

1 **WUSCHEL-Related Homeobox Genes Cooperate with Cytokinin Signalling to Promote**  
2 **Bulbil Formation in *Lilium lancifolium***

3  
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28 **Supporting information**

29 **Table S1.** Primers used in full length and promoter sequence cloning of *LIWOX9* and *LIWOX11*.

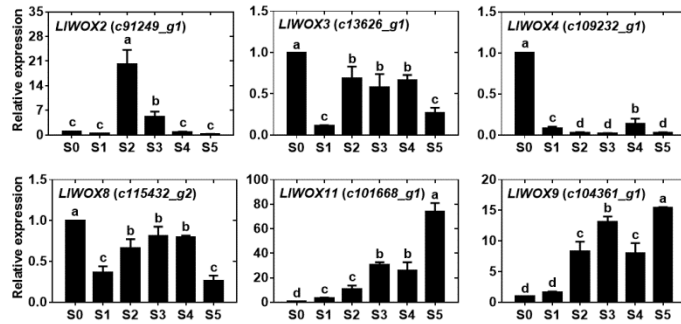
30 **Table S2.** Primers used in qRT-PCR.

31 **Table S3.** Primers used in vectors construction.

32 **Fig. S1.** The expression of *WOX* genes during bulbil formation.

33 **Fig. S2.** The phenotype of abnormal proliferation in leaf axil after overexpression of *LIWOX9*  
34 and *LIWOX11*.

35



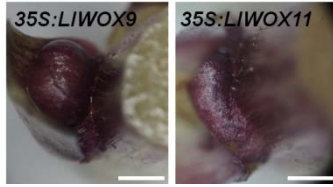
## 36 Summary

37 The bulbil is an important vegetative reproductive organ in triploid *Lilium lancifolium*. Based on  
38 our previously obtained transcriptome data, we screened two *WUSCHCEL-related homeobox*  
39 (*WOX*) genes closely related to bulbil formation, *LIWOX9* and *LIWOX11*. However, the  
40 biological functions and regulatory mechanisms of *LIWOX9* and *LIWOX11* are unclear. In this  
41 study, we cloned the full-length coding sequences of *LIWOX9* and *LIWOX11*. Transgenic  
42 *Arabidopsis* showed increased branch numbers, and the overexpression of *LIWOX9* and  
43 *LIWOX11* in stem segments promoted bulbil formation, while the silencing of *LIWOX9* and  
44 *LIWOX11* inhibited bulbil formation, indicating that *LIWOX9* and *LIWOX11* are positive  
45 regulators of bulbil formation. Cytokinins acting through type-B response regulators (type-B  
46 RRs) could bind to the promoters of *LIWOX9* and *LIWOX11* and promote their transcription.  
47 *LIWOX11* could enhance cytokinin pathway signalling by inhibiting the transcription of type-A  
48 *LIRR9*. Our study enriches the understanding of the regulation of plant development by the *WOX*  
49 gene family and lays a foundation for further research on the molecular mechanism of bulbil  
50 formation in lily.

51

52 **Key words** Bulbil formation, Cytokinin, *Lilium lancifolium*, Type-B response regulators,  
53 *WUS-related homeobox*

54



## 55 **Introduction**

56 *Lilium lancifolium*, also known as tiger lily, is an important *Lilium* species of the  
57 Liliaceae family. *L. lancifolium* shows high adaptability and is widely cultivated in China for its  
58 edible bulbs and medicinal applications (Liang & Tamura, 2000; China Pharmacopoeia  
59 Committee, 2005; Yu *et al.*, 2015), with a production value of approximately six billion Yuan per  
60 year. *L. lancifolium* is a natural triploid and cannot be propagated sexually, but its leaf axils can  
61 form a large number of purple-black bulbils (Bach & Sochacki, 2012; Chung *et al.*, 2015).  
62 Bulbils grow on leaf axils and can naturally fall off the mother plant and develop into a new  
63 complete individual after maturity (Yang *et al.*, 2017). The bulbil propagation strategy has the  
64 advantages of high efficiency and better retention of maternal genetic characteristics and is  
65 therefore the main reproductive strategy for *L. lancifolium*.

66 Bulbils are a special and important type of reproductive organ in plants and are only  
67 formed in a few plant species, such as *Dioscorea batatas*, *Allium sativum*, *Titanotrichum*  
68 *oldhamii*, *Pinellia ternate*, *Agave tequilana*, and *Lilium* species (Wang *et al.*, 2004; Bell & Bryan,  
69 2008; Abraham-Juárez *et al.*, 2010; Sandoval *et al.*, 2012; Yang *et al.*, 2017). The formation of  
70 bulbils is a complex developmental process that is regulated by genetic and environmental  
71 factors and phytohormones.

72 Plant hormones, especially auxin and cytokinin, have been proven to be involved in the  
73 regulation of bulbil formation, in which auxin inhibits bulbil formation, whereas cytokinin  
74 promotes the bulbil formation (Wang & Cronk, 2003; Peng *et al.*, 2005; Abraham-Juárez *et al.*,  
75 2015; Navarro *et al.*, 2015; He *et al.*, 2020). Before bulbil initiation in *D. polystachya*, auxin  
76 rapidly accumulates in the leaf axil, followed by the expression of auxin transport genes, such as  
77 *ARF9*, *ARF18*, *AX15A*, and *AUX22D*, resulting in auxin outflow from the leaf axil and bulbil  
78 initiation (Wu *et al.*, 2020). *AtqPIN1* and *AtqSoPIN1* participate in auxin outflow in *A. tequilana*

79 (Abraham-Juárez *et al.*, 2015). In *D. polystachya*, the expression of cytokinin  
80 oxidase/dehydrogenase genes (*CKX1*, *CKX3*, *CKX9* and *CKX11*) is decreased before bulblet  
81 initiation and leads to the accumulation of cytokinin in the leaf axil (Wu *et al.*, 2020). In a  
82 previous study, we revealed that iP-type cytokinins were the most important cytokinins during  
83 bulbil formation and showed that the accumulation of iP-type cytokinins was mainly due to the  
84 upregulation of cytokinin biosynthesis genes (*IPT1* and *IPT5*) and cytokinin activation genes  
85 (*LOG1*, *LOG3*, *LOG5* and *LOG7*) and the significant downregulation of cytokinin degradation  
86 gene (*CKX4*) expression (He *et al.*, 2020).

87 As a special type of axillary organ, bulbils originate from the axillary meristem (AM). A  
88 recent study revealed that cytokinins can promote AM initiation through cytokinin type-B  
89 response regulators (type-B RRs) (Wang *et al.*, 2017). Type-B RRs are positive regulatory  
90 transcription factors in cytokinin signalling and mostly modulate the transcription of  
91 cytokinin-regulated genes by directly binding target DNA sequences at their C-terminal MYB  
92 domains (Hosoda *et al.*, 2002; Kieber & Schaller, 2014). In *A. thaliana*, cytokinin signalling is  
93 mainly mediated by five members of type-B RR subfamily I: ARR1, ARR2, ARR10, ARR11 and  
94 ARR12 (Mason *et al.*, 2004, 2005; Schaller *et al.*, 2007; Yokoyama *et al.*, 2007; Ishida *et al.*,  
95 2008; Tsai *et al.*, 2012). In regulating axillary bud formation, type-B RRs act as key  
96 transcriptional regulators involved in AM initiation. ARR1 can directly bind to the *WUS*  
97 promoter and activate the transcription of *WUS*; ARR2, ARR10, ARR11 and ARR12 can also  
98 activate the expression of *WUS*, indicating that type-B ARRs show functional redundancy in  
99 regulating the expression of *WUS*, in which ARR1 is the key regulatory factor (Wang *et al.*,  
100 2014a,b, 2017).

101 Regarding the molecular regulation of bulbil formation, however, only a small number of  
102 genes related to bulbil formation have been identified to date, and the associated regulatory  
103 mechanism is not clear. In *A. tequilana*, *AtqKNOX1* and *AtqKNOX2* are expressed at the  
104 beginning of globular bud formation and are specifically expressed during meristem  
105 development (Abraham-Juárez *et al.*, 2010). The expression of some *AtqMADS* genes is

106 decreased during bulbil formation, indicating that *AtqMADS* genes may be negatively related to  
107 bulbil formation in this species (Sandoval *et al.*, 2012). In *T. oldhamii*, the expression of  
108 *Gesneriaceae-FLORICAULA (GFLO)* is also downregulated during bulbil formation, indicating  
109 that *GFLO* acts as a negative regulator during bulbil formation (Wang *et al.*, 2004). The AGO  
110 protein mediates the silencing of downstream genes through miRNA. In *L. lancifolium*, *LLAGO1*  
111 is specifically expressed in the bulbil and upregulated during bulbil formation, which indicates  
112 that the miRNA pathway may also be involved in the regulation of bulbil formation (Yang *et al.*,  
113 2018).

114 The WUSCHEL-related homeobox (WOX) proteins are a plant-specific family within the  
115 eukaryotic homeobox transcription actor superfamily characterized by a conserved N-terminal  
116 homeodomain (HD) consisting of 60-66 amino acids (Mayer *et al.*, 1998; Haecker *et al.*, 2004).  
117 Functional studies have revealed that the WOX transcription factors play important roles in  
118 promoting cell division, preventing immature cells from differentiating, embryonic development,  
119 stem cell niche maintenance in the meristem and organ formation (Stahl *et al.*, 2009; Van Der  
120 Graaff *et al.*, 2009; Yadav *et al.*, 2011). Based on the phylogenetic analysis and the distribution  
121 of *WOX* genes in the plant kingdom, they have been classified into three clades: a modern/WUS  
122 clade (found in seed plants), an intermediate/WOX9 clade (found in vascular plants including  
123 lycophytes), and an ancient/WOX13 clade (found in vascular and nonvascular plants, including  
124 mosses and green algae) (Nardmann *et al.*, 2009; Van Der Graaff *et al.*, 2009).

125 Some members of the WOX gene family have been shown to be involved in the  
126 regulation of AM. In *A. thaliana*, *WUS* is essential for the initiation and maintenance of AM  
127 (Wang *et al.*, 2014a,b). Unlike the situation in *A. thaliana*, the AM of *O. sativa* is coregulated by  
128 *OsWUS* and *OsWOX4*. *OsWUS* is expressed only before meristem formation and not in the  
129 established AM, and *OsWOX4* is expressed only in the established AM, indicating that *OsWOX4*  
130 functions only in maintaining meristem activity (Ohmori *et al.*, 2013; Lu *et al.*, 2015; Tanaka *et*  
131 *al.*, 2015). WOX9 and WOX11 are members of the intermediate clade and regulate the shoot  
132 meristem or AM. In the *A. thaliana wox9* mutant, the development of the embryo, apical

133 meristem and root meristem is abnormal, and the growth and development of the axillary buds  
134 and roots is significantly inhibited (Skylar *et al.*, 2010; Skylar & Wu, 2010). In addition, the loss  
135 of *WUS* expression in the *wox9* mutant indicates that *WOX9* can positively regulate the  
136 expression of *WUS* (Wu *et al.*, 2005). In *O. sativa*, *OsWOX9* (*Dwarfiller1*, *DWT1*) plays an  
137 important role in the development of rice tillers, and the *dwt1* mutant shows shorter tillers and a  
138 reduced tiller number (Wang *et al.*, 2014c). In *A. thaliana* and *O. sativa*, *WOX11* mainly  
139 regulates the lateral root or crown root primordium (Liu *et al.*, 2014; Hu & Xu, 2016). *wox11*  
140 mutants show crown root number and growth rate deficiencies, a dwarf phenotype and delayed  
141 flowering (Zhao *et al.*, 2009). In crown and root development, *OsWOX11* mediates the cytokinin  
142 pathway by inhibiting the expression of type-A *OsRR2*, thus enhancing cytokinin signalling to  
143 promote crown and root formation (Nardmann & Werr, 2006; Zhao *et al.*, 2009). A recent study  
144 revealed that in addition to its function in crown root development, *OsWOX11* is also required  
145 for rice shoot development and can activate gene expression during the development of the rice  
146 shoot apical meristem by recruiting the H3K27me3 demethylase *JMJ705* (Cheng *et al.*, 2018).

147 On the basis of transcriptome data (accession number: SRP103184), we screened the  
148 expression of all annotated *WOX* genes during bulbil formation and identified two *WOX* genes  
149 closely related to bulbil formation, *LIWOX9* and *LIWOX11* (Fig. S1). In this study, our results  
150 showed that *LIWOX9* and *LIWOX11* were members of the intermediate clade and that their  
151 expression increased continuously during bulbil formation. The overexpression of *LIWOX9* and  
152 *LIWOX11* promoted bulbil formation, while the silencing of *LIWOX9* and *LIWOX11* inhibited  
153 bulbil formation, indicating that *LIWOX9* and *LIWOX11* are positive regulators of bulbil  
154 formation. Cytokinin type-B LIRRs can bind to the promoters of *LIWOX9* and *LIWOX11* to  
155 promote their transcription. In addition, *LIWOX11* can enhance cytokinin signalling by  
156 inhibiting the transcription of type-A *LIRR9*. Our study enriches the understanding of the roles of  
157 the *WOX* gene family in regulating plant development. We also show for the first time that *WOX*  
158 genes cooperate with cytokinins to regulate the formation of bulbils. Our study lays a foundation  
159 for further research on the molecular mechanism of bulbil formation in lily.

160

## 161 **Materials and methods**

162

### 163 *Plant materials and treatments*

164 Bulbs of *Lilium lancifolium* of uniform size were harvested and buried in soil at 4°C at  
165 the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS),  
166 Beijing, China, in November 2019. Well-grown stems with a height of 10 cm were selected  
167 according to an *in vitro* bulbil induction system (He *et al.*, 2020), and stem segments were  
168 cultured on Murashige and Skong medium for bulbil induction. The stages of bulbil formation  
169 were divided into the bulbil initiation stage (S0–S2), bulbil primordium formation stage (S3–S4),  
170 and bulbil structure formation stage (S5) (He *et al.*, 2020). Different stages of developing bulbils  
171 and different tissues (leaf axils at stage S4, shoot apex, leaf, stem, root, scale, stigma, ovary,  
172 anther and petal tissues) were collected for RNA extraction.

173 To determine whether *LIWOX9* and *LIWOX11* are immediately induced by cytokinins, 4  
174 mM 6-BA was added to MS medium during bulbil formation, and stem segments at the S4 stage  
175 were treated with 10 mM 6-BA or with 0.05 mM NaOH as a control. Leaf axils were harvested at  
176 the S0-S5 stages and after 0, 0.5, 1.0, 1.5, 2.0 or 2.5 h of treatment.

177

### 178 *Isolation of LIWOX9 and LIWOX11 genes and promoters*

179 According to our transcriptome data (accession number: SRP103184), we designed  
180 primers by using Primer 6 to clone the full-length sequences and promoters of *LIWOX9* and  
181 *LIWOX11*. The full-length sequences of *LIWOX9* and *LIWOX11* were cloned via RLM-RACE  
182 using the GeneRacer™ Kit (Invitrogen, US) according to the kit protocol. To obtain the promoter  
183 sequences of *LIWOX9* and *LIWOX11*, three gene-specific reverse primers were designed and a  
184 nested PCR program was used according to the protocol of a genome walking kit (Takara, Japan).  
185 The sequences of the primers used for amplification are shown in Table S1. Conserved protein  
186 domains were analyzed using SMART (<http://smart.embl.de/>). Phylogenetic analysis was



187 performed using MEGA6 (<http://mega6.software.informer.com/>). Multiple sequence alignments  
188 were analysed using the DNAMAN software package. New PLACE  
189 (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) and PlantCARE  
190 (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) were used to analyse the *LIWOX9*  
191 and *LIWOX11* promoters.

