Functional verification of the *JmLFY* gene associated with the flowering of *Juglans mandshurica* Maxim.

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Abstract

In this study, a pBI121-*JmLFY* plant expression vector was constructed on the basis of obtaining the full-length sequence of the *JmLFY* gene from *Juglans mandshurica*, which was then used for genetic transformation via *Agrobacterium* inflorescence infection using wild-type *Arabidopsis thaliana* and *lfy* mutants as transgenic receptors. Seeds of positive *A. thaliana* plants with high expression of *JmLFY* were collected and sowed till the homozygous T3 regeneration plants were obtained. Then the expression of flowering-related genes (*AtAP1*, *AtSOC1*, *AtFT* and *AtPI*) in T3 generation plants were analyzed and the results showed that *JmLFY* gene overexpression promoted the expression of flowering-related genes and resulted in earlier flowering in *A. thaliana*. The *A. thaliana* plants of *JmLFY*-transformed and *JmLFY*-transformed *lfy* mutants appeared shorter leaves, longer fruit pods, and fewer cauline leaves than those of wild-type and the *lfy* mutants plants, respectively. In addition, some secondary branches in the transgenic plants converted into inflorescences, which indicated that the overexpression of *JmLFY* promoted the transition from vegetative growth to reproductive growth, and compensate the phenotypic defects of *lfy* mutant partially. The results provides a scientific reference for formulating reasonable genetic improvement strategies such as shortening childhood, improving yield and quality, and breeding desirable varieties, which have important guiding significance in production.

Introduction

Flowering is an important process in the life cycle of higher plants. The developmental processes of flowers are divided into flowering decisions, flower initiation, and flower organ development

40   (Zhang and Liu, 2003). During plant flower induction, the external environment and genetic
41   factors together form a complex regulatory network (Zeng, 2018; Hassankhah et al., 2018). The
42   model plant *Arabidopsis thaliana* controls flowering through the interaction of temperature,
43   vernalization, photoperiod, gibberellin, and autonomous pathways (Martina et al., 2015; Jian et
44   al., 2019; Jin et al., 2019).
45   In the flowering regulatory network of plants, the *LEAFY* (*LFY*) gene is a key transcription factor
46   in the flowering signaling pathway, which is expressed in the early stage of flower primordia
47   initiation. The *LFY* gene determines the floral meristem properties of the flower primordia by
48   activating the expression of floral meristem characteristic genes and floral organ characteristic
49   genes, such as *AP1* and *CAL*. Therefore, the *LFY* gene is an important floral meristem
50   characteristic gene (Alvarez-Buylla et al. 2006; He et al., 2018). *LFY* and other genes related to
51   flower formation together form a flower development regulatory network and jointly control
52   plant flower formation, *AtAP1* gene is involved in the formation of flower meristem, which is a
53   characteristic gene of flower organ and plays an important role in the process of flower
54   formation. *AP1* and *LFY* genes are positively regulated each other. *SOC1* gene is the upstream
55   gene of *LFY* gene, which can integrate multiple flowering pathways; *FT* gene is also the
56   upstream gene o*f LFY* gene and the target gene of CO protein; *PI* gene is the downstream gene of
57   *LFY* gene, and *LFY* gene promotes the expression of *PI* gene (Zhao, 2020).
58   *LFY* gene plays an important role not only for controlling the transition from inflorescence
59   meristem to floral meristem but also for regulating flowering time in *A. thaliana* (Weigel et al.,
60   1993; Weigel et al., 1995; He et al., 2011; He et al., 2017). When the *LFY* gene was transferred
61   into wild-type *A. thaliana*, it was found that all lateral shoots were transformed into flowers
62   (Blazquez et al., 1997). In a functional study of *LFY* genes in plants such as *Populus simonii*,
63   *Nicotiana tabacum*, and *Oryza sativa*, it was found that transfection and overexpression of *LFY*
64   gene could lead to earlier flowering (He et al., 2000; Ahearn et al., 2001; Peña et al., 2001). The
65   activity of the *LFY* gene is conserved even in distantly related species (Shao et al., 1999;).
66   Therefore, *LFY* is not only a key regulatory gene in the plant flowering pathway but also a key
67   regulator of downstream floral meristem and floral organ-determining genes (Feng et al., 2016).
68   Although the *LFY* gene has been isolated and cloned in many plants, functional studies have
69   shown that it plays an important role in the reproductive and growth processes of plants.
70   However, no studies on the *LFY* homologous gene in *J. mandshurica* have been conducted.
71   *Juglans mandshurica* is a dichogamous and monoecious plant with two main mating types,
72   protandrous and protogynous. The asynchronous development and the imbalanced ratios of the
73   female and male flowers affect the efficiency of pollination and fruit setting (Zhang, 2019). For
74   the walnut species, the fruit production is also limited by late-spring frost in many countries
75   (Hassankhah et al., 2017), thus the late-leafing and early-harvesting varieties have been studied
76   in *Juglans regia* by targeted hybridization, and a significant variation between seedlings in terms
77   of leafing date (45 days) were observed, which indicated that it is possible to get late leafing and
78   early-harvesting genotypes with desirable nut traits (Hassankhah et al., 2017; Fallah et al., 2022).
79   Currently, there are few studies focus on the development of male and female flower buds and

