency conditions, the stomatal areas of
210 miR2105-ox, *crbzip86*, and *bZIP86*-RNAi plants were smaller whereas those of STTM2105 and
211 *bZIP86*-ox plants were larger than those of ZH11 (Figure **3A,B**). In addition, more stomata were
212 completely closed and fewer stomata were completely open in STTM2105 and *bZIP86*-ox leaves
213 compared with ZH11. The opposite tendency was observed for miR2105-ox, *crbzip86*, and
214 *bZIP86*-RNAi (Figure **3C,D**). These results indicate that miR2105 and *OsbZIP86* plays positive and
215 negative roles in stomata movement under water deficiency, respectively (Figure **3**).

216

217 **OsbZIP86 directly binds to the promoter of *OsNCED3***

218 Given that OsbZIP86 had been demonstrated to bind to the G-box motif of the wheat *Em* promoter
219 (Nantel & Quatrano, 1996), and promoters of ABA biosynthetic and metabolic genes (Huang *et al.*,
220 2018) including *OsNCED1–5* and *OsABA8ox1–3* harbored G-box *cis*-elements, we checked whether

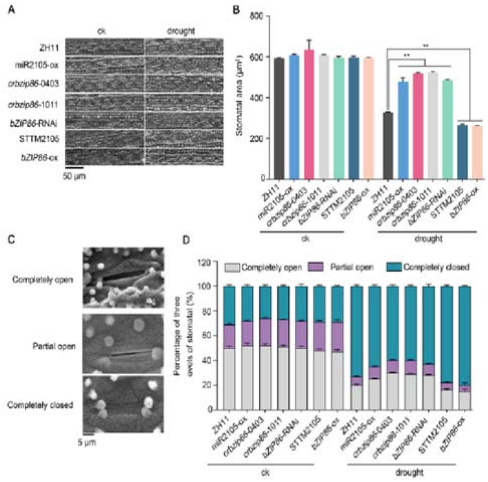

Figure 3. miR2105 and *OsbZIP86* mediate leaf stomatal opening in rice.

(A) Stomatal arrangement in abaxial leaf blade. (B) Area per stomatal pore. (C) SEM images of three levels of stomatal opening. (D) Statistics of stomatal opening state. Eight-week-old seedling leaves of ZH11, miR2105, and *OsbZIP86* transgenic rice plants were measured before (ck) and after drought stress. All images were continuously observed by SEM. Six seedlings of each line were used for measurement, and 180 stomata per line were measured (B and D). The experiments were performed in three biological replicates with similar results. Values shown are means ± SD of six independent seedlings. $*p < 0.05$, $**p < 0.01$ according to student's *t*-test.

221 overexpression of *OsbZIP86* could change the expression of these genes. Under normal conditions,

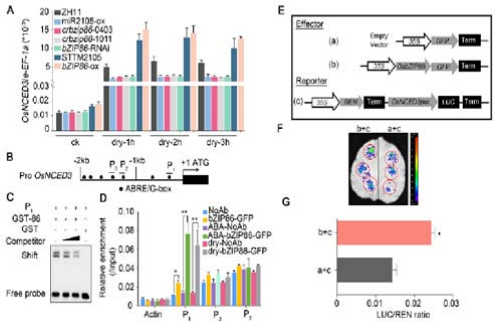

Figure 4. OsbZIP86 binds to the G-box of *OsNCED3* promoter to regulate its expression.

(A) Expression levels of *OsNED3* in miR2105 and *OsbZIP86* transgenic rice under normal conditions (ck) and drought stress. The rice *e-EF-1a* gene was used as the internal control. Data represent means ± SD (n = 3). All qRT-PCR analyses for gene expression were performed in three biological replicates with similar results. (B) G-box elements (black dot) in 2-kb *OsNCED3* promoter region. P_1, P_2, and P_3 represent probe positions for EMSA and amplification regions for ChIP-qPCR. (C) *In vitro* EMSA using G-box sequences from promoter of *OsNCED3* as probes. The P_1 probe was a biotin-labelled fragment of the *OsNCED3* promoter, and the competitor was a non-labelled competitive probe. GST-tagged OsbZIP86 was purified, and 2 μg protein was used. The gradient indicates the increasing amount of competitor. GST-86, fusion protein GST-OsbZIP86; GST, negative control. (D) *In vivo* ChIP-qPCR using ZH11 (NoAb, no antibody) and OsbZIP86-GFP overexpressing line (bZIP86-GFP). The anti-GFP antibody was used to precipitate DNA bound to OsbZIP86. Precipitated DNA was amplified with primers overlapping the G-box motif (P_1, P_2, and P_3). For drought and ABA treatment, ZH11 and *OsbZIP86*-6HA-GFP lines were grown in boxes filled with Yoshida solution for 2 weeks, then water was poured out or they were treated with 50 μm ABA for 2 h. Values are means ± SD from three parallel repeats. NoAb served as a negative control. Rice *Actin* was used as an internal control. (E) Schematic diagram of various constructs for *in vivo* luciferase transient transcriptional activity assay. *35S: OsbZIP86-GFP* was constructed as the effector. *35S: REN-OsNCED3pro: LUC* was constructed as the reporter. Free GFP (empty vector) was used as a negative control. (F–G) *In vivo* luciferase activity assay in tobacco leaves. D-luciferin was used as the substrate of luciferase. The expression level of *REN* was used as an internal control. The LUC/REN ratio represents the relative activity of the *OsNCED3* promoter. Error bars indicate SD with biological triplicates (n = 3). *$p < 0.05$, **$p < 0.01$, according to student's *t*-test in (D and G).

222 none of these genes showed significant differences in expression between ZH11 and *bZIP86*-ox; only

223 *OsNCED3* and *OsABA8ox2* showed slight upregulation in *bZIP86*-ox compared with ZH11
224 (Supplemental Figure **S5C,G**). However, under drought treatment, expression of *OsNCED3* and
225 *OsABA8ox2* was significantly upregulated, especially that of *OsNCED3* (Supplemental Figure **S5C,G**).
226 By contrast, the expression of *OsNCED5* was downregulated in *bZIP86*-ox compared with ZH11
227 (Supplemental Figure **S5E**). To investigate whether miR2105 affected *OsNCED3* expression,
228 *OsNCED3* mRNA levels were further analyzed in leaves of miR2105 transgenic rice under normal and
229 drought stress conditions. Under normal conditions, *bZIP86*-ox and STTM2105 showed slight
230 upregulation of *OsNCED3* compared with ZH11 (Figure **4A**). However, levels of the *OsNCED3*
231 transcript were significantly increased after drought treatment in STM2105 and *bZIP86*-ox seedlings,
232 but significantly decreased in miR2105-ox, *crbzip86*, and *bZIP86*-RNAi seedlings compared with those
233 in ZH11 (Figure **4A**). These results indicate that OsbZIP86 could promote *OsNCED3* transcription
234 under drought conditions.