192

### 193 ***Real-time RT-PCR (qRT-PCR)***

194 Total RNA from the different tissue and leaf axil specimens was extracted with an  
195 RNAPrep Pure Plant Kit (TIANGEN, China) according to the kit protocol, and DNA  
196 contamination was removed with RNase-free DNase I. First-strand cDNA was synthesized with  
197 a Hifair<sup>®</sup> II 1st Strand cDNA Synthesis Kit (gDNA digester plus) (Yeasen, China) according to  
198 the kit protocol. Gene-specific primers for qRT-PCR were designed with Primer 6.0 (Table S2).  
199 The *LilyActin* primer was used as an internal control (Xu *et al.*, 2017), and SYBR<sup>®</sup> Green Master  
200 Mix (No Rox) (Yeasen, Shanghai, China) was used in the reaction mixture according to the  
201 manufacturer's instructions. qRT-PCR was conducted using the CFX96 Real-Time System  
202 (Bio-Rad, USA), with an initial denaturation step at 95°C for 3 min, followed by 40 cycles of  
203 denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 1 min. The  
204  $2^{-\Delta\Delta C_t}$  method was used to calculate the relative expression levels of the different genes (Livak &  
205 Schmittgen, 2001). Three biological and three technical replicates were performed to reduce  
206 error.

207

### 208 ***Subcellular localization***

209 The full-length cDNAs of *LIWOX9* and *LIWOX11* under the control of the 35S  
210 cauliflower mosaic virus promoter were cloned into the pCAMBIA 2300 vector using the  
211 pEASY<sup>®</sup>-Basic Seamless Cloning and Assembly Kit (Transgen Biotech, China). The sequences  
212 of the primer pairs used for amplification are shown in Table S3. The resulting plasmids were  
213 transferred into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cells were collected

214 and suspended in infiltration buffer (10 mM methylester sulfonate, 10 mM MgCl<sub>2</sub>, and 150 mM  
215 acetosyringone, pH 5.7) at OD<sub>600</sub>=0.8 and infiltrated into *Nicotiana benthamiana* leaves. 3 days  
216 after infiltration, the leaves were harvested and treated with 0.5 mg/ml DAPI  
217 (4',6-diamidino-2-phenylindole; Sigma). A Zeiss LSM 510 confocal scanning microscope was  
218 used to collect images.

219

### 220 ***RNA fluorescence in situ hybridization***

221 Leaf axils in S1-S5 were fixed with FAA, and after dehydration, clearing and embedding,  
222 paraffin sections of the leaf axils were sliced at a thickness of 0.8 μm. The obtained slides were  
223 rehydrated with xylene, digested with protease K (20 μg/mL) at 37°C, blocked with a 3%  
224 methanol-H<sub>2</sub>O<sub>2</sub> solution for 25 min, with avidin (0.07%) at 37°C for 25 min and with biotin  
225 (0.005%) at 37°C for 15 min. Hybridization with the probes was performed at 37°C overnight in  
226 a moist chamber. After hybridization, the slides were washed in 2× SSC for 10 min, three times  
227 in 1× SSC for 5 min and once in 0.5× SSC for 10 min at 37°C. The avidin-labelled probe was  
228 detected with a streptavidin Alexa Fluor 405 conjugate (1:250) (Invitrogen). Antibodies were  
229 diluted in PBS containing 3% (w/v) BSA, and the slides were incubated with the antibodies for  
230 30 min at 37°C. After antibody incubation, the slides were washed three times with 4× SSC  
231 containing 0.1% Tween 20, stained with 100 ng/mL DAPI in PBS for 30 min and dehydrated  
232 with ethanol. Confocal images were obtained using a Zeiss LSM 510 confocal scanning  
233 microscope.

234

### 235 ***Transformation of A. thaliana and L. lancifolium***

236 The full-length cDNAs of *LIWOX9* and *LIWOX11* were amplified by PCR and inserted  
237 into the pCAMBIA 3301 vector using the pEASY<sup>®</sup>-Basic Seamless Cloning and Assembly Kit  
238 (Transgen Biotech, China). All primers used are listed in Table S1. *A. thaliana* was transformed  
239 using *A. tumefaciens* strain GV3101 and the floral dip method (Clough & Bent, 1998).  
240 Transgenic *A. thaliana* plants were selected on 1/2 Murashige and Skoog (MS) medium with 30

241 mg/L kanamycin. Transgenic *A. thaliana* plants were grown in climate-controlled boxes at 24°C  
242 under a 12/12 h light/dark cycle.

243 *L. lancifolium* was transformed using *A. tumefaciens* strain EHA105 via  
244 *Agrobacterium*-mediated vacuum infiltration. *Agrobacterium* cells were collected and suspended  
245 in infiltration buffer that contained 10 mM MgCl<sub>2</sub>, 200 mM acetosyringone and 10 mM MES  
246 (pH 5.6). The small stem segments of *L. lancifolium* were submerged in infiltration solution and  
247 then subjected to -50 kPa vacuum for 10 min. The infiltrated segments were washed with  
248 distilled water three times and then were grown on MS medium with 30 g/L sucrose and 6 g/L  
249 agar (pH 5.8) in the dark at 20°C for 1 d, followed by growth at 22°C under a 16/8 h light/dark  
250 cycle. The rate of bulbil formation was assessed after one week of culture, and RNA was  
251 extracted from leaf axils to measure the expression of the target genes. Each treatment consisted  
252 of three experimental replicates, with 30 leaf axils per replicate.

253

#### 254 ***Virus-induced gene silencing (VIGS)***

255 For the generation of pTRV2-LIWOX9 and pTRV2-LIWOX11, gene-specific fragments  
256 of ~300 bp were cloned into the pTRV2 vector using the pEASY<sup>®</sup>-Basic Seamless Cloning and  
257 Assembly Kit (Transgen Biotech, China). Five pTRV2-LIRR vectors were constructed as  
258 previously described (He *et al.*, 2021). The primer pairs used to generate the TRV vectors are  
259 shown in Table S3. VIGS was performed using *A. tumefaciens* strain EHA105 and vacuum  
260 infiltration method (He *et al.*, 2021). The rate of bulbil formation was assessed after two weeks  
261 of culture, and RNA was extracted from leaf axils to measure the expression of the target genes.  
262 Each treatment consisted of three experimental replicates, with 30 leaf axils per replicate.

263

#### 264 ***GUS staining***

265 A 1318 bp fragment upstream of the start codon of *LIWOX9* and a 2351 bp fragment  
266 upstream of the start codon of *LIWOX11* were introduced into the pCAMBIA 3301 vector, and  
267 the 35S promoter was replaced using the pEASY<sup>®</sup>-Basic Seamless Cloning and Assembly Kit

268 (Transgen Biotech, China). The constructed plasmids were transferred into *A. tumefaciens* strain  
269 EHA105. The method of *N. benthamiana* leaf infiltration was the same as that used in the  
270 subcellular localization assay. Stem segments at S0 and S5 were used for vacuum infiltration  
271 according to the method described above. Three days after infiltration, the leaves and stem  
272 segments were harvested and treated with GUS staining solution (Solarbio, China) according to  
273 the kit protocol. After staining, the leaves and stem segments were washed and cleared with 70%  
274 ethanol for more than 24 h before image capture using a Leica Microsystems DM5500B  
275 instrument (Wetzlar, Germany).

276

### 277 ***Yeast one-hybrid assay***

278 Y1H analysis was performed according to the method described by Lin *et al.* (2013).  
279 Briefly, the full-length coding regions of five *LIRRs* and *LIWOX11* were cloned into the pGADT7  
280 vector to generate the pGADT7-LIRRs and pGADT7-LIWOX11 constructs. Various truncated  
281 versions of the promoter regions of *LIWOX9* and *LIWOX11* were amplified and ligated into the  
282 pABAI reporter vector. The constructs were then cotransformed into the yeast strain EGY48.  
283 Transformants were grown on SD-Trp/-Ura plates for 3 d at 28°C. The interactions were  
284 determined based on the growth ability of the cotransformants on medium supplemented with  
285 aureobasidin A (AbA).

286

### 287 ***Dual-luciferase reporter assay***

288 The coding sequence of *LIWOX11* was cloned into the pCAMBIA 3301 vector using the  
289 pEASY<sup>®</sup>-Basic Seamless Cloning and Assembly Kit (Transgen Biotech, China). Five pCAMBIA  
290 3301-LIRR vectors were constructed as previously described (He *et al.*, 2021). A 1318 bp  
291 fragment upstream of the start codon of *LIWOX9* and a 2351 bp fragment upstream of the start  
292 codon of *LIWOX11* were introduced into the pluc-35Rluc vector using the pEASY<sup>®</sup>-Basic  
293 Seamless Cloning and Assembly Kit (Transgen Biotech, China). The primers used to generate  
294 the constructs are listed in Table S3. The constructed plasmids were transformed into *A.*

295 *tumefaciens* strain GV3101. Different effectors were subsequently coinfiltrated with the reporter  
296 into *N. benthamiana* leaves using a syringe. At 3 d after infiltration, 2-cm-diameter leaf discs  
297 were harvested and ground in liquid nitrogen. The activities of firefly and Renilla luciferase were  
298 measured with a Dual-Luciferase Reporter Assay System (Promega) using a GloMax 20/20  
299 luminometer (Promega).

300

### 301 **EMSAs**

302 To construct plasmids for the expression of the recombinant LIWOX11 protein in  
303 *Escherichia coli*, the full-length cDNA was amplified and cloned into the pMal-c2X vector,  
304 which was expressed in the *Escherichia coli* strain BL21 (DE3) cell line. The pET32a-LIRR1  
305 vector was constructed as previously described (He *et al.*, 2021). The primers are listed in Table  
306 S3. Protein expression was induced by incubation in 1 mM IPTG at 16°C at 160 rpm for 24 h.  
307 Protein purification was carried out using an amylose resin purification system (NEB) following  
308 the manufacturer's instructions. Double-stranded oligonucleotide probes were synthesized and  
309 labelled with biotin at the 5' end. EMSA was carried out using the LightShift<sup>®</sup> Chemiluminescent  
310 EMSA Kit (Thermo Fisher Scientific, USA). Competition experiments were performed with  
311 different amounts of nonlabelled oligonucleotides. The mutated competitors were generated by  
312 replacing eight base pairs in the WOX binding elements (TTAATGAG to AAAAAAAAA).

313

314

315 **Results**

316

317 ***Full-length cloning and sequence analysis of LIWOX9 and LIWOX11***

318 On the basis of transcriptome data (accession number: SRP103184), we cloned the  
319 full-length sequences of *LIWOX9* (1008 bp) and *LIWOX11* (699 bp) by RLM-RACE and found  
320 that they encoded 335 and 232 amino acids, respectively (Fig. **1a**). Amino acid sequence analysis  
321 showed that both *LIWOX9* and *LIWOX11* contained HOX domains at the N-terminus (Fig. **1a**).  
322 Sequence alignment confirmed a conserved HOX domain at the N-terminus in *LIWOX9* and  
323 *LIWOX11* (Fig. **1b,c**). A phylogenetic tree of *LIWOX9*, *LIWOX11* and the members of the  
324 WOX transcription factor family in *A. thaliana* was constructed, and the results showed that  
325 *LIWOX9* and *LIWOX11* belonged to the intermediate evolutionary branch of the WOX family  
326 (Fig. **1d**). Phylogenetic tree of *WOX9* and *WOX11* from different species showed that *LIWOX9*  
327 and *LIWOX11* were clustered with the sequences of other monocotyledonous species and were  
328 closely related to the *WOX9* and *WOX11* amino acid sequences of *Palmaceae* plants (Fig. **1e, f**).



**Fig. 1.** Full-length cloning, sequence alignment and phylogenetic tree of *LIWOX9* and *LIWOX11*.

**a:** Full-length cloning and domain prediction of *LIWOX9* and *LIWOX11*. **b:**

Multiple sequence alignment of *LIWOX9* with sequences of other species. **c:**

Multiple sequence alignment of *LIWOX11* with sequences of other species. The red

boxes in B and C represent the HOX domain. *Ll*: *Lilium lancifolium*, *Eg*: *Elaeis*

*guineensis* (EgWOX9, XP 029121206.1; EgWOX11, XP 010938138.1), *Pe*:

*Phalaenopsis equestris* (PeWOX9, XP 020596429.1; PeWOX11, XP 020573379.1),

*Jc*: *Jatropha curcas* (JcWOX9, XP 012092417.1; JcWOX11, XP 012070529.1), *Vv*:

*Vitis vinifera* (VvWOX9, RVW37990.1; VvWOX11, XP 019077126.1), *At*:

*Arabidopsis thaliana* (AtWOX9, NP 180994.2; AtWOX11, NP 187016.2). **d:**

Neighbour-joining tree of the *LIWOX9* and *LIWOX11* amino acid sequences of *L.*

*lancifolium* and WOX family amino acid sequences from *A. thaliana*. **e:**

Neighbour-joining tree of the *LIWOX9* amino acid sequence of *L. lancifolium* and

WOX9 amino acid sequences from other species. **f:** Neighbour-joining tree of the

*LIWOX11* amino acid sequence of *L. lancifolium* and WOX11 amino acid

sequences from other species. Bootstrap values from 1,000 replicates were used to

assess the robustness of the tree.

329

### 330 ***Expression pattern and subcellular localization of LIWOX9 and LIWOX11***

331 To study the subcellular localization of *LIWOX9* and *LIWOX11*, we fused the *LIWOX9*

332 and *LIWOX11* proteins with a green fluorescent protein (GFP) tag and introduced them into the

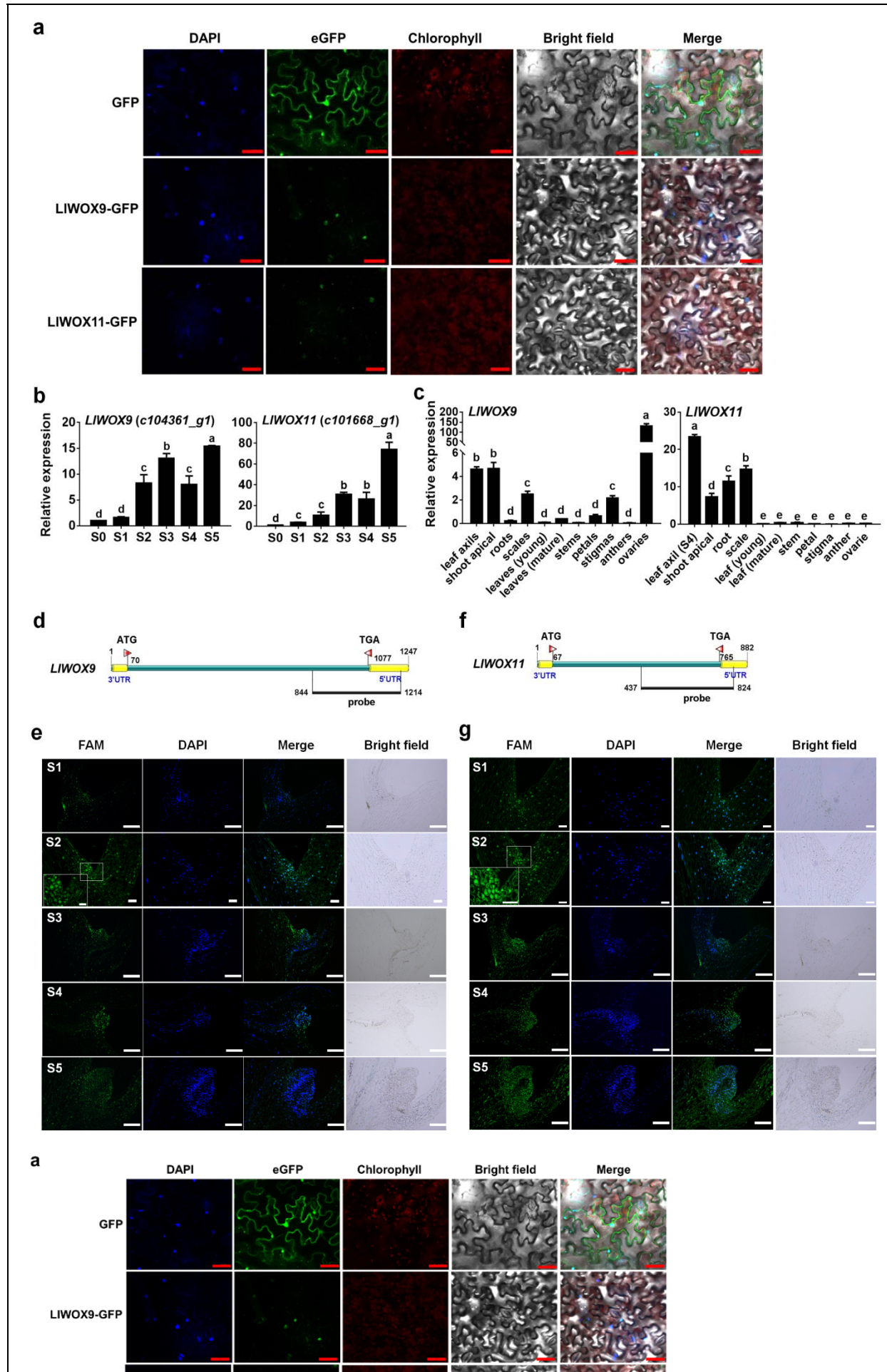
333 leaves of *Nicotiana benthamiana*. The subcellular localization results showed that the GFP

334 signals of the *LIWOX9*-GFP and *LIWOX11*-GFP fusion proteins were located in the nuclei of

335 tobacco leaf epidermal cells (Fig. **2a**), indicating that *LIWOX9* and *LIWOX11* may function as

336 transcription factors in the nucleus.