80   the asynchrony of flowering period. In *J. regia*, the flowering pattern was altered by spraying
81   GA$_3$ (the best concentration is 100 mg/L), which increased the total number of flowers, the male
82   flowers, and male: female flower ratio significantly (Hassankhah et al., 2018). However, the
83   molecular mechanism of flower formation in the process of male and female differentiation has
84   not been fully analyzed.
85   Therefore, based on the successful cloning of the *JmLFY* gene in the early stage of the
86   experiment, a plant expression vector of the *JmLFY* gene of *J. mandshurica* was successfully
87   constructed and transformed with *Agrobacterium* to obtain transgenic *A. thaliana* plants, and the
88   T0–T3 generations of the transgenic plants were verified and analyzed for the function of the
89   *JmLFY* gene. This study lays a foundation for the exploration of the molecular mechanism of the
90   flowering of *J. mandshurica*, and provides a scientific basis for the shortening of the juvenile
91   period, early flowering, yield and quality improvement of *J. mandshurica*, selection of improved
92   varieties, and rational formulation of genetic improvement strategies.
93

Materials & Methods

95   **Plant Material**
96   The test materials were obtained from the *JmLFY* gene strain stored in the Forest Genetics and
97   Breeding Laboratory of Shenyang Agricultural University and stored at -80 °C; wild-type *A.*
98   *thaliana* seeds (Columbia type) and *lfy* mutant seeds were purchased from TAIR website
99   (https://www.arabidopsis.org/index.jsp).
100   **Experimental reagents**
101   BM Seamless Cloning Kit (Biomed, China), DH5 α and Agrobacterium GV3101(Biomed,
102   China), Small plasmid extraction kit(TIANGEN, China), Gel recovery kit and DL2000 Marker
103   and High fidelity enzyme and LA Taq (TaKaRa, China), X-gal and IPTG(Real Times, China),
104   Bis, Tris Hcl, SDS, TEMED, ammonium persulfate, ammonium persulfate, kana, sucrose, and
105   agar powder(Sinopharm, China), LB medium (ShengGong, China)
106   **Acquisition of the *JmLFY* gene of *J. mandshurica***
107   The plasmid was extracted from the *JmLFY* cDNA bacterial solution of *J. mandshurica* stored at
108   -80 °C, and the full-length PCR gene was amplified using high-fidelity enzymes and specific
109   primers, the primer sequences are shown in Table 1. The PCR products were recovered by
110   cutting the gel using a gel recovery kit and then were sent to Goldwisdom for sequencing.
111   **Construction of *JmLFY* gene expression vector**
112   The plasmid pBI121 was double-digested with XbaI and SmaI, and the gel-recovered product
113   was ligated into the pBI121 vector. The recombinant plasmid pBI121-*JmLFY* was transformed
114   into DH5α cells and detected by PCR. The positive bacterial liquid that was successfully verified
115   by sequencing was subjected to plasmid extraction, transformed into *Agrobacterium* GV3101
116   competent cells, and sent to Goldwisdom for sequencing after PCR. The successfully detected
117   bacterial solution was used to prepare an infection solution.
118   **Validation of *lfy* mutants**