235 To further investigate whether OsbZIP86 could directly bind to the promoter of *OsNCED3*, we
236 performed Electrophoretic mobility shift assay (EMSA) and Chromatin immunoprecipitation-
237 quantitative PCR (ChIP-qPCR) to test the DNA-binding ability of OsbZIP86 to the promoter of
238 *OsNCED3*. Based on the G-box motifs present in the *OsNCED3* promoter (Figure **4B**), we designed
239 three pairs of specific primers for ChIP-qPCR and three labeled probes for EMSA (Supplemental Table
240 **S2** and Figure **4B**). We found that OsbZIP86 could specifically bind to the probe 1 (P_1) region of the
241 *OsNCED3* promoter (Figure **4C** and Supplemental Figure **S6A**). EMSA binding was substantially
242 weakened by non-labeled competitive probe in a dosage-dependent manner (Figure **4C**). Subsequently,
243 ChIP-qPCR was performed to validate this binding *in vivo*, using an anti-GFP antibody and specific
244 primers. Consistent with the EMSA results, OsbZIP86 was enriched in the P_1 region of the *OsNCED3*
245 promoter (Figure **4D**). Moreover, the enrichment was significantly enhanced under drought conditions
246 and ABA treatment (Figure **4D**). These results showed that OsbZIP86 could specifically bind to the P_1
247 fragment of the *OsNCED3* promoter, which is 387 bp upstream of the ATG of the *OsNCED3* coding
248 region (Figure **4B–D** and Supplemental Figure **S6A**). Finally, a dual-luciferase reporting system in
249 tobacco leaves was used to determine the regulatory effect of OsbZIP86 on *OsNCED3* transcription
250 (Figure **4E–G**). In comparison with the empty effector, LUC activity was significantly enhanced when
251 *OsNCED3pro: LUC* was co-transfected with *35S: OsbZIP86* (Figure **4F–G**). Therefore, we concluded
252 that OsbZIP86 could bind to the *OsNCED3* promoter to active its transcription, and it was activated by

253 drought and ABA to promote the transcription of *OsNCED3*.

254

255 **OsbZIP86 interacts with OsSAPK10 to positively regulate *OsNCED3* expression**

256 The SAPKs, which are components of ABA signaling, have been reported to activate bZIP TFs in
257 plants (Banerjee & Roychoudhury, 2017; Fu *et al.*, 2021). To test whether OsbZIP86 interacted with
258 OsSAPK1-10, luciferase complementation imaging (LCI) in tobacco was performed (Supplemental
259 Figure **S6B**). We found that OsbZIP86 could interact with OsSAPK4, OsSAPK6, OsSAPK7, and
260 OsSAPK10. Then, we performed a dual-luciferase transient transcriptional activity assay to examine
261 whether the four OsSAPKs could function cooperatively with OsbZIP86 to positively regulate
262 *OsNCED3* expression. We detected a significant increase in *OsNCED3* promoter activity when
263 *OsSAPK4*, *OsSAPK7*, and *OsSAPK10* were co-transformed with *OsbZIP86* (Figure **5A–C** and
264 Supplemental Figure **S6C,D**). The transcription level of the *OsNCED3* promoter was most strongly
265 increased by OsSAPK10 (Figure **5A–C**). The interaction between OsbZIP86 and OsSAPK10 was
266 further confirmed by a pull-down assay *in vitro* and by bimolecular fluorescence complementation
267 (BiFC) and Co-immunoprecipitation (Co-IP) assays *in vivo* (Figure **5D–F**). These results indicate that
268 OsSAPK10 may activate OsbZIP86 and thus cooperatively regulate *OsNCED3* expression. OsSAPK10
269 has been reported to positively regulate ABA sensitivity (Min *et al*., 2007; Wang *et al*., 2020).
270 Together, we suggested that OsSAPK10 facilitates OsbZIP86 to positively regulate the transcript
271 expression of *OsNCED3*.

272

273 **miR2105 and *OsbZIP86* mediate ABA biosynthesis**

274 To test whether endogenous ABA biosynthesis was mediated by miR2105 and *OsbZIP86*, we
275 measured the ABA contents of leaves of transgenic rice (Figure **6A**). When the rice seedlings were
276 grown under normal conditions, the ABA contents of transgenic rice did not show any difference
277 compared with those of ZH11 rice (Figure **6A**). However, when they were treated by drought for 4 h,
278 the ABA contents of miR2105-ox, *crbzip86*, and *bZIP86*-RNAi plants were lower compared with
279 ZH11, whereas those of STTM2105 and *bZIP86*-ox were higher (Figure **6A**). To investigate whether
280 exogenous ABA treatment could complement the change in ABA biosynthesis in these transgenic rice,
281 we performed ABA treatment (Figure **6B–D**). The seedling growth of these transgenic rice plants did
282 not show any difference compared with that of ZH11 under normal conditions (Figure **6B–D**).