**Fig. 2.** Subcellular localization, expression patterns and fluorescence in situ hybridization of *LIWOX9* and *LIWOX11*. **a:** Subcellular localization of *LIWOX9*-GFP and *LIWOX11*-GFP proteins in *Nicotiana benthamiana* leaf epidermal cells with 4',6-diamidino-2-phenylindole (DAPI) staining. Scale bars = 50  $\mu$ m. **b:** *LIWOX9* and *LIWOX11* expression during bulbil formation. **c:** *LIWOX9* and *LIWOX11* expression in different tissues. Values are means  $\pm$  SDs (n=3). Lowercase letters (a-d in B; a-e in C) indicate statistically significant differences at  $P < 0.05$ . **d:** Gene-specific probe of *LIWOX9* used in fluorescence in situ hybridization. **e:** Fluorescence in situ hybridization of *LIWOX9* during bulbil formation. **f:** Gene-specific probe of *LIWOX11* used in fluorescence in situ hybridization. **g:** Fluorescence in situ hybridization of *LIWOX11* during bulbil formation. Scale bar in A (S2) and B (S1, S2), 100  $\mu$ m. Scale bar in A (S1, S3-S5) and B (S3-S5), 500  $\mu$ m.

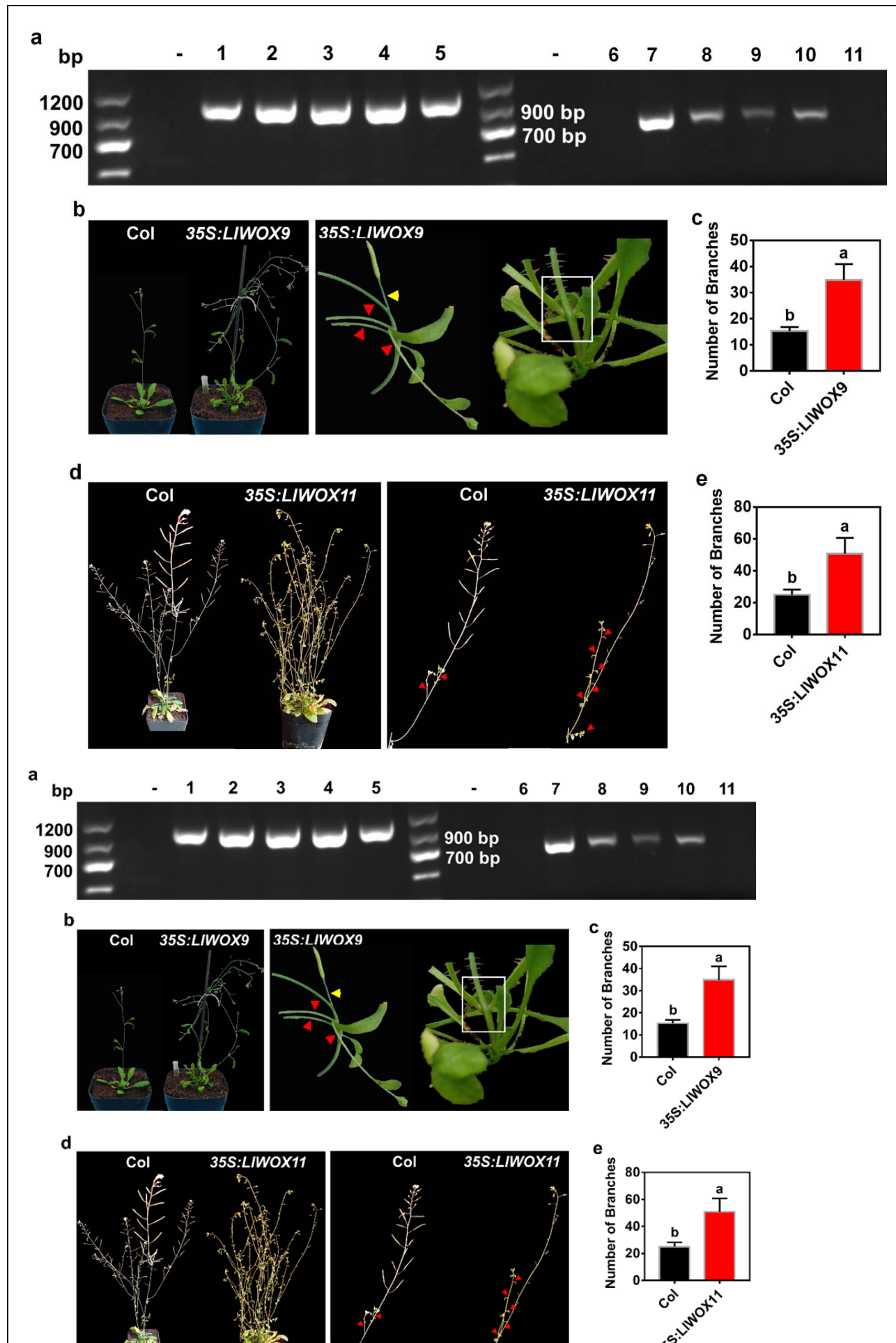
337 The expression of *LIWOX9* and *LIWOX11* increased continuously during bulbil formation  
338 (Fig. **2b**). *LIWOX9* was mainly expressed in the leaf axil (S4 stage), shoot apical meristem, scale,  
339 stigma and ovary, with the highest relative expression in the ovary and the second highest in the  
340 leaf axil (Fig. **2c**). *LIWOX11* was mainly expressed in the leaf axil (S4 stage), shoot apical tissue,  
341 root and scale, and the highest relative expression was found in the leaf axil (S4 stage) (Fig. **2c**).  
342 The relatively high expression of *LIWOX9* and *LIWOX11* in leaf axils further indicated that  
343 *LIWOX9* and *LIWOX11* might be involved in bulbil formation.

344 We further detected the expression of *LIWOX9* and *LIWOX11* during bulbil formation by  
345 fluorescence in situ hybridization (FISH). Gene-specific sequences containing the 3'-UTRs of  
346 *LIWOX9* and *LIWOX11* were selected to synthesize the FAM -labelled fluorescent probes (Fig.  
347 **2d,f**). Our results showed that although the *LIWOX9* and *LIWOX11* fluorescent signals could be  
348 detected throughout the analysed tissue, the fluorescent signals of *LIWOX9* and *LIWOX11* were  
349 mainly located in the leaf axil and gradually increased during bulbil formation (Fig. **2e,g**). In  
350 addition, the fluorescent signals of *LIWOX9* and *LIWOX11* appeared on a differentiated scale (S5  
351 stage) (Fig. **2e,g**). These results further indicated that *LIWOX9* and *LIWOX11* are involved not  
352 only in the formation of the bulbil primordium but also in the differentiation of the bulbil scale.

353

354 ***Overexpression of LIWOX9 and LIWOX11 increases the number of branches in A. thaliana***

355 Bulbils can be considered a special type of branch. To investigate the function of  
356 *LIWOX9* and *LIWOX11* in *A. thaliana* branches, we generated transgenic *A. thaliana* lines. The  
357 transgenic lines were identified using the 35S-F and LIWOX9-R or LIWOX11-R primers. An  
358 ~1200 or ~800 bp band was amplified from the genomic DNA of the transgenic lines, and no  
359 corresponding bands were amplified from control plants (Fig. **3a**). Our results demonstrated that  
360 overexpression of *LIWOX9* or *LIWOX11* in *A. thaliana* increased the number of branches and  
361 promoted the formation of accessory buds on inflorescences (Fig. **3b,d**). The number of branches  
362 was significantly higher in the *35S::LIWOX9* and *35S::LIWOX11* transgenic lines than in the  
363 wild type (Fig. **3c,e**). Interestingly, we found that the *35S::LIWOX9* transgenic lines showed  
364 some abnormal phenotypes, such as the development of the inflorescence branches into a single  
365 flower and the abnormal elongation of stem internodes in rosette leaves (Fig. **3b**).

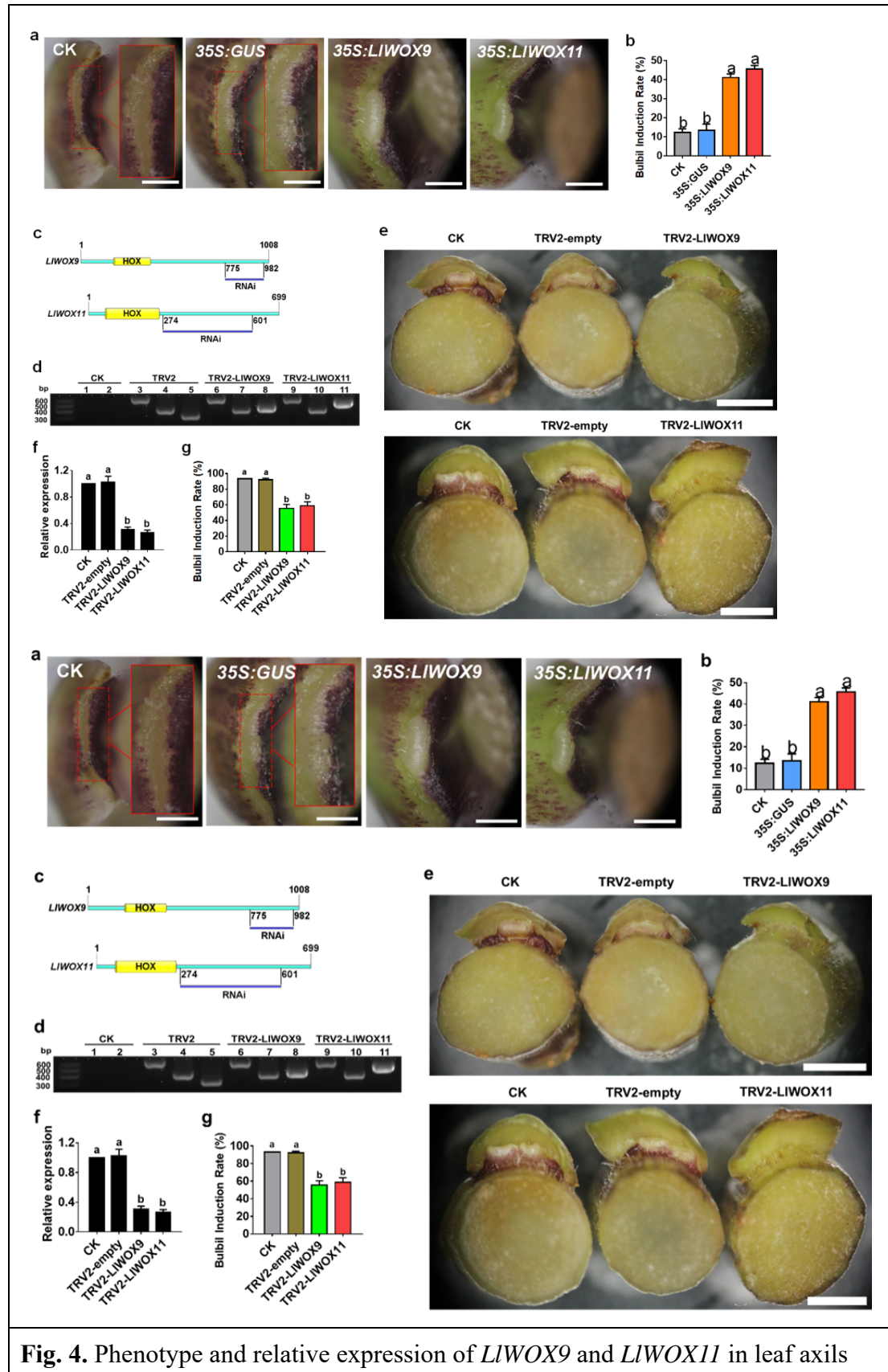


**Fig. 3.** The phenotypes of *35S::LIWOX9* and *35S::LIWOX11* transgenic lines and wild-type *Arabidopsis thaliana* plants. **a:** The transgenic plants of the T3 generation of *A. thaliana* were detected by PCR. ‘+’ indicates the positive control and ‘-’ indicates the negative control. 1-5 represent different transgenic lines overexpressing *LIWOX9*, 6-11 represent different transgenic lines overexpressing *LIWOX11*. **b:** The branching phenotypes of wild-type Col and transgenic plants overexpressing *LIWOX9*. **c:** The numbers of branches on wild-type Col and transgenic plants overexpressing *LIWOX9*. **d:** The branching phenotypes of wild-type Col and transgenic plants overexpressing *LIWOX11*. **e:** The numbers of branches on wild-type Col and transgenic plants overexpressing *LIWOX11*.

366

### 367 ***LIWOX9 and LIWOX11 overexpression promotes bulbil formation***

368 To preliminarily understand the functions of *LIWOX9* and *LIWOX11* during bulbil  
369 formation, we further evaluated the functions of *LIWOX9* and *LIWOX11* via their transient  
370 overexpression in leaf axils through an *in vitro* bulbil induction system. Our results showed that  
371 after 6 d of culture, most of the developing leaf axils in the control group and the *35S::GUS*  
372 treatment group were still in the S3 stage (Fig. 4a), but the overexpression of *LIWOX9* and  
373 *LIWOX11* could significantly promote the formation of bulbils (Fig. 4a), and the rate of bulbil  
374 induction was significantly higher than that in the control group and the *35S::GUS* treatment  
375 group (Fig. 4b). These results indicated that *LIWOX9* and *LIWOX11* play important roles during  
376 bulbil formation.



**Fig. 4.** Phenotype and relative expression of *LIWOX9* and *LIWOX11* in leaf axils

after overexpressing or silencing *LIWOX9* and *LIWOX11*. **a:** The phenotype of the leaf axil after the transient overexpression of *LIWOX9* and *LIWOX11*. **b:** The bulbil induction rate after the transient overexpression of *LIWOX9* and *LIWOX11*. The red box in figure A shows an enlargement of the indicated portion of the leaf axil. Values are means  $\pm$  SDs (n=3). Scale bar in A, 1 mm. **c:** Specific fragments of genes used in VIGS experiments. **d:** PCR was used to detect the presence of the TRV1 and TRV2 viruses in the leaf axils. CK is the negative control, TRV2 is the positive control. Lanes 1, 3, 6 and 9 show TRV1 detection; 2, 4, 7 and 10 show the detection of coat proteins in TRV2; and lanes 5, 8 and 11 show the detection of inserts in TRV2. **e:** The phenotype of the leaf axil after silencing *LIWOX9* and *LIWOX11*. **f:** The relative expression of *LIWOX9* and *LIWOX11* in leaf axils after silencing *LIWOX9* and *LIWOX11*. **g:** The bulbil induction rate after silencing *LIWOX9* and *LIWOX11*. Values are means  $\pm$  SDs (n=3). Scale bar in C, 50 mm. Lowercase letters (a-b in D, E) indicate statistically significant differences at  $P < 0.05$ .