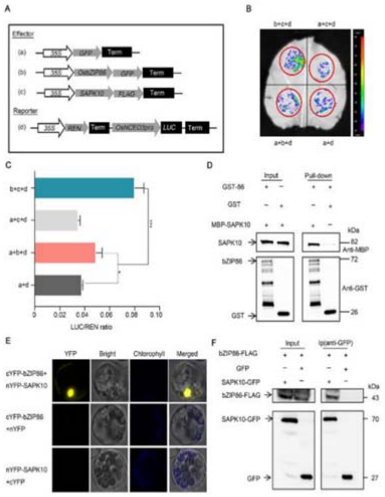

Figure 5. OsbZIP86 interacts with and functions cooperatively with OsSAPK10 to positively regulate *OsNCED3* expression. (A) Schematic diagram of various constructs used in the transient transformation assay. Free GFP was used as a negative control. (B–C) *In vivo* luciferase activity assay in tobacco leaves. D-luciferin was used as the substrate of luciferase. The expression level of *REN* was used as an internal control. The LUC/REN ratio represents the relative activity of the *OsNCED3* promoter. Error bars indicate SD with biological triplicates (n = 3). $*p < 0.05$, $***p < 0.001$ according to student's *t*-test. (D) BiFC analysis of interaction between OsbZIP86 and OsSAPK10 *in vivo*. CDS of *OsbZIP86* and *OsSAPK10* fused with the C-terminus and the N-terminus of yellow fluorescent protein (YFP) were co-transformed into rice protoplasts. Overlaid images show signals for YFP (yellow) and chloroplasts (blue). nYFP alone was used as a negative control. (E) Pull-down analysis of interaction between OsbZIP86 and OsSAPK10 *in vitro*. MBP-OsSAPK10 was incubated with GST or GST-OsbZIP86 proteins, and the immunoprecipitated proteins were detected by an anti-GST antibody. Free GST was used as the negative control. (F) Co-IP analysis of interaction between OsbZIP86 and SAPK10 *in vivo*. GFP, OsSAPK10-GFP, and OsbZIP86-FLAG were co-expressed in tobacco leaves by *Agrobacterium* injection. Total protein extracts were immunoprecipitated with the immobilized anti-GFP antibody (Ip), and the immunoprecipitated protein was then detected by using an anti-FLAG antibody. Input OsSAPK10-GFP and OsbZIP86-FLAG proteins were detected with anti-GFP and anti-FLAG antibodies, respectively. The molecular weights (kDa) and proteins are indicated in the left and right panels, respectively.

283 However, after ABA treatment, the seedlings of miR2105-ox, *crbzip86*, and *bZIP86*-RNAi plants had

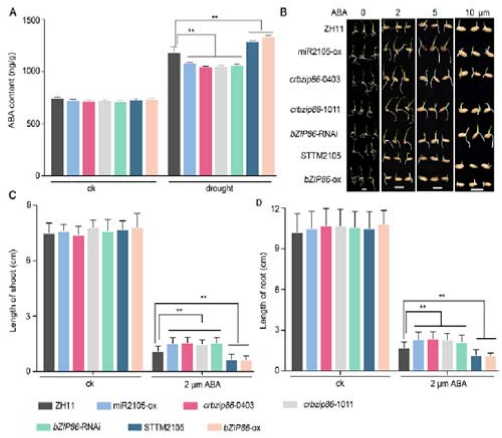

Figure 6. miR2105 and *OsbZIP86* mediate leaf ABA biosynthesis of rice under drought conditions.

(A) Leaf ABA content of miR2105 and OsbZIP86 transgenic rice under normal (ck) and drought stress conditions. Error bars indicate SD for biological triplicates (n = 3). (B) Germination performance of miR2105 and OsbZIP86 transgenic rice seedlings under ABA treatment. Seeds were placed on double sheets of filter paper in a 9-cm Petri dish and moistened with distilled water or 2, 5, or 10 μM ABA for 7 d. Scale bar, 0.5 cm. (C–D) Lengths of shoots (C) and main roots (D) of miR2105 and *OsbZIP86* transgenic rice seedlings treated with distilled water or 2 μM ABA for 7 d. Experiments were performed using three biological replicates with similar results. Each repeat was measured in 30 independent seedlings. Values show means ± SD of 30 independent plants. $*p < 0.05$, $**p < 0.01$ according to student's *t*-test (A, C, and D).

284 longer shoots and roots, and those of STTM2105 and *bZIP86*-ox plants had shorter shoots and roots

compared with ZH11 (Figure **6B–D**). This may indicate that exogenous ABA treatment strongly repressed ZH11 growth and slightly complemented the endogenous ABA insufficiency of miR2105-ox, *crbzip86*, and *bZIP86*-RNAi plants. Conversely, there was too much ABA in STTM2105 and *bZIP86*-ox plants after ABA treatment, and this further repressed the growth of these seedlings. Therefore, we propose that miR2105 and *OsbZIP86* regulate ABA biosynthesis only in the presence of drought stress.

Discussion

Plants quickly accumulate ABA to activate a series of stress responses when subjected to abiotic stresses. When environmental conditions are optimal, the amount of ABA is reduced to basic levels that promote optimal growth. When plants encounter a non-optimal environment, the regulation of ABA level in tissues and cells is essential for balancing defense and growth (Chen *et al.*, 2020). Therefore, excessive ABA levels under normal conditions will adversely affect the normal growth of plants. Here, we found that miR2105 targeted *OsbZIP86*, regulating its expression at post-transcriptional levels. OsbZIP86 directly activates *OsNCED3* transcription by binding to the G-box in the promoter, and interacts with OsSAPK10, resulting in enhanced-*OsNCED3* expression to control ABA biosynthesis. Therefore, the drought tolerance of the stable miR2105-overexpressing or *OsbZIP86*-downregulated transgenic rice plants was enhanced without any penalty with respect to major agronomic traits under normal conditions. Our findings suggest a molecular breeding strategy for improving the drought resistance of rice without affecting agronomic traits by using miR2105 or *OsbZIP86*.

miR2105/*OsbZIP86* regulates drought-induced ABA biosynthesis through *OsNCED3*

Under water stress, the ABA content in rice leaves can be rapidly induced within 1 h and quickly decrease to the basal line within 1 h during rehydration (Ye *et al.*, 2011). NCEDs have been reported to be involved in the rate-limiting step in the ABA biosynthetic pathway (Nambara & Marion-Poll, 2005; Chen *et al.*, 2020). However, how to regulate expression of *NCEDs* only under abiotic stresses was not completely clear. Our data showed that the expression of miR2105 in ZH11 plants was repressed by drought, ABA, and salt treatments; conversely, the expression of *OsbZIP86* was induced (Figure **1A–C**). We found that miR2105 could repress the expression of *OsbZIP86*, and miR2105 could direct to cleave *OsbZIP86* mRNA (Figure **1D,E**). In addition, the transgenic rice plants with changed expression of *OsbZIP86* and miR2105 showed the expected phenotypic changes under drought conditions (Figure **2, 3** and **6**): miR2105 negatively regulated the drought/salt tolerance and ABA sensitivity of the seedlings, whereas *OsbZIP86* positively regulated these responses (Figure **2, 6** and Supplemental Figure **S4**). Therefore, we concluded that *OsbZIP86* is a target gene of miR2105 and its expression level is regulated by miR2105 under drought salt and ABA treatment.

Rice is predicted to have five *OsNCED*s (Huang *et al.*, 2018; Hwang *et al.*, 2018; Huang *et al.*,

2019), which are tightly regulated in response to developmental or stress conditions (Chen *et al*., 2020). *OsNCED3*, which encodes a key enzyme in ABA synthesis, has the G-box core sequence 5′-ACGT-3′ in its promoter, and its expression is significantly induced by NaCl, PEG, and H_2O_2 stresses (Huang *et al*., 2018). *OsbZIP86* is predicted to be a G-box binding factor, and it can bind to and activate the wheat *Em* promoter (Nantel & Quatrano, 1996). Our EMSA, ChIP-qPCR, and luciferase activity assays showed that *OsbZIP86* could directly bind to the *OsNCED3* promoter (Figure **4**). We also demonstrated that drought and ABA treatment could enhance the binding ability of *OsbZIP86* to the *OsNCED3* promoter (Figure **4D**), suggesting that the function of OsbZIP86 might be dependent on the ABA signaling pathway. However, the stable transgenic rice plants with altered expression of *OsbZIP86* and miR2105 showed significant differences in *OsNCED3* expression (Figure **4A**), ABA content (Figure **6A**), and drought tolerance (Figure **2**) only when the plants were treated by drought. Of the five rice *OsNCEDs*, only *OsNCED3* showed significant upregulation of expression in *OsbZIP86*-ox plants under drought conditions (Supplemental Figure **S5**). Therefore, we concluded that *OsbZIP86* is a direct regulator of *OsNCED3*, and that *OsNCED3* expression is regulated under drought stress.

Like *OsNCED3*, *OsNCED5* is expressed in all tissues and regulates ABA biosynthesis and tolerance of rice to salt and water stresses (Huang *et al*., 2019). However, overexpression of *OsbZIP86* could cause upregulation of *OsABA8ox2* and downregulation of *OsNCED5* under drought treatment (Supplemental Figure **S5**). *OsbZIP86* has ABRE *cis*-elements for ABA signaling (Figure **1F**), suggesting that *OsbZIP86* may positively regulate ABA signaling. The strongly induced expression of *OsNCED3* in *OsbZIP86*-ox (Supplemental Figure **S5C**) under drought conditions in turn may negatively regulate the expression of *OsNCED5* and positively regulate expression of *OsABA8ox2* to maintain the balance of endogenous ABA.

OsSAK10 interacts with OsbZIP86 to regulate the transcript expression of *OsNCED3*

SnRK2 kinases have been reported to play essential parts in ABA signaling through phosphorylating AREB/ABF TFs at the post-translational level in *Arabidopsis* (Banerjee & Roychoudhury, 2017). Several OsSAPKs have been found to phosphorylate rice bZIPs via protein–protein interactions, for instance, OsSAPK6 can interact with OsbZIP10 (Chae *et al*., 2007) and OsbZIP46 (Tang *et al*., 2012; Kim *et al*., 2015), OsSAPK2 can interact with OsbZIP23 and OsbZIP46 (Zong *et al*., 2016), OsbZIP72 can interact with OsSAPK10 and OsSAPK1 (Wang *et al*., 2020; Fu *et al*., 2021). We found that

OsbZIP86 interacted with OsSAPK10 (Figure **5D–F** and Supplemental Figure **S6B**). Overexpression of *OsSAPK10* reduced water loss in detached leaves (Min *et al.*, 2019) and increased sensitivity to ABA (Wang *et al.*, 2020). These phenotypes were similar to those resulting from overexpression of *OsbZIP86* (Figure **2** and **6**). Moreover, the expression pattern and subcellular localization of OsSAPK10 (Wang *et al.*, 2020) overlapped with those of OsbZIP86 (Supplemental Figure **S2B** and Supplemental Figure **S3**). OsSAPK10 could function cooperatively with OsbZIP86 to positively regulate *OsNCED3* expression (Figure **5A–C**). These results indicate that OsbZIP86 is activated by OsSAPK10. Taken together, our results suggest that OsbZIP86 may regulate ABA biosynthesis through *OsNCED3* in an OsSAPK10-dependent manner. Further research is needed to investigate how OsSAK10 enhances the activity of OsbZIP86.

Overexpression of *OsbZIP86* and downregulation of miR2105 can improve drought tolerance without affecting the main agronomic traits of rice

ABA can improve plant tolerance to abiotic stresses, but ABA induces leaf senescence and negatively affects the yield of rice. Direct overexpression of ABA biosynthetic genes (such as *OsNCED3*) or its biosynthetic regulators (such as *OsNAC2*) caused negative effects on leaves and yield, while these transgenic plants could improve the stress tolerance (Mao *et al.*, 2017; Huang *et al.*, 2018). Therefore, when the ABA synthesis is promoted only under stress (such as drought), the resistance of plants can be improved without affecting their growth and development under normal conditions. Thus, these genes could be used in molecular breeding to improve stress tolerance without negative effects.

The transgenic rice with altered expression of miR2105 or *OsbZIP86* showed changes in ABA contents associated with overexpression of *OsNCED3* only when drought was present (Figure **6A**), leading to changes in the drought resistance in rice seedlings (Figure **2** and **3**). These data indicate that miR2105 and *OsbZIP86* regulate ABA biosynthesis to enhance drought tolerance through *OsNCED3* only under drought conditions, while not affecting aspects of rice growth including plant height, tiller numbers, seed setting percentage, and thousand seed weight under normal conditions (Supplemental Figure **S7**). However when treated with lacunar drought and salt, the seed setting percentage, thousand seed weight, and grain yield per panicle of *crbzip86*, *bZIP86*-RNAi, and miR2105-ox were lower than those of ZH11 (Figure **2E–G** and Supplemental Figure **S4G–I**). miR2105-ox and *bZIP86*-ox showed no significant differences compared with ZH11, possibly owing to the insufficient drought and salt

383 treatments. These results imply that altered expression of miR2105 or *OsbZIP86* had no impact on rice
384 growth and development under normal conditions but could improve the stress resistance of rice under
385 drought and salt stresses; this is different from other genes for they may exert negative effects on
386 normal rice growth when promoting ABA biosynthesis (Kasuga *et al*., 2004; Ito *et al*., 2006).
387 Therefore, miR2105 and OsbZIP86 may have potential for use in promoting drought tolerance without
388 penalty to major agronomic traits under normal conditions.

389 In summary, we have proposed a working model of how OsbZIP86 is regulated by miR2105 and
390 OsSAK10 to control ABA biosynthesis through *OsNCED3* and functions in regulating drought
391 resistance in rice (Figure **7**). Under drought stress, the expression of miR2105 is repressed, whereas the
392 expression of *OsbZIP86* is induced. Then, OsbZIP86 is activated by OsSAPK10 through direct
393 interaction, thereby promoting the expression of *OsNCED3* by directly binding to the promoter. The
394 upregulated *OsNCED3* plays an important part in the regulation of ABA biosynthesis to modulate
395 drought resistance.