377

### 378 ***LIWOX9 and LIWOX11 silencing inhibits bulbil formation***

379 To further understand the functions of *LIWOX9* and *LIWOX11* during bulbil formation,  
380 we constructed the TRV2-*LIWOX9* and TRV2-*LIWOX11* silencing vectors by selecting specific  
381 fragments of the *LIWOX9* and *LIWOX11* genes (Fig. 4c). After 12 d of infection with the empty  
382 TRV2 vector and the recombinant TRV2-*LIWOX9* or TRV2-*LIWOX11* vector, leaf axil cDNAs  
383 were obtained, and TRV1-F/R and TRV2-F/R were used for PCR-based detection. The results  
384 showed that in leaf axils infected with the empty TRV2 vector, TRV2-*LIWOX9* or  
385 TRV2-*LIWOX11*, the target bands of pTRV1, the coat protein in pTRV2 and the insert fragment  
386 in pTRV2 could be detected (Fig. 4d). These results indicated that TRV2, TRV2-*LIWOX9* and  
387 TRV2-*LIWOX11* were successfully inserted and expressed in the genome of *L. lancifolium*.

388 The silencing of the *LIWOX9* and *LIWOX11* genes in leaf axils was detected by qRT-PCR.

389 The results showed that the expression of *LIWOX9* and *LIWOX11* in leaf axils infected with  
390 TRV2-*LIWOX9* or TRV2-*LIWOX11* was significantly lower than that in the control and the leaf  
391 axils infected with TRV2 (Fig. 4f). These findings indicated that *LIWOX9* and *LIWOX11* were  
392 effectively silenced in TRV2-*LIWOX9*- and TRV2-*LIWOX11*-infected leaf axils, respectively.

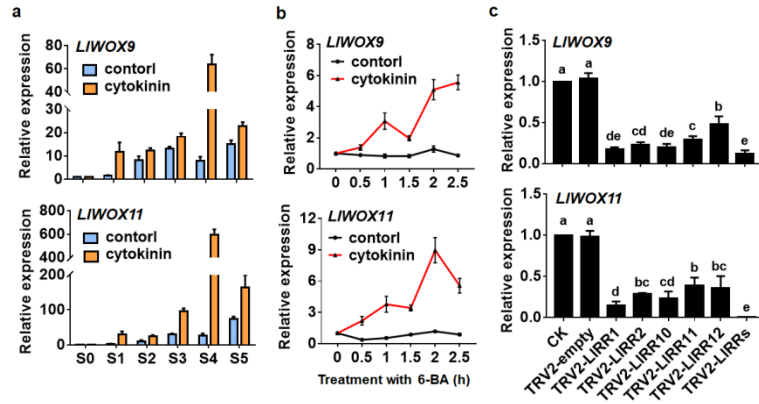
393 The silencing experiment results showed that after *LIWOX9* and *LIWOX11* silencing, the  
394 formation of bulbils was inhibited compared to that in the control group and the empty TRV2  
395 treatment group (Fig. 4e) and the rate of bulbil induction decreased significantly (Fig. 4g). These  
396 results indicated that *LIWOX9* and *LIWOX11* play important roles by positively regulating bulbil  
397 formation.

398

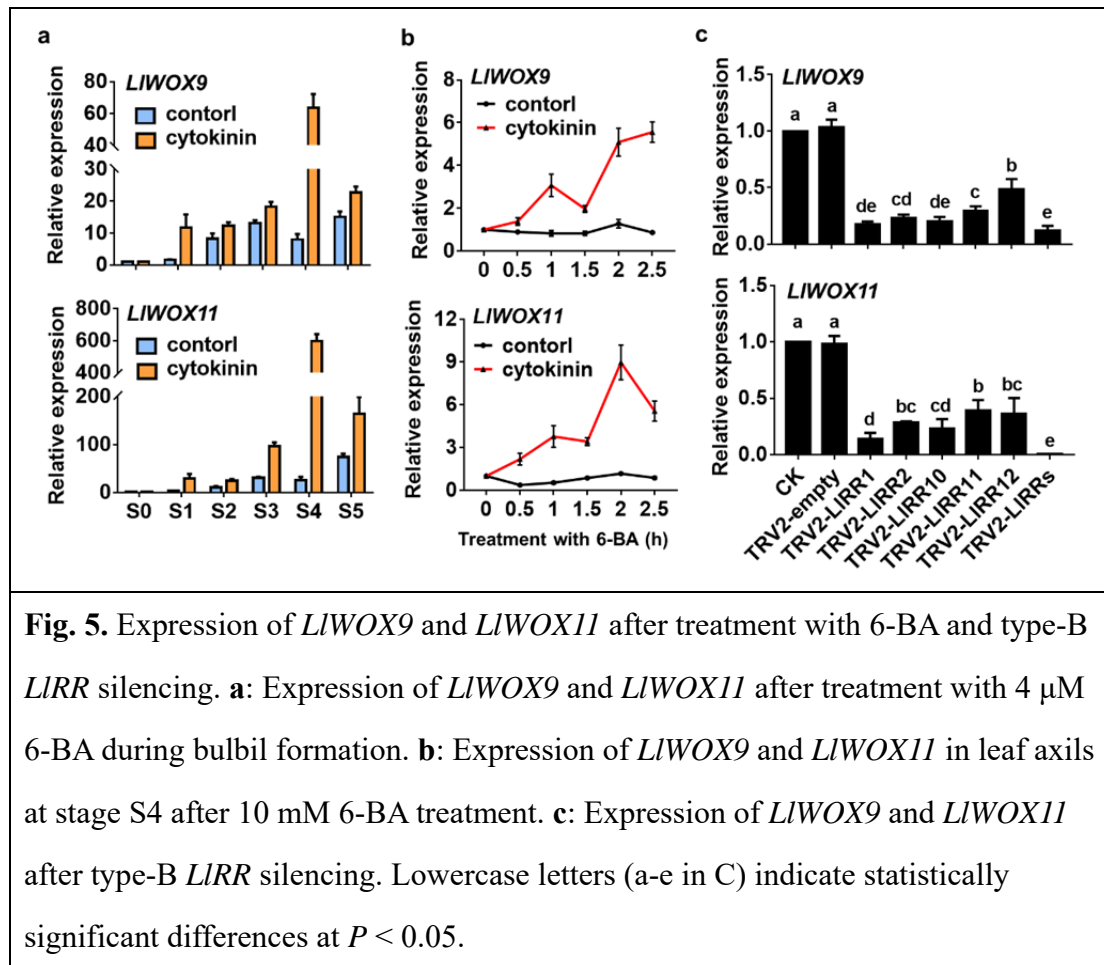
### 399 ***Cytokinins induce the expression of LIWOX9 and LIWOX11***

400 Our previous studies have revealed that cytokinins can promote bulbil formation through  
401 type-B RRs. To study whether the expression of *LIWOX9* and *LIWOX11* is regulated by  
402 cytokinins, we detected the expression of *LIWOX9* and *LIWOX11* after exogenous cytokinin  
403 treatment and the silencing of type-B *LIRRs*. The results showed that after treatment with 6-BA,  
404 the expression of *LIWOX9* and *LIWOX11* during bulbil formation was significantly higher than in  
405 the control group (Fig. 5a). To further study whether the expression of *LIWOX9* and *LIWOX11*  
406 was directly induced by exogenous cytokinins, we treated leaf axils at the S4 stage with 6-BA.  
407 The results showed that after exogenous 6-BA treatment, the expression of *LIWOX9* and  
408 *LIWOX11* was rapidly induced (Fig. 5b), indicating that exogenous cytokinins could induce the  
409 expression of *LIWOX9* and *LIWOX11*. In addition, because cytokinins regulate downstream  
410 genes through type-B RRs, we detected the expression of *LIWOX9* and *LIWOX11* after the  
411 silencing of five type-B *LIRRs* in leaf axils. The results showed that the expression of *LIWOX9*  
412 and *LIWOX11* decreased significantly after the silencing of a single type-B *LIRR* gene, while  
413 after the silencing of five type-B *LIRRs*, the relative expression of *LIWOX9* and *LIWOX11* was  
414 almost undetectable (Fig. 5c). These results suggest that cytokinins can induce the expression of  
415 *LIWOX9* and *LIWOX11* and that type-B *LIRRs* may directly regulate the expression of *LIWOX9*





416 and *LIWOX11*.

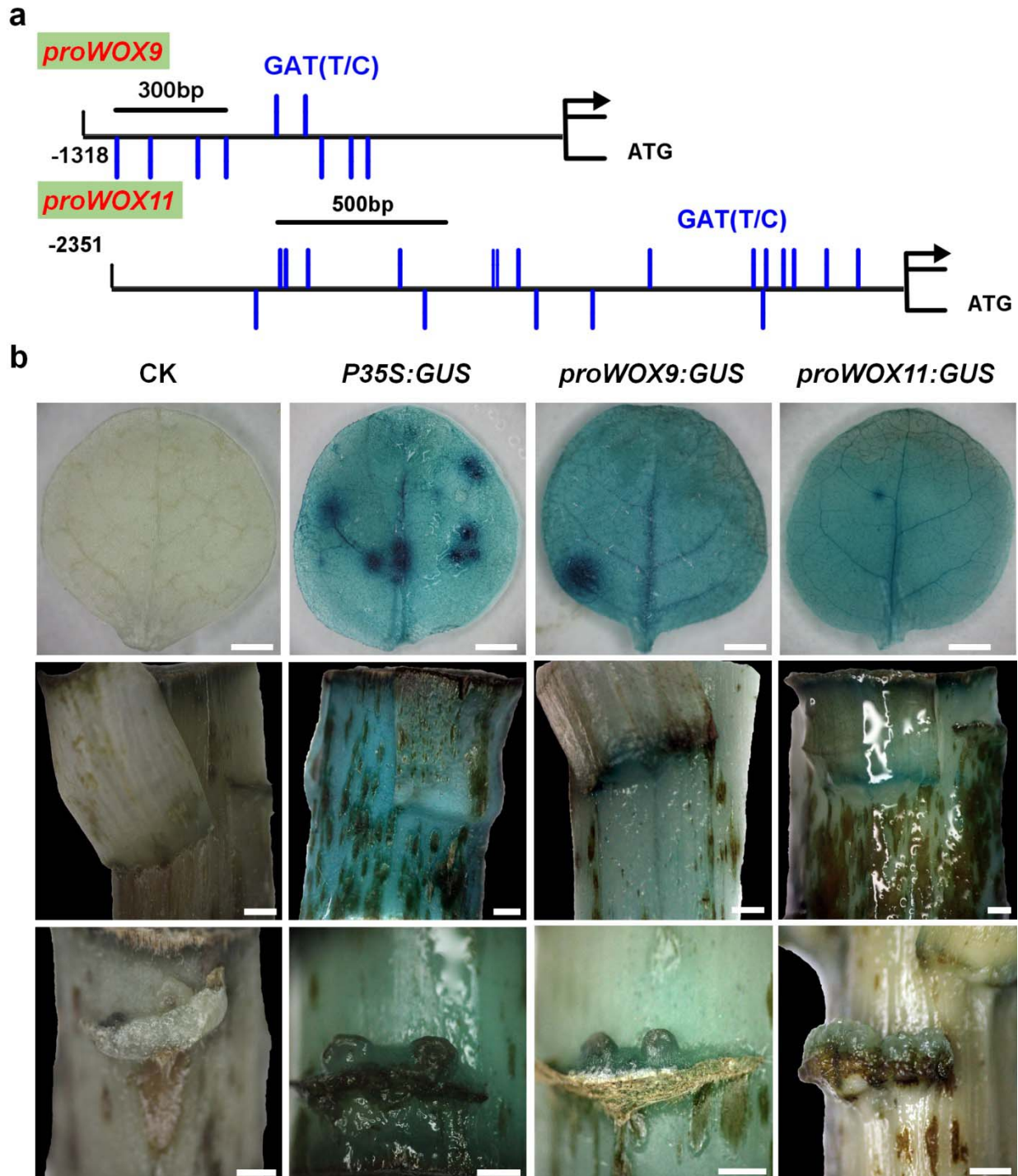


417

418 ***Type-B LIRRs promote the transcription of LIWOX9 and LIWOX11***

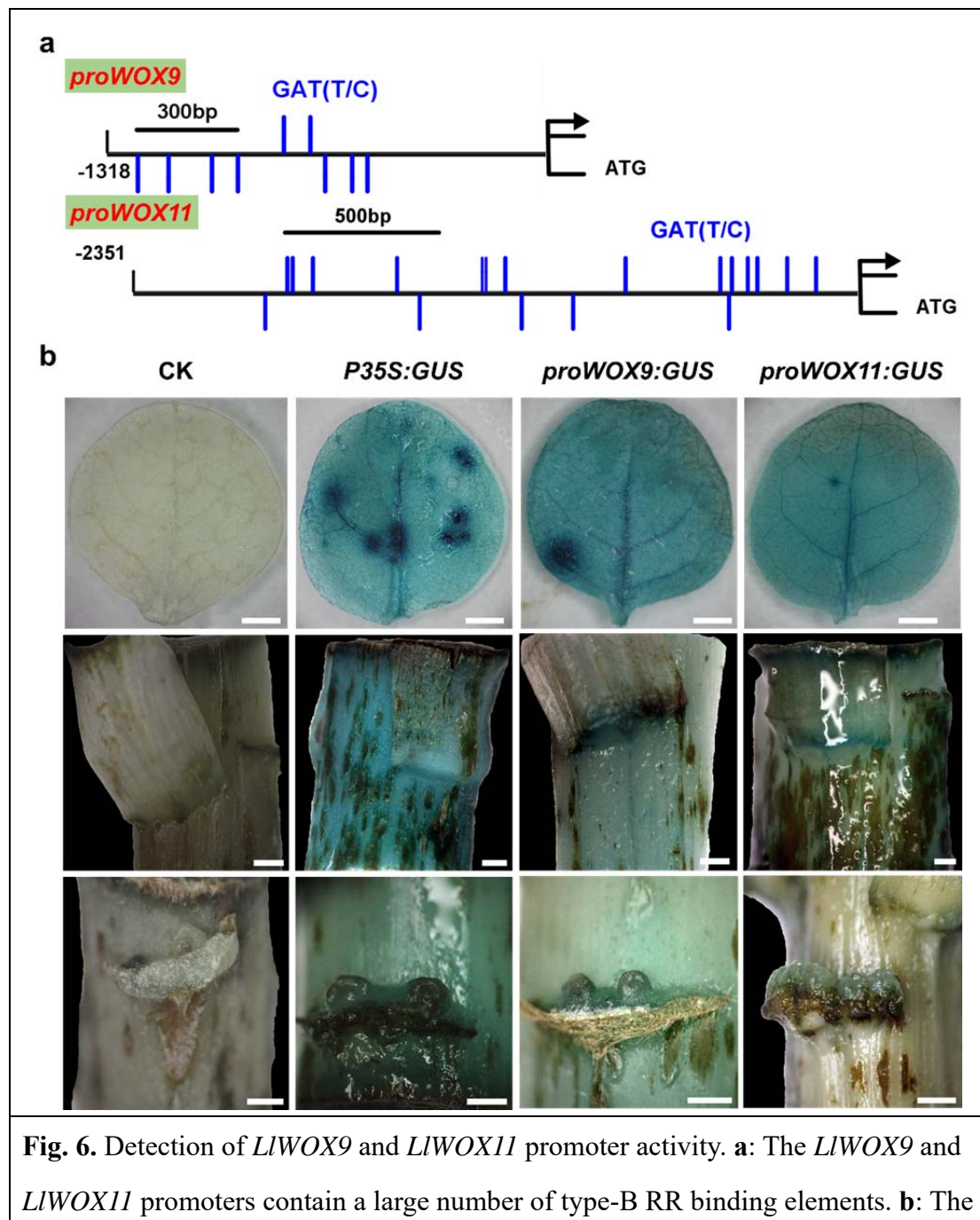
419 To study whether type-B LIRRs regulate the transcription of *LIWOX9* and *LIWOX11*, we

420 first cloned the promoter sequences of *LIWOX9* and *LIWOX11* via the chromosome walking



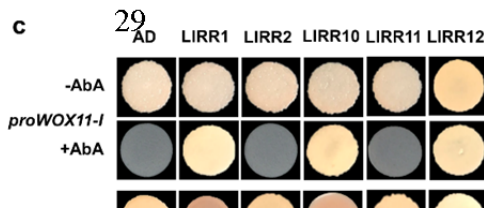
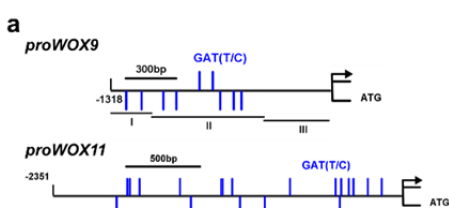
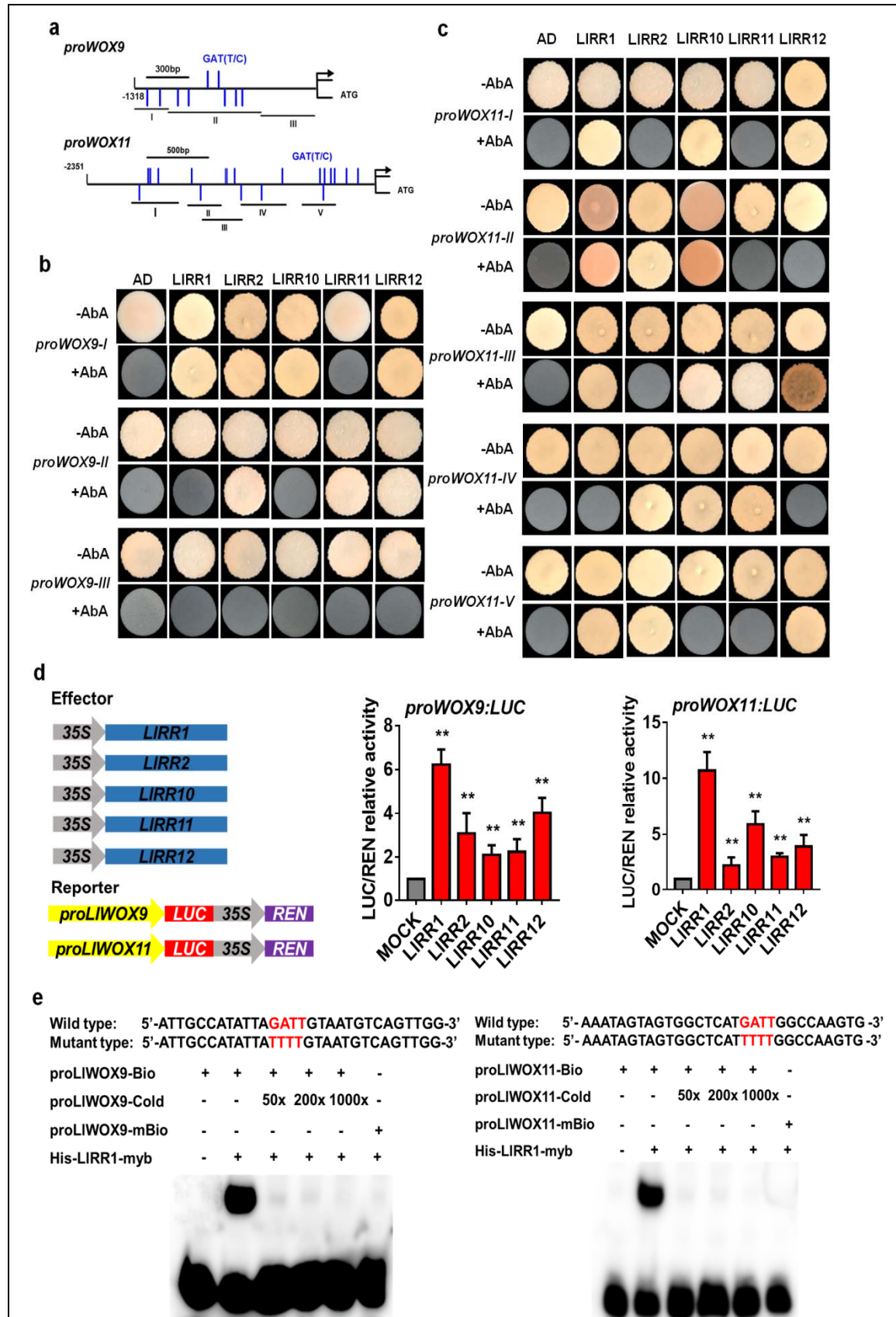
421 technique and obtained promoters with lengths of 1318 bp and 2351 bp, respectively (Fig. 6a).  
422 The use of the New Place and PlantCARE online element prediction tools showed that the  
423 promoters of *LIWOX9* and *LIWOX11* contained a large number of type-B RR binding elements  
424 (NGATT/C) (Fig. 6a). Furthermore, we studied the promoter activities of *LIWOX9* and *LIWOX11*

425 in tobacco leaves and *L. lancifolium* stem segments. GUS staining results showed that both the  
426 *LIWOX9* and *LIWOX11* promoters were active and that their activity was weaker than that of the  
427 35S promoter (Fig. 6b). In the stem segments, we observed stronger GUS staining of the  
428 promoters of *LIWOX9* and *LIWOX11* in the leaf axils (Fig. 6b), indicating that the promoters of  
429 *LIWOX9* and *LIWOX11* may show tissue specificity.



expression of *GUS* was driven by the 35S, *LlWOX9* and *LlWOX11* promoters, and *GUS* staining was performed in *Nicotiana benthamiana* leaves and *Lilium lancifolium* stems. In B, the *N. benthamiana* leaf scale bar is 50 mm, and the *L. lancifolium* stem scale bar is 1 mm.

430           Then, we divided the *LlWOX9* and *LlWOX11* promoters into three or five segments,  
431 respectively, according to the positions of GATT/C elements to construct yeast bait vectors (Fig.  
432 **7a**). Yeast one-hybrid results showed that five type-B LIRRs could strongly bind the promoter  
433 sequences of *LlWOX9* and *LlWOX11*. Among these sequences, the *proWOX9-I* fragment could be  
434 bound by LIRR1, LIRR2, LIRR10 and LIRR12 (Fig. **7b**); the *proWOX9-II* fragment could be  
435 bound by LIRR2, LIRR11 and LIRR12 (Fig. **7b**); and the *proWOX9-III* fragment could not be  
436 bound by any LIRR because it contained no predicted binding element (Fig. **7b**). The  
437 *proWOX11-I* fragments could be bound by LIRR1, LIRR10 and LIRR12 (Fig. **7c**); the  
438 *proWOX11-II* fragments could be bound by LIRR1, LIRR2 and LIRR10 (Fig. **7c**); the  
439 *proWOX11-III* fragments could be bound by LIRR1, LIRR10, LIRR11 and LIRR12 (Fig. **7c**); the  
440 *proWOX11-IV* fragments could be bound by LIRR2, LIRR10 and LIRR11 (Fig. **7c**); and the  
441 *proWOX11-V* fragments could be bound by LIRR1, LIRR2 and LIRR12 (Fig. **7c**).



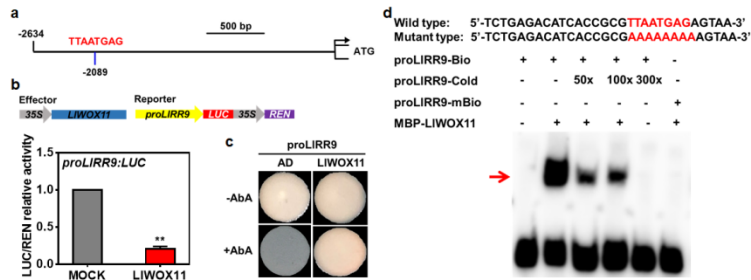
**Fig. 7.** Yeast one-hybrid and dual-luciferase reporter assays and EMSAs of type-B LIRRs with the *LIWOX9* and *LIWOX11* promoters. **a:** Division of the *LIWOX9* and *LIWOX11* promoters into fragments according to the location of type-B RR binding elements (GATT/C). **b:** Yeast one-hybrid assays between five type-B LIRRs and *LIWOX9* promoter fragments. **c:** Yeast one-hybrid assays between type-B LIRRs and *LIWOX11* promoter fragments. **d:** The transient activation test in tobacco leaves verified the transcriptional activation ability of the five type-B LIRRs toward the *LIWOX9* and *LIWOX11* promoters. **e:** The binding ability of His-LIRR1 protein toward the *proLIWOX9-1* and *proLIWOX11-2* fragments was verified by EMSAs. The binding element GATT was mutated to TTTT in the mutant probe. Asterisks in A indicate significant differences compared with the control, with two asterisks indicating  $P < 0.01$ .

442 Furthermore, we studied the transcriptional activation ability of five type-B LIRRs  
443 toward the *LIWOX9* and *LIWOX11* promoters in tobacco leaves. The results showed that  
444 compared with the control group, all five type-B LIRRs could significantly activate the  
445 transcription of the *LIWOX9* and *LIWOX11* promoters (Fig. 7d). In addition, we selected 30 bp  
446 fragments of the *proWOX9-I* and *proWOX11-II* fragments containing GATT/C elements to  
447 synthesize biotin-labelled probes for electrophoretic mobility shift assays (EMSAs). The results  
448 showed that the His-LIRR1 protein could directly bind the *proWOX9-I* and *proWOX11-II*  
449 fragments (Fig. 7e).

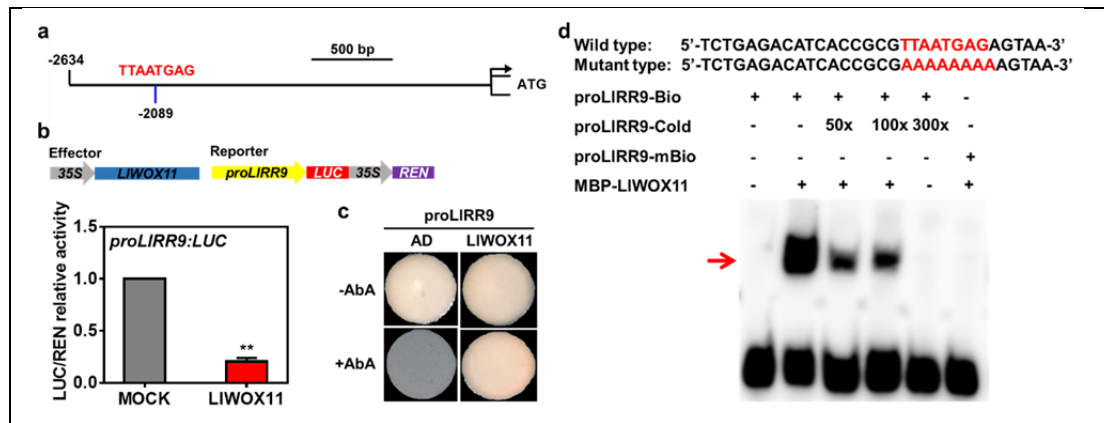
450

#### 451 ***LIWOX11 mediates the cytokinin pathway by inhibiting the transcription of LIRR9***

452 *LIRR9* is a type-A response regulator gene whose product is a negative regulator of  
453 cytokinin signalling. Our previous studies have revealed that LIRR9 is involved in bulbil  
454 formation and transcriptional regulation by LIRR1. In this study, we found a WOX binding  
455 element (TTAATGAG) 2097 bp upstream of ATG in the promoter of *LIRR9* (Fig. 8a). To  
456 determine whether *LIRR9* is a downstream gene directly regulated by *LIWOX9* or *LIWOX11*, we



457 carried out dual-luciferase reporter and yeast one-hybrid assays and EMSAs. Our results showed  
 458 that LIWOX9 did not affect transcription from the *LIRR9* promoter (data not shown), but  
 459 LIWOX11 significantly inhibited transcription from the *LIRR9* promoter (Fig. 8b). The results of  
 460 yeast one-hybrid assays showed that LIWOX11 could bind the *LIRR9* promoter fragment  
 461 containing the TTAATGAG element (Fig. 8c), while LIWOX9 did not show any binding  
 462 capacity (data not shown). Furthermore, a biotin-labelled probe was synthesized by selecting a  
 463 30 bp fragment of the *LIRR9* promoter containing the TTAATGAG element for EMSA. The  
 464 EMSA results showed that LIWOX11 could directly bind the *LIRR9* promoter sequence (Fig.  
 465 8d).



**Fig. 8.** The interaction between LIWOX11 and the *LIRR9* promoter was verified by dual-luciferase reporter and yeast one-hybrid assays and EMSA.

**a:** The transient activation test in tobacco leaves verified the transcriptional activation ability of LIWOX11 toward the *LIRR9* promoter. **b:** The binding ability of LIWOX11 toward the *LIRR9* promoter was verified by a yeast one-hybrid assay. **c:** The binding ability of the MBP-LIWOX11 protein toward the *proLIRR9*

fragment was verified by EMSA. The binding element TTAATGA was mutated to AAAAAAA in the mutant probe. **d**: The transient activation test in tobacco leaves verified the transcriptional activation ability of LIWOX11 toward the *LIWOX9* promoter. Asterisks in C indicate significant differences compared with the control, with two asterisks indicating  $P < 0.01$ .

466

467



468 **Discussion**

469

470 ***LIWOX9 and LIWOX11 are widely expressed in L. lancifolium***

471 *WOX9* and *WOX11* show broad expression profiles in different species (Wu *et al.*, 2005;  
472 Zhao *et al.*, 2009; Cheng *et al.*, 2014; Li *et al.*, 2018). In this study, we showed that *LIWOX9* was  
473 mainly expressed in the leaf axil (S4 stage), shoot apical tissue, scale, stigma and ovary and that  
474 *LIWOX11* was mainly expressed in the leaf axil (S4 stage), shoot apical tissue, root and scale.  
475 Through in situ hybridization and the analysis of GUS reporter promoter fusions, *WOX9* could  
476 be detected in the vegetative SAM, leaf primordia, floral meristems, early floral organs and root  
477 meristematic zone (Wu *et al.*, 2005). *OsWOX11* mRNA was detected in calli, roots, 7-d-old  
478 seedlings, SAM, leaf primordia, and young leaves (Zhao *et al.*, 2009). Our results similarly  
479 showed that *LIWOX9* and *LIWOX11* were highly expressed in leaf axils (S4 stage) and apical  
480 shoots. In accord with the reported relationships of *WOX11* with the development of lateral roots  
481 and crown roots in *A. thaliana* and *O. sativa* (Liu *et al.*, 2014; Hu & Xu, 2016), our results  
482 showed that *LIWOX11* was highly expressed in roots. Unlike previous research results, our  
483 results showed that *LIWOX9* presented the highest relative expression in female reproductive  
484 organs but was almost undetectable in male reproductive organs. In *M. truncatula*, *MtWOX9* is  
485 mainly expressed in nodules, leaves and flowers but is expressed at lower levels in ovaries (Li *et*  
486 *al.*, 2018). *OsWOX9* is expressed in axillary tillers, panicles, stamen primordia and pistil  
487 primordia but can only be detected in anthers after flower development maturity (Cheng *et al.*,  
488 2014).

489

490 ***LIWOX9 and LIWOX11 are positive regulators during bulbil formation***

491 Members of the intermediate clade are widely expressed in plants and usually play a role  
492 in maintaining meristem cell division (Wu *et al.*, 2007; Breuninger *et al.*, 2008; Zhao *et al.*,  
493 2009). In *A. thaliana*, when *WOX9* function is lost, cells divide abnormally, and the development  
494 of the shoot meristem is defective (Wu *et al.*, 2005; Skylar *et al.*, 2010). In the *Oswox11* mutant,

495 the crown root number and plant height are decreased, and the growth rate and flowering time  
496 are delayed, while in *OsWOX11*-overexpressing lines, the number of crown roots is increased,  
497 ectopic crown roots form at the base of the spikelets, and the growth rate increases significantly  
498 (Zhao *et al.*, 2009). In this study, the overexpression of *LIWOX9* and *LIWOX11* in *A. thaliana*  
499 significantly increased branch numbers and promoted bulbil formation in *L. lancifolium*. We  
500 found that the rate of bulbil induction decreased after the silencing of *LIWOX9* and *LIWOX11*  
501 expression but increased significantly after *LIWOX9* and *LIWOX11* overexpression, indicating  
502 that *LIWOX9* and *LIWOX11* are positive regulators of bulbil formation. After the overexpression  
503 of *LIWOX9* and *LIWOX11*, we also observed the abnormal proliferation of axillary tissue cells  
504 and the development of large purple-black bulbils on leaf axils (Fig. S2), which indicated that  
505 *LIWOX9* and *LIWOX11* maintain the normal division of the meristem.