396

397 **Materials and Methods**

398 **Vector construction and rice transformation**

399 To generate osa-miR2105-overexpression rice (miR2105-ox), a mature 20-base-pair (bp) sequence
400 (MI0010566) of miR2105 was downloaded from miRBase (http://www.mirbase.org). The cloning
401 procedure for miR2105-ox followed the description at
402 http://wmd3.weigelworld.org/cgi-bin/webapp.cgi. The artificial osa-miR2105 was inserted downstream
403 of the *35S* promoter in pCAMBIA1301 (http://www.cambia.org). To downregulate osa-miR2105
404 (STTM2105), the short tandem target mimic method was used as described previously (Yan *et al*.,
405 2012). The fragment
406 (GAAGCTTTTTGTGATGTGAATGATTCATGTTGTTGTTGTTATGGTCTAGTTGTTGTTGTTAT
407 GGTCTAATTTAAATATGGTCTAAAGAAGAAGAATATGGTCTAAAGAAGAAGAATTTGTGA
408 TGTGAATGATTCATGGATCCA) was synthetized by Invitrogen™ (China) and inserted downstream
409 of the *Ubi-1* promoter in pXU1301 (modified from pCAMBIA1301; the *35S* promoter was replaced by
410 the *Ubi-1* promoter). Therefore, the fragment contained two target mimic sequences (underlined).

411 For overexpression of *OsbZIP86* (*bZIP86*-ox), the CDS of *OsbZIP86* was amplified from *Oryza*
412 *sativa* L. cv "Zhonghua 11" (ZH11) and subcloned into pXU1301 to produce a 6×HA–GFP fusion

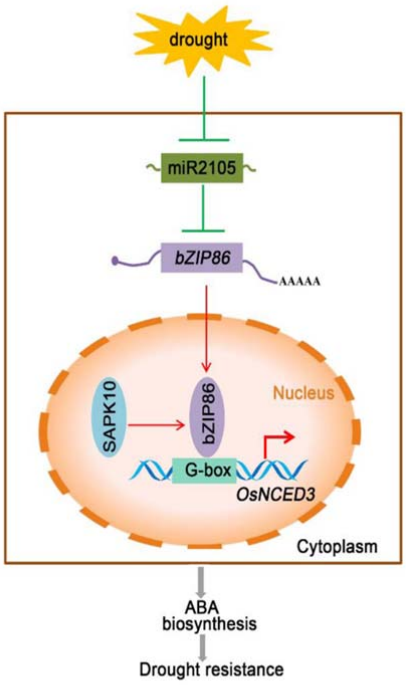

Figure 7. Working model of 'miR2105-OsbZIP86-*OsNCED3*' module functions in regulating drought resistance

Under drought stress, the expression of miR2105 is repressed, whereas the expression of *OsbZIP86* is induced. Then, OsbZIP86 is activated by OsSAPK10 through direct interaction, thereby promoting the expression of *OsNCED3*. The upregulated *OsNCED3* has an important role in regulating ABA biosynthesis to modulate drought resistance. Arrowheads show positive regulation; flat-ended lines show negative regulation.

413 protein expressed from the *Ubi* promoter. To generate *OsbZIP86*-RNAi, two fragments of the

OsbZIP86 CDS (391 bp) were inserted downstream of the *Ubi-1* promoter in the rice RNA interference (RNAi) vector pTCK303 (Wang *et al.*, 2004). The *osbzip86* mutants (cr*bzip86*) were generated by a CRISPR/Cas9 genome-editing system (Ma *et al.*, 2015). The targets selected are listed in Supplemental Figure **S1E**. Individual T_0 transformants were analyzed by sequencing their *OsbZIP86* target regions, which were amplified by PCR. To generate *OsbZIP86pro: GUS* transgenic plants, an approximately 2-kb promoter sequence of *OsbZIP86* was cloned and inserted into pCAMBIA1301.

All the above-mentioned constructs were introduced into *Agrobacterium tumefaciens* strain *EHA*105, and ZH11 was transformed by *Agrobacterium*-mediated transformation. All the primers used in this study are listed in Supplemental Table **S2**.

5′-RLM-RACE assay

Total RNA from tillering stage leaf sheaths of ZH11 was directly ligated to a synthesized RNA adaptor (GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA). The cDNA was amplified by nested PCR, and the final PCR product was gel-purified and subcloned into the pGEM-T Easy Vector (Promega, Guangzhou, China) for sequencing. Other processes for 5′-RLM-RACE were as previously described (Xia *et al.*, 2015a). The primers are listed in Supplemental Table **S2**.

Plant growth conditions and stress treatments

All the rice used in this study was generated from ZH11. The seeds were surface-sterilized in 70% (v/v) ethanol for 30 s and then in 2.5% NaClO (w/v) solution for another 40 min, followed by several rinses with sterile water. Then, seeds were incubated in darkness at 28 °C for 2 d to induce germination. The rice were grown under controlled field conditions in Guangzhou, China, or in boxes filled with Yoshida solution at 30 °C with a 14 h/10 h light/dark period.

To determine the effects on gene expression of ABA, water deficiency, and salt treatments, 2-week-old ZH11 seedlings grown in Yoshida solution were supplied with 0.5 μM ABA for different times, exposed to air for 4 h, or treated with 150 mM NaCl for 4 h. The seedlings were then transferred to Yoshida solution again for recovery.

For drought tolerance test, 2-week-old rice seedlings were grown in boxes filled with sandy soil and grown for 2 weeks; then, all the water in the boxes was poured out and watering was stopped for 2 weeks until the seedling wilted for 3 d and recovery 1 week. Photographs were taken and the surviving

seedlings were counted.

For the ABA sensitivity test at the seedling stage, transgenic rice seeds were placed on double sheets of filter paper in a 9-cm Petri dish and moistened with distilled water or different concentrations of ABA (2, 5, or 10 μM) for 7 d. Lengths of shoots and roots were measured. Three replicates were tested for each plant line. Thirty seeds were measured for each replicate. Leaf ABA contents were measured using a Phytodetek competitive ELISA kit (Mlbio, Shanghai, China). ABA was extracted as described previously (Fukumoto *et al*., 2013).

To examine the effects of salt on seedling growth, 4-week-old-seedlings were transferred to Yoshida solution with 150 mM NaCl and allowed to grow for 10 d. Photographs were taken and the surviving seedlings were counted.

Water loss rate and stomatal closure

To detect the water loss rate under dehydration conditions, leaves of 2-week-old rice seedlings were cut into 1-cm lengths, exposed to air at room temperature in the dark, and weighed at different times; the water loss rate was calculated as the percentage of total weight lost compared with the initial weight at each time point (Miao *et al*., 2020). Stomatal closure was examined according to the method described previously (Miao *et al*., 2020). Leaves from 8-week-old rice grown under normal or drought stress conditions were cut in 0.1-cm^2 pieces and fixed in 2.5% glutaraldehyde solution with 0.1 M phosphate-buffered saline (pH 7.2). Stomatal status was monitored by scanning electron microscopy (SEM) (SU8010, Hitachi, Japan); the SEM analysis was performed as described previously (Xia *et al*., 2015b). Six individual plants from the ZH11 and transgenic rice lines were used for measurements of the stomatal aperture, with 30 stomata measured per plant.

RNA extraction and reverse transcription PCR (RT-PCR)

The extraction of small RNA and total RNA from rice, reverse transcription and quantitative RT-PCR (qRT-PCR) amplification were performed as previously described (Xia *et al*., 2015a). *OsbZIP86* expression during the plants' entire growth life was verified using data from RiceXpro (http://ricexpro.dna.affrc.go.jp/).

β-glucuronidase (GUS) assay

The GUS assay was carried out as described previously (Liu *et al.*, 2021) with minor modifications. Transgenic plant tissues were incubated in GUS staining solution (Sangon, Shanghai, China). Following vacuum infiltration, the samples were incubated at 37 °C for 16 h. After staining, the tissues were bleached with 70% ethanol and photographed under a light microscope (Leica DVM6, Leica Microsystems, Germany).

Subcellular localization of OsbZIP86

To determine the subcellular localization of OsbZIP86, the CDS of *OsbZIP86* was cloned into the pUC18 vector, which we had modified to produce an OsbZIP86-GFP fusion construct driven by the CaMV*35S* promoter (*35S: OsbZIP86-GFP*). Rice protoplasts prepared from etiolated shoots were co-transformed with *35S: OsbZIP86-GFP* and *NSL-mCherry* (a nuclear marker) using polyethylene glycol (PEG). In addition, the CDS of *OsbZIP86-GFP* was cloned into the pXU1301 vector, and the fusion construct (*Ubi: OsbZIP86-GFP*) was introduced into *EHA105* to create transgenic rice. The fluorescence signal was observed with a confocal laser scanning microscope (Leica SP8 STED 3X, Leica Microsystems, Germany). The primers are listed in Supplemental Table **S2**.

Dual-luciferase assay of transiently transformed tobacco leaves

To generate the luciferase reporter construct for the dual-luciferase assay, the 2-kb promoter sequence of *OsNCED3* was amplified from rice and inserted into a pGreenII0800-LUC vector (Hellens *et al.*, 2005) using the *Hind*III and *BamH*I sites, respectively. The CDS of *OsbZIP86* and *OsSAPKs* were cloned into the pCAMBIA1300-GFP and pHB-3×FLAG vectors, respectively, as the effectors. The tobacco leaves were used for dual-luciferase assays as described previously (Hellens *et al.*, 2005). The effector construct (*35S: OsbZIP86*, *35S: OsSAPKs* or *35S: GFP*) and the reporter construct (*35S: REN-OsNCED3pro: LUC*) were introduced into *A. tumefaciens* strain GV3101 and then infiltrated into 3-week-old tobacco leaves by *Agrobacterium* injection. The activities of firefly luciferase (LUC) and Renilla luciferase (REN) were measured using a Dual-Luciferase Reporter Assay System (E2920, Promega, USA). The LUC activity was normalized to the REN activity. The primers are listed in Supplemental Table **S2**.

EMSA and pull-down assay

The EMSA and pull-down assays were performed as described previously (Wang *et al.*, 2020). The CDS of *OsbZIP86* and *OsSAPK10* were cloned into the pGEX4T-1 and pMAL-c2x vectors to generate GST-OsbZIP86 and MBP-OsSAPK10 fusion proteins, respectively. These recombinant vectors were transformed into *Escherichia coli* strain BL21. Fusion proteins were induced with 0.5 mM Isopropyl β-D-Thiogalactoside (IPTG) at 20°C for 12 h. The fusion proteins (GST-OsbZIP86 and MBP-OsSAPK10) were purified using Glutathione Sepharose 4B (GE Healthcare, USA) and Amylose Magnetic Beads (New England Biolabs, USA) according to the manufacturer's protocols, respectively. For the EMSA assay, complementary single-stranded oligonucleotides derived from 40 bp of the G-box region of the *OsNECD3* promoter were synthesized as DNA probes. The probes were synthesized by Invitrogen (Shanghai, China). EMSA was performed using a LightShift™ Chemiluminescent EMSA Kit (Thermo, No. 20148) following the manufacturer's protocol. For the pull-down assay, GST-OsbZIP86 and MBP-OsSAPK10 were incubated with Glutathione Sepharose 4B (GE Healthcare, USA) in pull-down buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.25% Triton X-100, 35 mM β-mercaptoethanol) at 4 °C for 3 h. After washing six times with pull-down buffer, the pulled proteins were eluted by boiling and further analyzed by immunoblotting using anti-GST. The primers and EMSA probes are listed in Supplemental Table **S2**.

ChIP-qPCR assay

The ChIP-qPCR assay was performed as described previously (Wang *et al.*, 2020). Briefly, 3 g of leaves from 2-week-old seedlings of ZH11 and *OsbZIP86*-6HA-GFP lines grown under normal conditions or treated with 50 μM ABA or subjected to water deficiency for 2 h were cross-linked twice by 3% formaldehyde for chromatin isolation under vacuum for 15 min and stopped using 2 M glycine. Then the samples were ground to powder in liquid nitrogen prior to isolating chromatin. After sonication, the chromatin complexes were incubated with GFP-Trap Agarose (Chromotek, USA). DNA was purified using a PCR purification kit (DNA Clean & Concentrator-5, Tianmo Biotech) and recovered in water for qPCR analysis. Primers were selected in the promoter regions of *OsNCED3* (Supplemental Table **S2**). The amount of precipitated DNA was calculated relative to the total input chromatin and expressed as a percentage of the total according to the formula: % input = $2^{\Delta Ct} \times 100\%$, where ΔCt = Ct (input) – Ct (IP), and Ct is the mean threshold cycle of the corresponding PCR reaction. The ChIP experiments were repeated three times with the similar results.