506 A recent study showed that *OsWOX11* can recruit the H3K27me3 demethylase JM1705  
507 to activate the expression of related genes during rice shoot development (Cheng *et al.*, 2018).  
508 We speculate that *LIWOX11* may regulate the expression of downstream genes through a similar  
509 mechanism to promote the formation of bulbils.

510

### 511 ***Cytokinins induce the transcription of LIWOX9 and LIWOX11 through type-B LIRRs***

512 Many studies have shown that *WOX* family genes can be induced by plant hormones,  
513 such as auxin, cytokinin and gibberellin (Gonzali *et al.*, 2005; Leibfried *et al.*, 2005; Weijers *et*  
514 *al.*, 2006; Sarkar *et al.*, 2007; Skylar *et al.*, 2010). Cheng *et al.* (2014) analysed the promoters of  
515 rice *WOX* family genes and found that there are abundant hormone response elements in these  
516 promoters, with the promoter regions of all family members including cytokinin response  
517 elements (NGATT/C) and auxin response elements (TGTATC or GAGACA). Further study  
518 showed that *OsWOX5*, *OsWOX11*, *OsWOX12A* and *OsWOX12B* could be rapidly induced by  
519 NAA and 6-BA (Cheng *et al.*, 2014). In our study, we also found a large number of plant  
520 hormone response elements, especially cytokinin response elements, in the *LIWOX9* and  
521 *LIWOX11* promoters. Then, we demonstrated that the expression of *LIWOX9* and *LIWOX11* could

522 be induced by cytokinin, during which the expression of *LIWOX9* reached a peak after 2.5 h of  
523 induction, and the expression of *LIWOX11* reached its highest level after 2 h of induction.

524

### 525 ***LIWOX11 mediates cytokinin signalling by inhibiting the transcription of LIRR9***

526 Some studies have shown that both *WOX9* and *WOX11* can mediate cytokinin pathways  
527 to regulate plant development (Wu *et al.*, 2005, 2007; Zhao *et al.*, 2009; Wang *et al.*, 2014c;  
528 Jiang *et al.*, 2017). In rice crown root formation, *OsWOX11* can directly bind and inhibit the  
529 transcription of *OsRR2* to mediate cytokinin signalling (Zhao *et al.*, 2009). *OsRR2* is specifically  
530 expressed in the crown root and is a member of the type-A RRs, which are negative regulators of  
531 cytokinin signalling. Therefore, after the transcription of *OsRR2* is inhibited, cytokinin signalling  
532 is enhanced to induce crown root formation (Zhao *et al.*, 2009). The ERF3 protein can bind to  
533 the *OsWOX11* protein and further enhance the transcriptional inhibition of *OsRR2* by *OsWOX11*  
534 (Zhao *et al.*, 2015). We identified a similar mechanism, as our results showed that *LIWOX11* can  
535 directly bind the promoter of *LIRR9*, a type-A *LIRR* gene, and inhibit its transcription to enhance  
536 cytokinin signalling and thus promote bulbil formation.

537 *WOX9* mediates cytokinin pathway signalling in a different way. During *A. thaliana*  
538 seedling development, *WOX9* seems to promote the expression of type-A RRs. In the *wox9*  
539 mutant, *ARR5* expression is decreased, which causes shoot meristem development termination  
540 (Skylar *et al.*, 2010; Skylar & Wu, 2010). In rice, *OsWOX9* plays a negative role in regulating  
541 the expression of type-A RRs (Wang *et al.*, 2014c). A study in rice revealed that *OsWOX9*  
542 modulates the cytokinin pathway to regulate the growth height and flowering time of tillers and  
543 main branches (Wang *et al.*, 2014c). In the *Oswox9* mutant, tiller elongation is inhibited, and in  
544 the shortened internodes, the expression of *OsCKX4*, *OsCKX9* and several type-A *OsRR* genes  
545 (*OsRR6*, *OsRR9*, *OsRR10*) is increased (Wang *et al.*, 2014c). However, in our study, we did not  
546 find an effect of *LIWOX9* on the expression of *LIRR9*.

547

### 548 ***LIWOX9 may mediate gibberellin signalling***

549 In the *Oswox9* mutant, the tillers have shorter internodes with cells that are fewer in  
550 number and unelongated relative to those of the wild type, and *OsWOX9* activity in internode  
551 elongation is directly or indirectly associated with GA signalling (Wang *et al.*, 2014c). The organ  
552 boundary gene *ARABIDOPSIS THALIANA HOMEBOX GENE 1 (ATH1)* and the gibberellin  
553 signalling *DELLA* genes maintain the compressed rosette growth habit of *Arabidopsis*. The loss  
554 of *ATH1* and *DELLA* function causes a change from a rosette to caulescent growth habit (Ejaz *et*  
555 *al.*, 2021). The phenotypes of *LIWOX9*-overexpressing *Arabidopsis* lines show elongated  
556 internodes, and we speculate that *LIWOX9* may regulate the internode elongation associated with  
557 GA signalling.

558

### 559 ***LIWOX9 and LIWOX11 may be involved in scale development and anthocyanin synthesis***

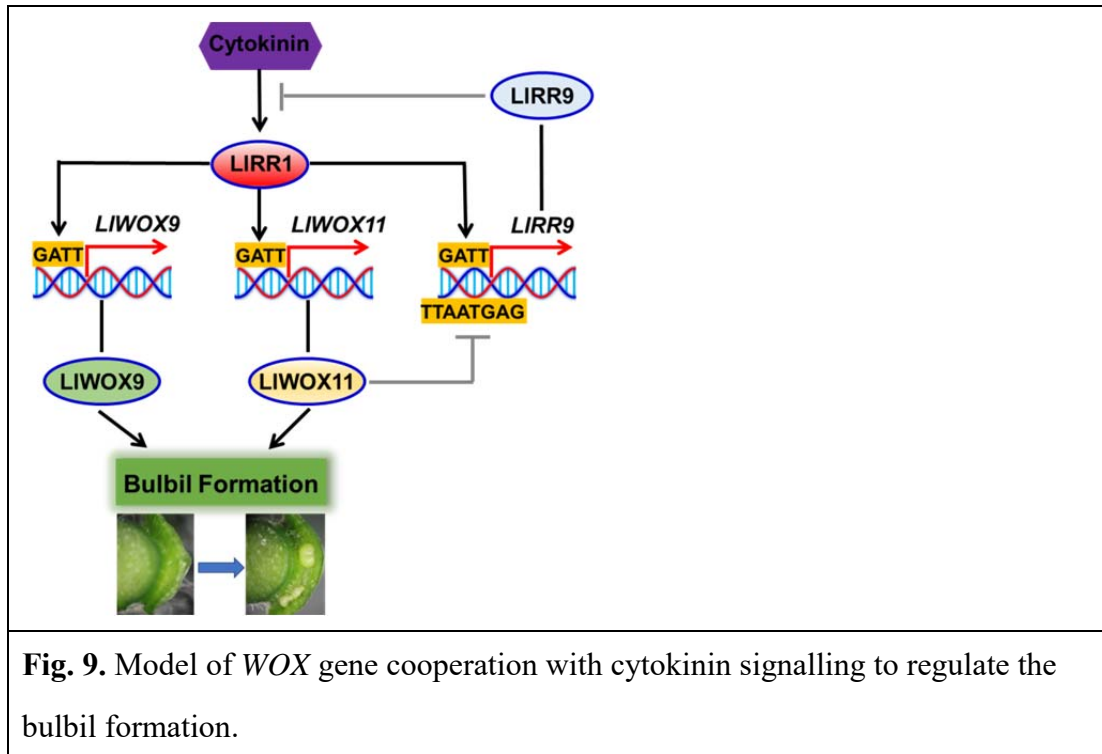
560 A recent study on the genome of garlic bulb plants showed that two *WOX* family genes  
561 (*Asa7G00799.1* and *Asa3G03517.1*) are involved in bulb development, among which  
562 *Asa7G00799.1* is expressed specifically in bulbs and positively correlated with bulb weight (Sun  
563 *et al.*, 2020). Our results showed that *LIWOX9* and *LIWOX11* were highly expressed in scales and  
564 that the expression of *LIWOX9* and *LIWOX11* reached the highest level at the stage of bulbil  
565 scale development (S5), indicating that *LIWOX9* and *LIWOX11* may be involved in the  
566 development of lily bulbs.

567 Interestingly, we found that the overexpression of *LIWOX9* and *LIWOX11* not only  
568 promoted bulbil formation but also resulted in abnormal purple-black bulbils on the leaf axils  
569 (Fig. S2). Although bulbils may gradually turn purple-black during development under normal  
570 circumstances, the overexpression of *LIWOX9* and *LIWOX11* significantly advanced this change.  
571 A recent study showed that *PgWOX11* in *Panax ginseng* can positively regulate the expression of  
572 *ERF1B* (an *AP2/ETHYLENE-RESPONSIVE FACTOR*) and thus regulate the biosynthesis of  
573 ginsenosides (Liu *et al.*, 2020a,b). Therefore, we speculate that *LIWOX9* and *LIWOX11* may be  
574 involved in the synthesis of anthocyanins.

575

## 576 Conclusion

577 In conclusion, we revealed the molecular mechanism by which WOX genes cooperate  
578 with cytokinin signalling to regulate bulbil formation. Type-B LIRRs promote the transcription  
579 of *LIWOX9* and *LIWOX11*, and *LIWOX11* inhibits the transcription of type-A *LIRR9* to enhance  
580 cytokinin signalling, thus promoting bulbil formation (Fig. 9).



581

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588

## 589 Author contribution

590 JM and PY designed the research. GH, YC, YT, LW, MS, JW, and LX conducted the experiments.

591 GH analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

592

593

594 **Accession numbers**

595 RNA-seq raw reads from this article can be found in the NCBI SRA data under accession

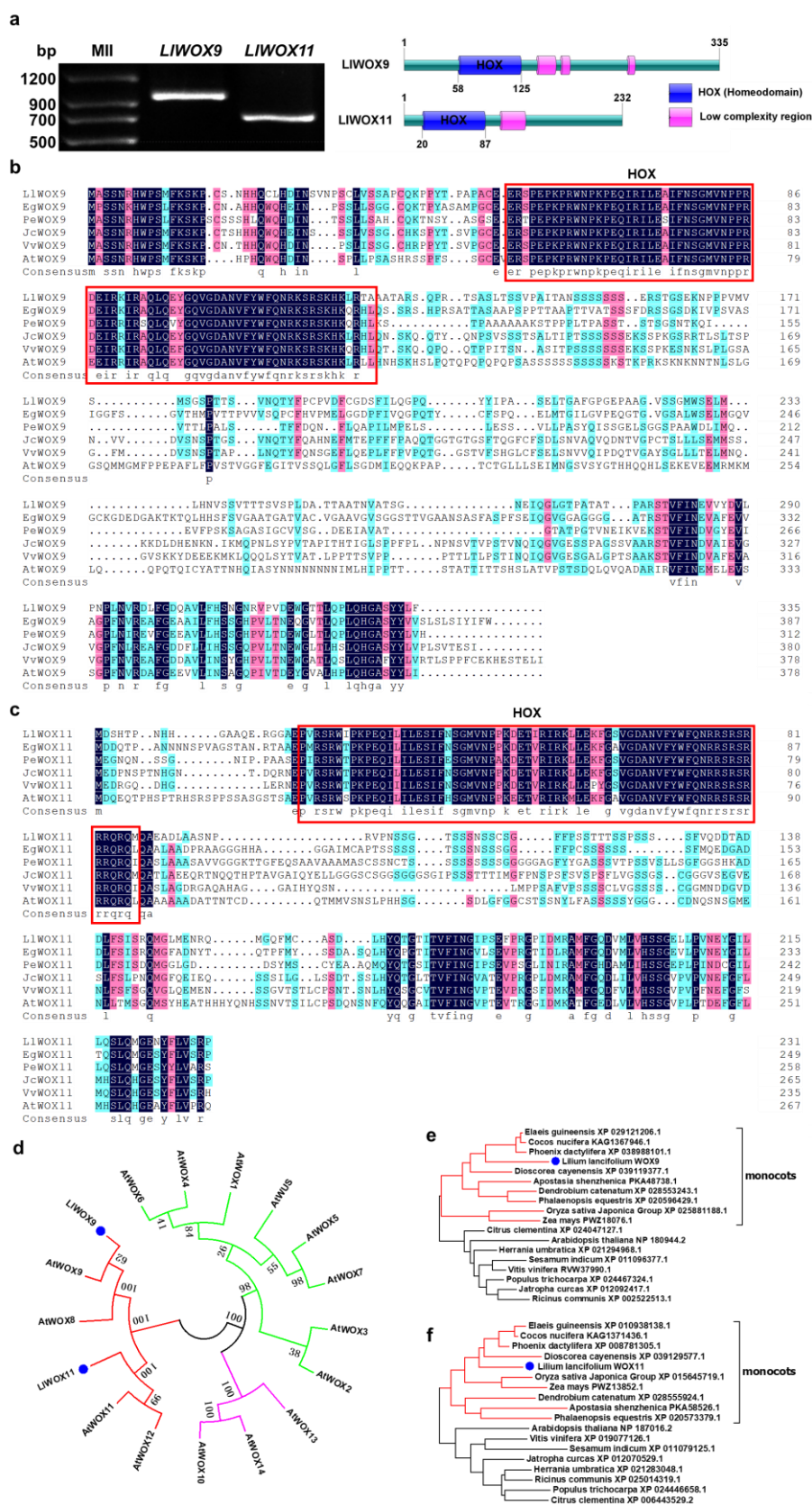
596 number SRP103184. Gene accession numbers used in this study: LIRR1 (MW509629), LIRR2

597 (MW509630), LIRR10 (MW509631), LIRR11 (MW509632), LIRR12 (MW509633) and LIRR9

598 (MW509634).