534

535 **LCI assay**

536 The CDS of *OsbZIP86* and *OsSAPKs* were cloned into the pCAMBIA1300-NLuc and
537 pCAMBIA1300-CLuc LUC vectors (Chen *et al.*, 2008), respectively. All of the constructs were
538 introduced into *A. tumefaciens* strain GV3101, which was then co-transformed into 3-week-old tobacco
539 leaves by *Agrobacterium* injection. One millimolar luciferin was sprayed onto leaves to detect the
540 fluorescence signal after 2.5 d infiltration under a plant *in vivo* imaging system (LB985 NightSHADE).
541 The details were examined according to the method described previously (Chen *et al*., 2008).

542

543 **BiFC assay**

544 The CDS of *OsbZIP86* and *OsSAPK10* were cloned into the pSATN-cYFP-C1 and pSATN-nYFP-C1
545 vectors (Citovsky *et al.*, 2006), respectively. The constructed vectors were transiently cotransformed
546 into rice protoplasts using PEG. Transfected cells were imaged using a confocal spectral microscope
547 imaging system (Leica, SP8 STED 3X, Germany).

548

549 **Co-IP assay**

550 Co-IP was performed as described previously (Wang *et al.*, 2020). To generate epitope-tagged
551 expression vectors for Co-IP, the CDS of *OsbZIP86* and *OsSAPK10* were fused with sequences
552 encoding a FLAG tag (vector:pHB-3×FLAG) and GFP tag (vector:pCAMBIA1300-GFP) driven by the
553 *35S* promoter, respectively. The fusion proteins OsbZIP86-FLAG (bZIP86-FLAG) and
554 OsSAPK10-GFP (SAPK10-GFP) were co-transformed into 3-week old tobacco leaves by
555 *Agrobacterium* injection. Total protein was extracted 2.5 d after infiltration with extraction buffer (50
556 mM Tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 1% NP-40, 0.2 mM PMSF,
557 with complete mini tablet) and then immunoprecipitated with GFP-Trap Agarose (Chromotek, USA)
558 according to the manufacturer's instructions. The immunoprecipitated proteins were separated via
559 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel) and analyzed by
560 immunoblotting analysis with anti-GFP or anti-FLAG.

561

562 **Statistical analysis**

563 All results were presented as mean ± standard deviation (SD) of three replicates. Statistical

564 analysis was performed using SPSS 19.0, including one-way ANOVA and the least significant
565 difference. Difference was considered significant at $p < 0.05$ and highly significant as $p < 0.01$.
566 Diagrams were prepared using GraphPad Prism 8.3.0 and Adobe Photoshop.
567
568 Supplemental Data
569 **Supplemental Table S1.** The description of the predicted 13 target genes of miR2105.
570 **Supplemental Table S2.** Primers used in this study.
571 **Supplemental Figure S1.** Identification of miR2105 and *OsbZIP86* transgenic rice.
572 **Supplemental Figure S2.** *OsbZIP86* is a target gene of miR2105 and the expression pattern analyse of
573 *OsbZIP86*.
574 **Supplemental Figure S3.** Subcellular localization of OsbZIP86.
575 **Supplemental Figure S4.** miR2105 and *OsbZIP86* mediate salt resistance and grain yield of rice under
576 salt treatment.
577 **Supplemental Figure S5.** Expression changes of ABA biosynthetic and metabolic genes in *OsbZIP86*
578 overexpression transgenic rice.
579 **Supplemental Figure S6.** OsbZIP86 binds to promoter fragments of *OsNCED3*, and OsbZIP86
580 interacts with OsSAPKs to regulate *OsNCED3* expression.
581 **Supplemental Figure S7.** Agronomic traits of miR2105 and *OsbZIP86* transgenic rice under normal
582 conditions.
583
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589
590 **Figure legends**
591 **Figure 1.** miR2105 regulates expression of *OsbZIP86* under ABA, drought, and salt treatments.
592 (A–C) Expression changes of *OsbZIP86* and miR2105 under ABA, drought, and salt treatments. RNA
593 was isolated from 2-week-old ZH11 rice seedlings grown in Yoshida solution supplied with 0.5 μM

ABA at the indicated time (A). For drought and salt treatments, 2-week-old seedlings grown in Yoshida solution were exposed to air (B) or treated with 150 mM NaCl (C) for 4 h, and then the seedlings were transferred to Yoshida solution again for recovery. (D) Expression changes of *OsbZIP86* in transgenic rice overexpressing osa-miR2105 (miR2105-ox) or with downregulation of osa-miR2105 (STTM2105) under normal growth conditions. (E) The cleavage site targeted by miR2105 in the *OsbZIP86* mRNA. The arrow on the miRNA:mRNA alignment indicates the cleavage site from 10 sequencing clones identified in ZH11 seedlings by 5′ -RLM-RACE. *U6* and *e-EF-1a* were used as miRNA and mRNA reference genes, respectively, and mean ± SD (n = 3) values are shown in (A–D). All qRT-PCR analyses for gene expression were performed in three biological replicates with similar results. (F) Main stress-related *cis*-acting elements in the 2-kb *OsbZIP86* promoter region. The *cis* elements are indicated.

Figure 2. miR2105 and *OsbZIP86* mediate drought-resistance and grain yield of rice under drought condition.

(A–B) Phenotypes (A) and survival rates (B) of transgenic rice seedlings under drought treatment. Two-week-old rice seedlings were grown in boxes with sandy soil, water was poured out, and irrigation was stopped for 2 weeks until the leaves wilted for 3 d (middle); then, irrigation was resumed for 1 week (bottom), and the seedlings were watered as the control (top). (C) Water loss rate in detached leaves of the transgenic rice seedlings. Values are means ± SD of 30 independent plants. (D–E) Grain weights per panicle under normal (D) and drought conditions (E). (F–G) Seed setting rate (F) and thousand grain weight (G) under drought treatment. All plants were grown in boxes filled with sandy soil. For drought treatment (F–G), all plants were grown under normal conditions until flowering, then all the water in the boxes was poured out and watering was stopped for 1 week, before plants were recovered with water for 3 d. Lacunar drought treatment was carried out from flowering to mature grain. The experiments were performed in three replicates with similar results, and two independent lines of each transgenic construction were tested. Each repeat was measured in at least 30 seedlings in (A–B) and in 20 independent plants in (D–G). Values are means ± SD; $*p < 0.05$, $**p < 0.01$ according to student's *t*-test in (B–G). miR2105-ox, miR2105 overexpression; *cribzip86-0403*/-1011, *OsbZIP86*-CRISPR; *bZIP86*-RNAi, *OsbZIP86* RNAi; STTM2105, miR2105 downregulation; *bZIP8686*-ox, *OsbZIP86* overexpression.