599

600

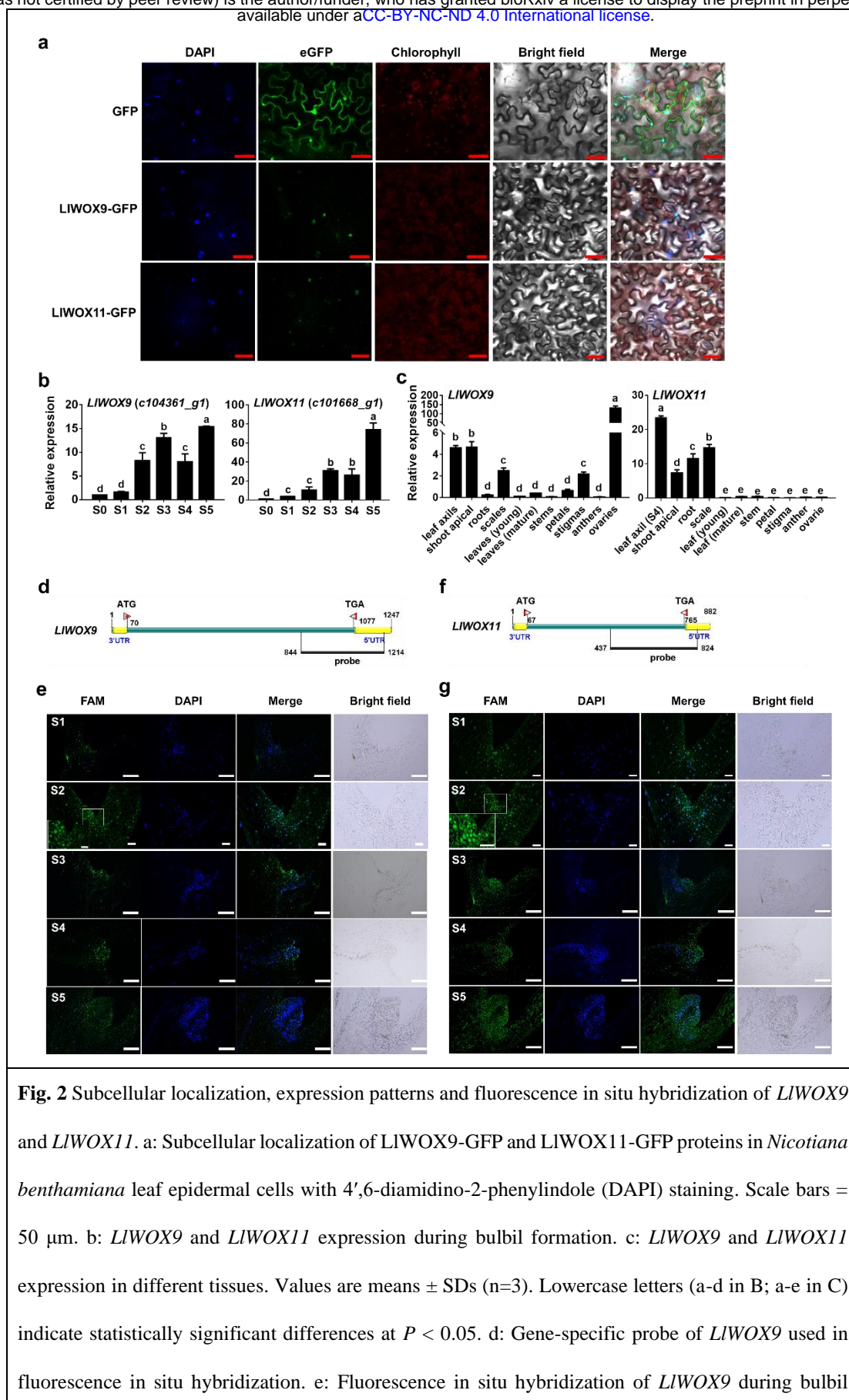


**Fig. 1** Full-length cloning, sequence alignment and phylogenetic tree of *LIWOX9* and *LIWOX11*.

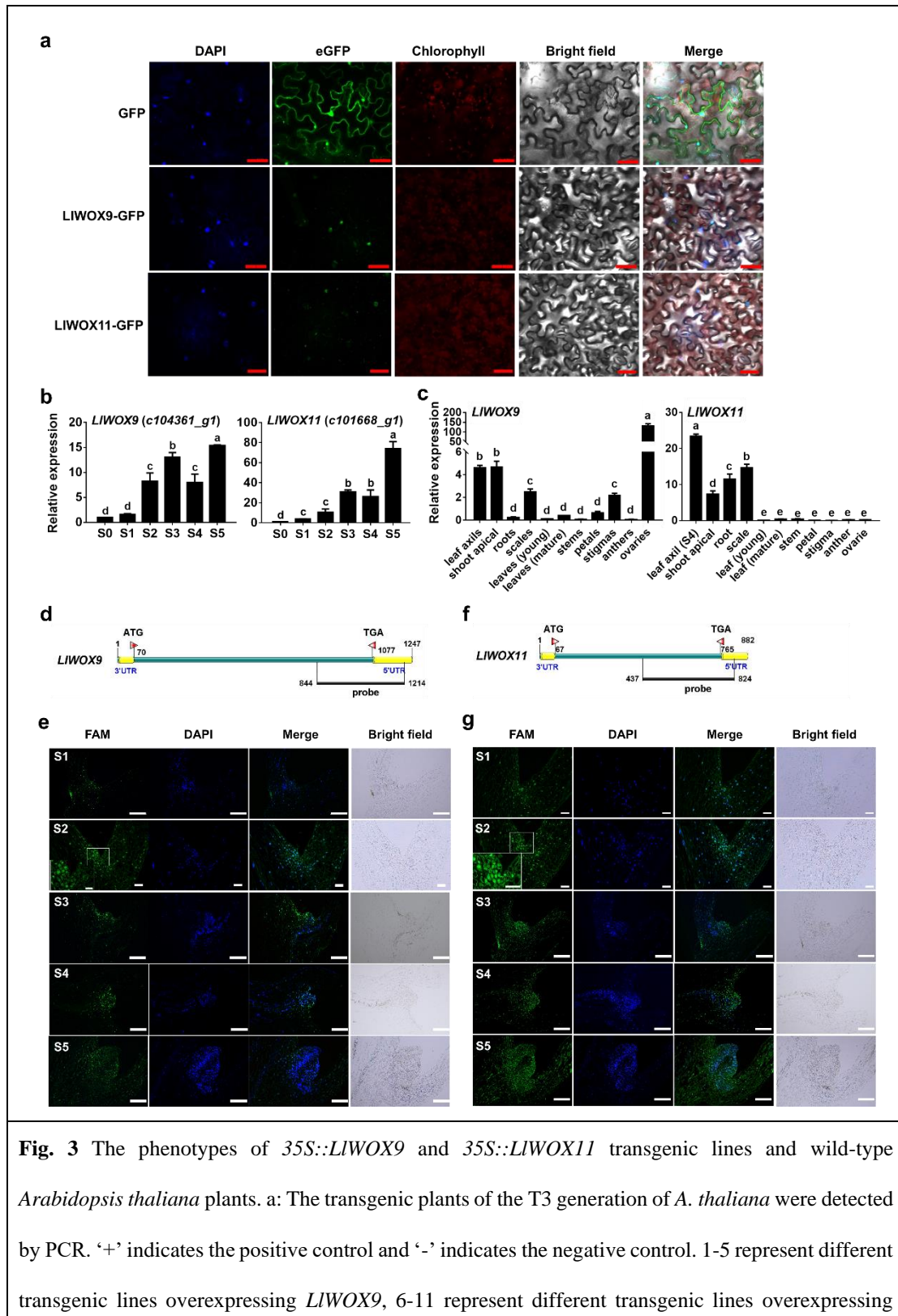
a: Full-length cloning and domain prediction of *LIWOX9* and *LIWOX11*. b: Multiple sequence



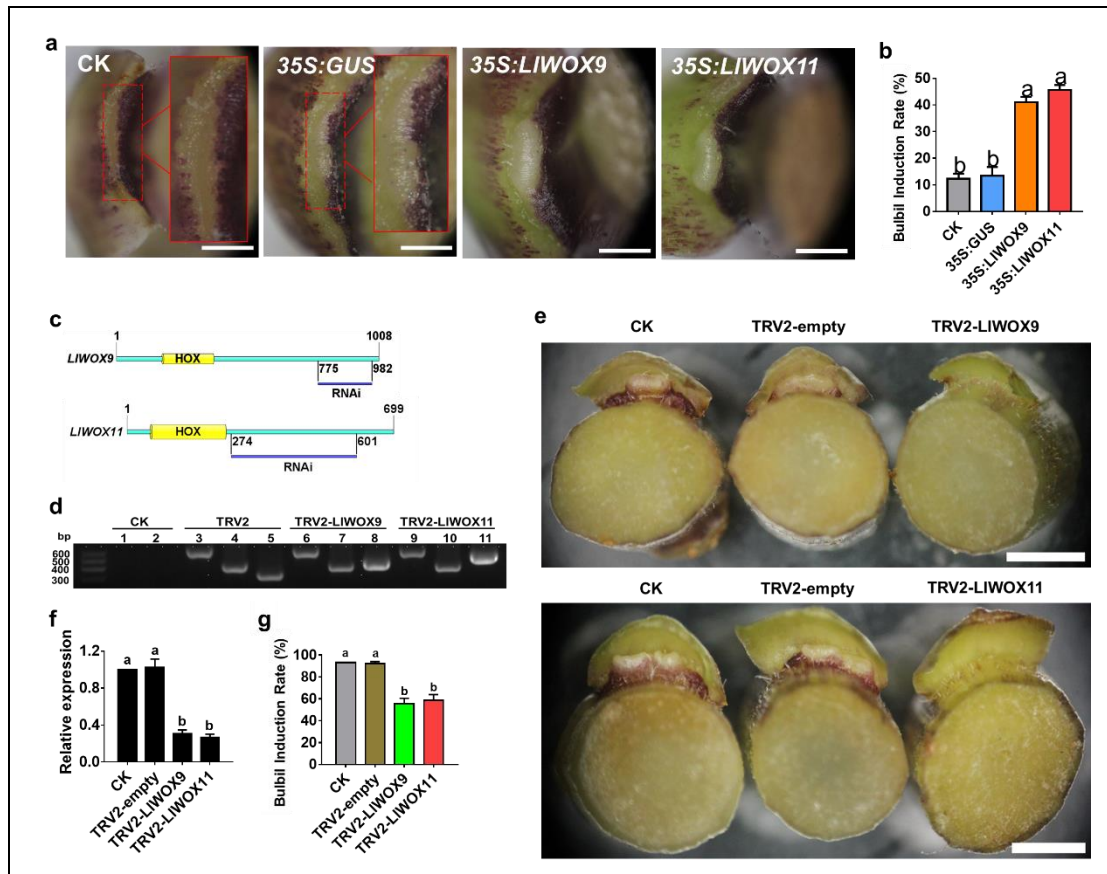
alignment of LIWOX9 with sequences of other species. c: Multiple sequence alignment of LIWOX11 with sequences of other species. The red boxes in B and C represent the HOX domain. *Ll*: *Lilium lancifolium*, *Eg*: *Elaeis guineensis* (EgWOX9, XP 029121206.1; EgWOX11, XP 010938138.1), *Pe*: *Phalaenopsis equestris* (PeWOX9, XP 020596429.1; PeWOX11, XP 020573379.1), *Jc*: *Jatropha curcas* (JcWOX9, XP 012092417.1; JcWOX11, XP 012070529.1), *Vv*: *Vitis vinifera* (VvWOX9, RVW37990.1; VvWOX11, XP 019077126.1), *At*: *Arabidopsis thaliana* (AtWOX9, NP 180994.2; AtWOX11, NP 187016.2). d: Neighbour-joining tree of the LIWOX9 and LIWOX11 amino acid sequences of *L. lancifolium* and WOX family amino acid sequences from *A. thaliana*. e: Neighbour-joining tree of the LIWOX9 amino acid sequence of *L. lancifolium* and WOX9 amino acid sequences from other species. f: Neighbour-joining tree of the LIWOX11 amino acid sequence of *L. lancifolium* and WOX11 amino acid sequences from other species. Bootstrap values from 1,000 replicates were used to assess the robustness of the tree.



formation. f: Gene-specific probe of *LIWOX11* used in fluorescence in situ hybridization. g: Fluorescence in situ hybridization of *LIWOX11* during bulbil formation. Scale bar in A (S2) and B (S1, S2), 100  $\mu$ m. Scale bar in A (S1, S3-S5) and B (S3-S5), 500  $\mu$ m.

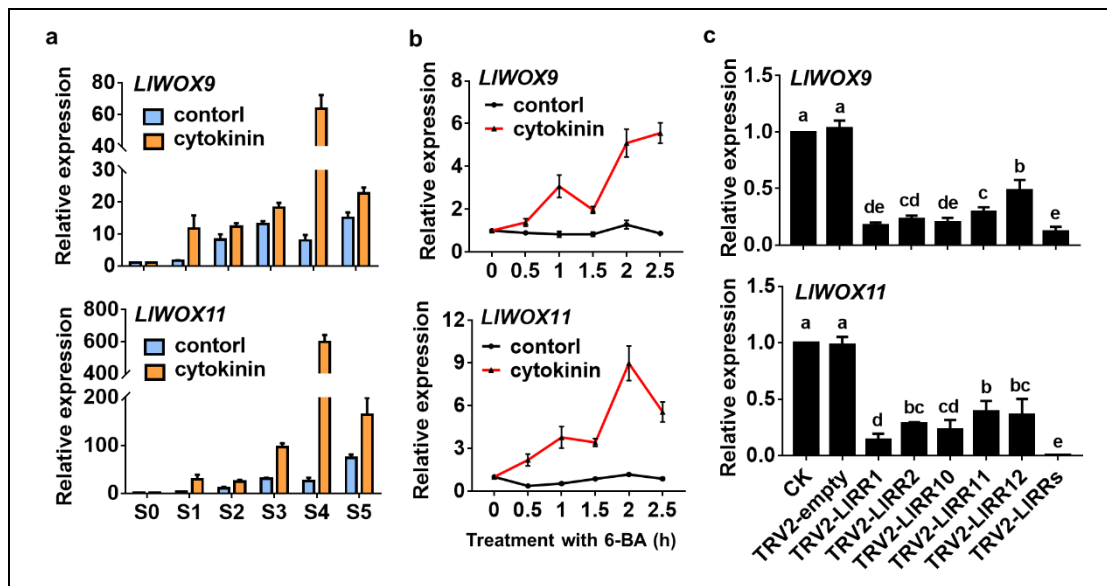


*LIWOX11*. b: The branching phenotypes of wild-type Col and transgenic plants overexpressing *LIWOX9*. c: The numbers of branches on wild-type Col and transgenic plants overexpressing *LIWOX9*. d: The branching phenotypes of wild-type Col and transgenic plants overexpressing *LIWOX11*. e: The numbers of branches on wild-type Col and transgenic plants overexpressing *LIWOX11*.

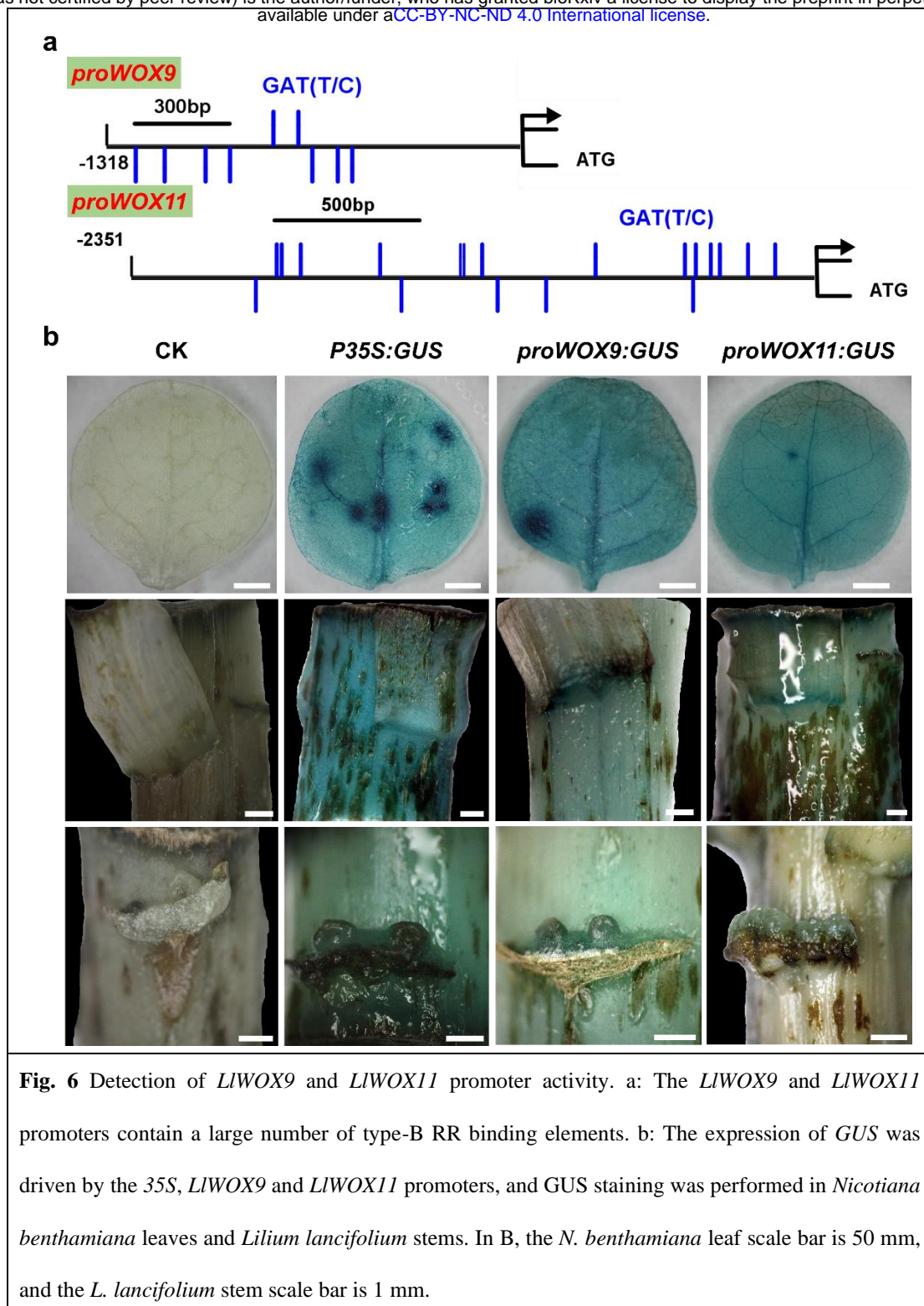


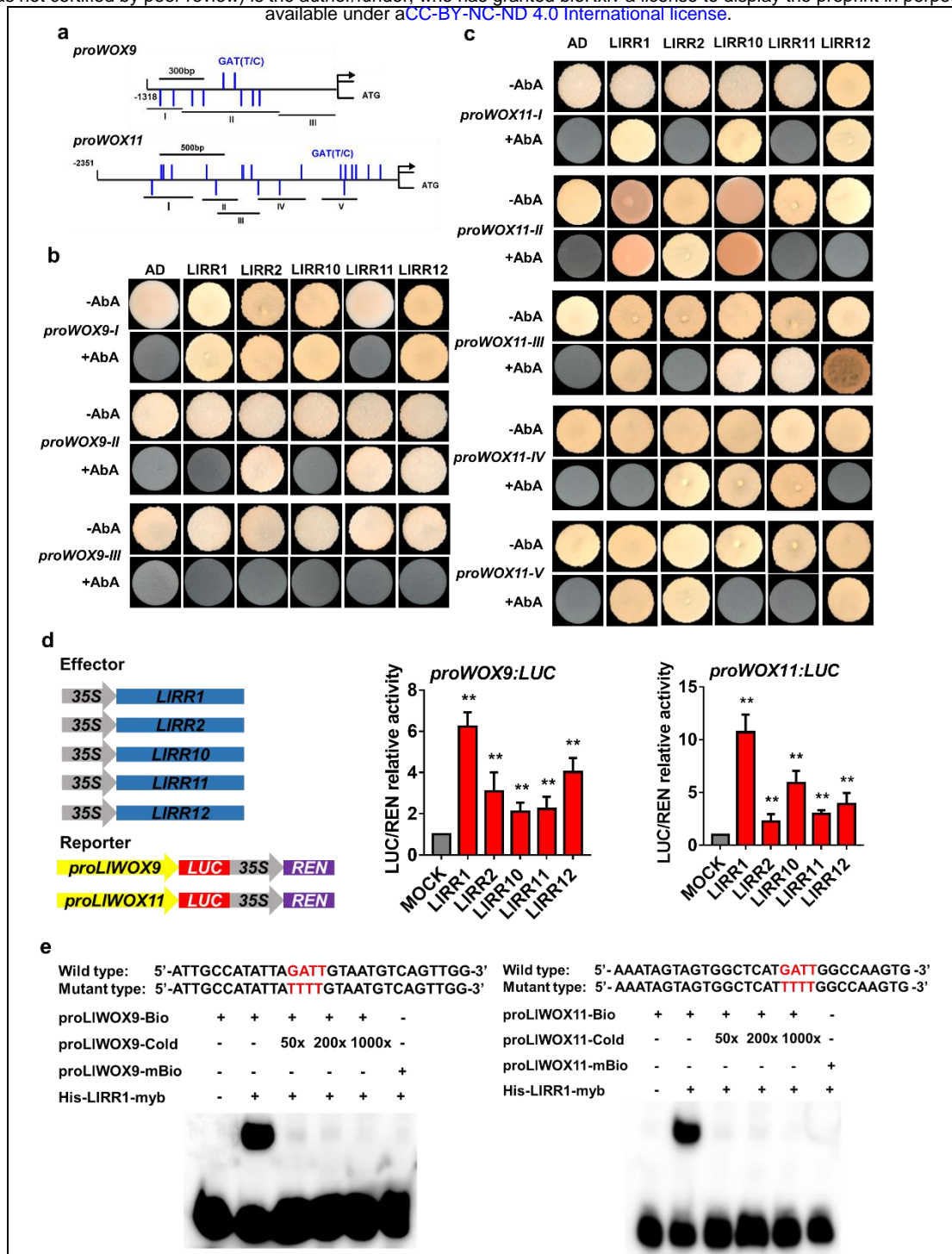
**Fig. 4** Phenotype and relative expression of *LIWOX9* and *LIWOX11* in leaf axils after overexpressing or silencing *LIWOX9* and *LIWOX11*. a: The phenotype of the leaf axil after the transient overexpression of *LIWOX9* and *LIWOX11*. b: The bulbil induction rate after the transient overexpression of *LIWOX9* and *LIWOX11*. The red box in figure A shows an enlargement of the indicated portion of the leaf axil. Values are means  $\pm$  SDs (n=3). Scale bar in A, 1 mm. c: Specific fragments of genes used in VIGS experiments. d: PCR was used to detect the presence of the TRV1 and TRV2 viruses in the leaf axils. CK is the negative control, TRV2 is the positive control. Lanes 1, 3, 6 and 9 show TRV1 detection; 2, 4, 7 and 10 show the detection of coat proteins in TRV2; and lanes 5, 8 and 11 show the detection of inserts in TRV2. e: The phenotype of the leaf axil after silencing *LIWOX9* and *LIWOX11*. f: The relative expression of *LIWOX9* and *LIWOX11* in leaf axils after silencing *LIWOX9* and *LIWOX11*. g: The bulbil induction rate after silencing *LIWOX9* and

*LIWOX11*. Values are means  $\pm$  SDs (n=3). Scale bar in C, 50  $\mu$ m. Lowercase letters (a-e in D, E) indicate statistically significant differences at  $P < 0.05$ .

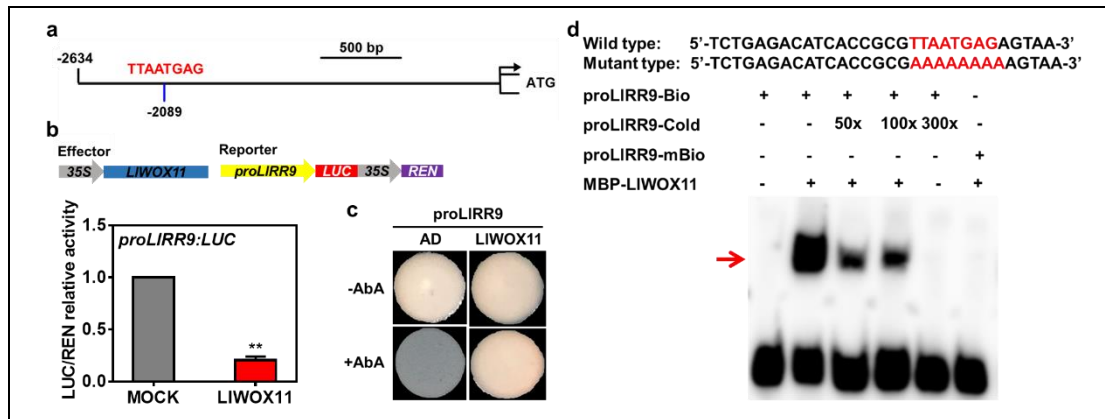


**Fig. 5** Expression of *LIWOX9* and *LIWOX11* after treatment with 6-BA and type-B *LIRR* silencing. a: Expression of *LIWOX9* and *LIWOX11* after treatment with 4  $\mu$ M 6-BA during bulbil formation. b: Expression of *LIWOX9* and *LIWOX11* in leaf axils at stage S4 after 10 mM 6-BA treatment. c: Expression of *LIWOX9* and *LIWOX11* after type-B *LIRR* silencing. Lowercase letters (a-e in C) indicate statistically significant differences at  $P < 0.05$ .





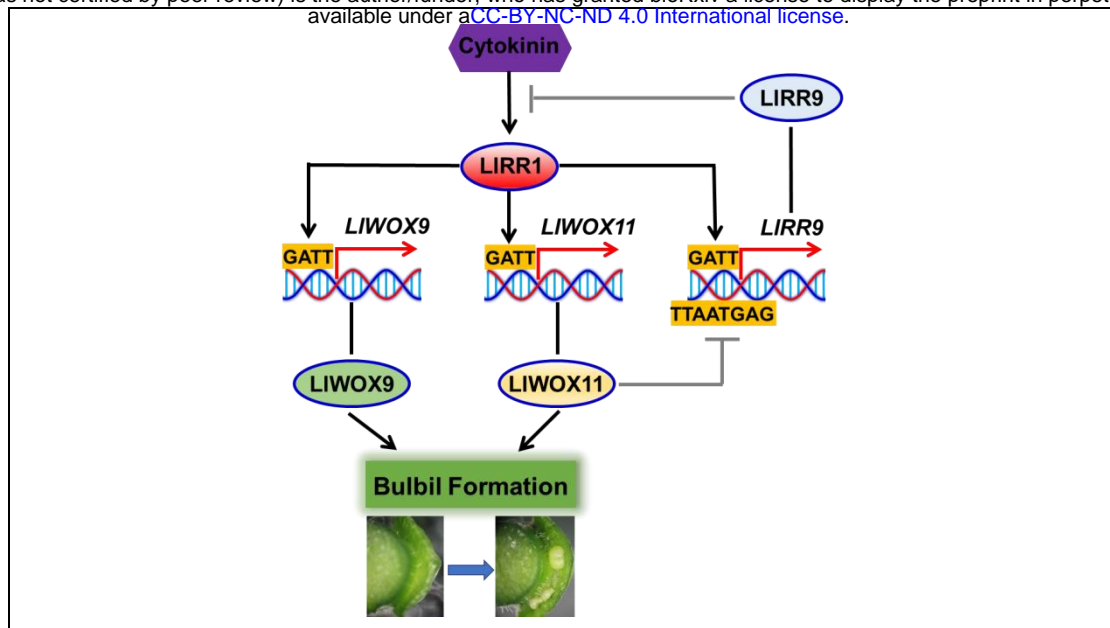
*LIWOX11* promoters. e: The binding ability of His-LIRR1 protein toward the *proLIWOX9-1* and *proLIWOX11-2* fragments was verified by EMSAs. The binding element GATT was mutated to TTTT in the mutant probe. Asterisks in A indicate significant differences compared with the control, with two asterisks indicating  $P < 0.01$ .



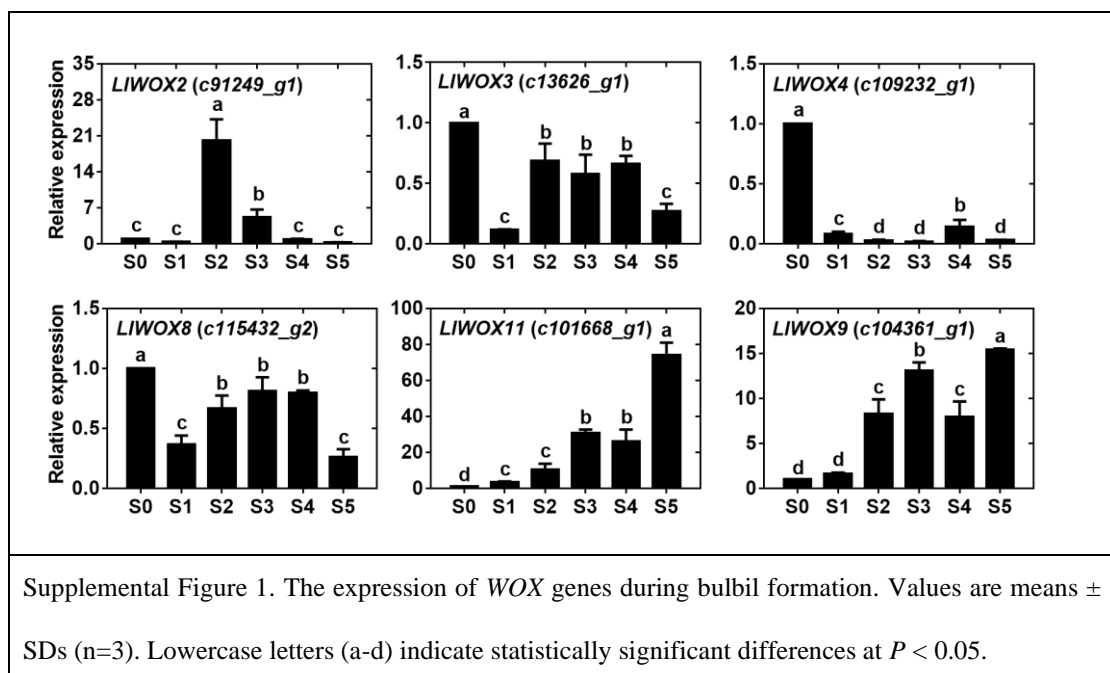
**Fig. 8** The interaction between LIWOX11 and the *LIRR9* promoter was verified by dual-luciferase reporter and yeast one-hybrid assays and EMSA.

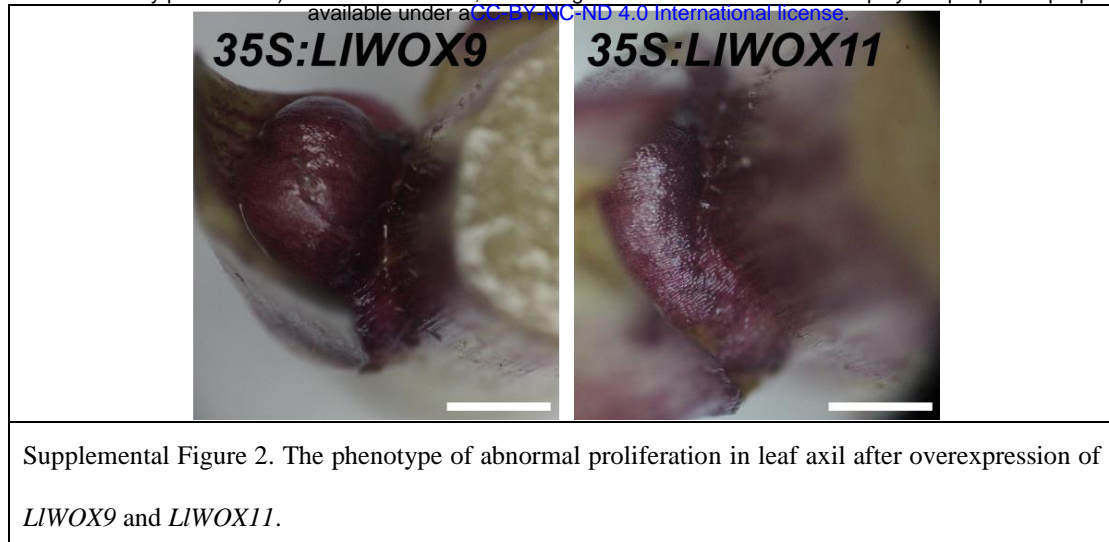
a: The transient activation test in tobacco leaves verified the transcriptional activation ability of LIWOX11 toward the *LIRR9* promoter. b: The binding ability of LIWOX11 toward the *LIRR9* promoter was verified by a yeast one-hybrid assay. c: The binding ability of the MBP-LIWOX11 protein toward the *proLIRR9* fragment was verified by EMSA. The binding element TTAATGA was mutated to AAAAAA in the mutant probe. d: The transient activation test in tobacco leaves verified the transcriptional activation ability of LIWOX11 toward the *LIWOX9* promoter. Asterisks in C indicate significant differences compared with the control, with two asterisks indicating  $P < 0.01$ .





**Fig. 9** Model of *WOX* gene cooperation with cytokinin signalling to regulate the bulbil formation.





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