Figure 3. miR2105 and *OsbZIP86* mediate leaf stomatal opening in rice.

(A) Stomatal arrangement in abaxial leaf blade. (B) Area per stomatal pore. (C) SEM images of three levels of stomatal opening. (D) Statistics of stomatal opening state. Eight-week-old seedling leaves of ZH11, miR2105, and *OsbZIP86* transgenic rice plants were measured before (ck) and after drought stress. All images were continuously observed by SEM. Six seedlings of each line were used for measurement, and 180 stomata per line were measured (B and D). The experiments were performed in three biological replicates with similar results. Values shown are means ± SD of six independent seedlings. $*p < 0.05$, $**p < 0.01$ according to student's *t*-test.

Figure 4. OsbZIP86 binds to the G-box of *OsNCED3* promoter to regulate its expression.

(A) Expression levels of *OsNED3* in miR2105 and *OsbZIP86* transgenic rice under normal conditions (ck) and drought stress. The rice *e-EF-1a* gene was used as the internal control. Data represent means ± SD (n = 3). All qRT-PCR analyses for gene expression were performed in three biological replicates with similar results. (B) G-box elements (black dot) in 2-kb *OsNCED3* promoter region. P_1, P_2, and P_3 represent probe positions for EMSA and amplification regions for ChIP-qPCR. (C) *In vitro* EMSA using G-box sequences from promoter of *OsNCED3* as probes. The P_1 probe was a biotin-labelled fragment of the *OsNCED3* promoter, and the competitor was a non-labelled competitive probe. GST-tagged OsbZIP86 was purified, and 2 μg protein was used. The gradient indicates the increasing amount of competitor. GST-86, fusion protein GST-OsbZIP86; GST, negative control. (D) *In vivo* ChIP-qPCR using ZH11 (NoAb, no antibody) and OsbZIP86-GFP overexpressing line (bZIP86-GFP). The anti-GFP antibody was used to precipitate DNA bound to OsbZIP86. Precipitated DNA was amplified with primers overlapping the G-box motif (P_1, P_2, and P_3). For drought and ABA treatment, ZH11 and *OsbZIP86*-6HA-GFP lines were grown in boxes filled with Yoshida solution for 2 weeks, then water was poured out or they were treated with 50 μm ABA for 2 h. Values are means ± SD from three parallel repeats. NoAb served as a negative control. Rice *Actin* was used as an internal control. (E) Schematic diagram of various constructs for *in vivo* luciferase transient transcriptional activity assay. *35S: OsbZIP86-GFP* was constructed as the effector. *35S: REN-OsNCED3pro: LUC* was constructed as the reporter. Free GFP (empty vector) was used as a negative control. (F–G) *In vivo* luciferase activity assay in tobacco leaves. D-luciferin was used as the substrate of luciferase. The expression

level of *REN* was used as an internal control. The LUC/REN ratio represents the relative activity of the *OsNCED3* promoter. Error bars indicate SD with biological triplicates (n = 3). *$p < 0.05$, **$p < 0.01$, according to student's *t*-test in (D and G).

Figure 5. OsbZIP86 interacts with and functions cooperatively with OsSAPK10 to positively regulate *OsNCED3* expression.

(A) Schematic diagram of various constructs used in the transient transformation assay. Free GFP was used as a negative control. (B–C) *In vivo* luciferase activity assay in tobacco leaves. D-luciferin was used as the substrate of luciferase. The expression level of *REN* was used as an internal control. The LUC/REN ratio represents the relative activity of the *OsNCED3* promoter. Error bars indicate SD with biological triplicates (n = 3). *$p < 0.05$, ***$p < 0.001$ according to student's *t*-test. (D) BiFC analysis of interaction between OsbZIP86 and OsSAPK10 *in vivo*. CDS of *OsbZIP86* and *OsSAPK10* fused with the C-terminus and the N-terminus of yellow fluorescent protein (YFP) were co-transformed into rice protoplasts. Overlaid images show signals for YFP (yellow) and chloroplasts (blue). nYFP alone was used as a negative control. (E) Pull-down analysis of interaction between OsbZIP86 and OsSAPK10 *in vitro*. MBP-OsSAPK10 was incubated with GST or GST-OsbZIP86 proteins, and the immunoprecipitated proteins were detected by an anti-GST antibody. Free GST was used as the negative control. (F) Co-IP analysis of interaction between OsbZIP86 and SAPK10 *in vivo*. GFP, OsSAPK10-GFP, and OsbZIP86-FLAG were co-expressed in tobacco leaves by *Agrobacterium* injection. Total protein extracts were immunoprecipitated with the immobilized anti-GFP antibody (Ip), and the immunoprecipitated protein was then detected by using an anti-FLAG antibody. Input OsSAPK10-GFP and OsbZIP86-FLAG proteins were detected with anti-GFP and anti-FLAG antibodies, respectively. The molecular weights (kDa) and proteins are indicated in the left and right panels, respectively.

Figure 6. miR2105 and *OsbZIP86* mediate leaf ABA biosynthesis of rice under drought conditions.

(A) Leaf ABA content of miR2105 and OsbZIP86 transgenic rice under normal (ck) and drought stress conditions. Error bars indicate SD for biological triplicates (n = 3). (B) Germination performance of miR2105 and OsbZIP86 transgenic rice seedlings under ABA treatment. Seeds were placed on double sheets of filter paper in a 9-cm Petri dish and moistened with distilled water or 2, 5, or 10 μM ABA for

7 d. Scale bar, 0.5 cm. (C–D) Lengths of shoots (C) and main roots (D) of miR2105 and *OsbZIP86* transgenic rice seedlings treated with distilled water or 2 μM ABA for 7 d. Experiments were performed using three biological replicates with similar results. Each repeat was measured in 30 independent seedlings. Values show means ± SD of 30 independent plants. $*p < 0.05$, $**p < 0.01$ according to student's *t*-test (A, C, and D).

Figure 7. Working model of 'miR2105-OsbZIP86-*OsNCED3*' module functions in regulating drought resistance

Under drought stress, the expression of miR2105 is repressed, whereas the expression of *OsbZIP86* is induced. Then, OsbZIP86 is activated by OsSAPK10 through direct interaction, thereby promoting the expression of *OsNCED3*. The upregulated *OsNCED3* has an important role in regulating ABA biosynthesis to modulate drought resistance. Arrowheads show positive regulation; flat-ended lines show negative regulation.

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