119 Resistance validation of *lfy* mutants of *A. thaliana* was conducted by kanamycin (KAN)
120 screening, for which seeds of *lfy* mutants were cultured on 1/2MS + 15 g/L sucrose + 10 g/L agar
121 + 50 μg/mL KAN. The seeds were cultured at 4℃ for 3 d and then transferred into climatic
122 cabinate, which maintained relative humidity of 60%, temperature of 20-22℃, light cycle of 24
123 hours and light intensity is 80-200 μmol/m²/s.
124 Homozygosity validation of *lfy* mutants of *A. thaliana* was conducted by three-primer method,
125 primers were designed using T-DNA Primer Design (http://signal.salk.edu/tdnaprimers.2.html), the
126 sequences of the three primers are listed in Table 1. DNA from leaves of wild type and *lfy*
127 mutants of *A. thaliana* were extracted using TIANGEN Plant Genomic DNA Kit, which were
128 then used for PCR. The PCR products were then detected by agarose gel electrophoresis.

129 ***Agrobacterium*-mediated genetic transformation of pBI121-*JmLFY* to *A. thaliana***
130 Genetic transformation of pBI121-*JmLFY* was conducted by Inflorescence Infection. Seeds of
131 wild-type and lfy mutants of *A. thaliana* were sterilized and cultured in 1/2MS medium, and the
132 seedlings were selected and transplanted after 7 to 10 days of germination into the culture soil
133 with robust and consistent growth (Fig. 1A). After bolting and flowering (Fig. 1B), the small
134 pots were inverted, and all the inflorescences were placed upside down in the prepared infection
135 solution, which described as in "Construction of a *JmLFY* gene expression vector"(Fig. 1C). The
136 transformation was performed every three days and the process was repeated four times, and the
137 mature seeds (Fig. 1D) were collected for obtained regenerated plants .

138 **Screening of positive plants**
139 The seeds received above were screened on 1/2 MS + KAN plates, and plants with KAN
140 resistance were preliminarily screened. The screened plants were transferred onto the soil, and
141 after 15 days of bolting, the DNA and RNA of the leaves were extracted using DNA and RNA
142 extraction kits (Tiangen), and reverse transcribed into cDNA using a cDNA synthesis kit
143 (Shenggong), using cDNA as the template for PCR detection, and successfully detected by qRT-
144 PCR using 2×SG Fast qPCR master mix (Lablead).

145 **Overexpression of *JmLFY* gene and regulation of other genes related to flowering**
146 The plants with high expression of *JmLFY* were harvested and cultured to the T3 generation
147 using the same method described above. The plants with the highest expression of *JmLFY* were
148 selected from the T3 generation plants, and the *actin* gene of *A. thaliana* was used as the internal
149 reference gene to determine the expression of the flowering-related genes *AtAP1*, *AtFT*, *AtSOC1*,
150 and *AtPI*. The qRT-PCR primers used are listed in Table 1.

151 **Morphological observation and statistical analysis**
152 The selected *LFY* transgenic *A. thaliana* plants were named L1-L10, and the *LFY* transgenic *lfy*
153 mutant plants were named C1-C10.The L- line and C-line plants with high expression of *JmLFY*
154 were selected and cultured simultaneously with the wild-type and *lfy* mutants, and the bolting,
155 flowering, and fruit pod formation times of the four lines were observed and recorded. The
156 number of rosette leaves, plant height, and leaf shape and size were recorded and statistical
157 analyses were performed using SPSS and Excel.
158

Results

Acquisition of the *JmLFY* gene related to the flowering of *J. mandshurica*

The plasmid was extracted from the *JmLFY* cDNA bacterial solution of *J. mandshurica* stored at -80 ℃ in our laboratory. Using the *JmLFY* gene plasmid as the template, the upstream and downstream primers of the *JmLFY* gene were designed according to the obtained CDS sequence of the *JmLFY* gene, and the full-length gene was amplified by PCR. The PCR amplification products were sent to Goldwisdom for sequencing, and the sequencing results showed that the *JmLFY* target gene was successfully obtained from the cDNA-glycerol bacteria. The *JmLFY* sequence is shown in Fig. 2.

Construction of the pBI121-*JmLFY* plant expression vector

The PCR-amplified product of the *JmLFY* gene was cut into a gel and ligated with the pBI121 vector. Sequencing and comparison showed that the homology between the two cDNA sequences reached 99.91%. The resulting plant expression vector was named pBI121-*JmLFY* and mapped (Fig. 3).

lfy mutant validation

The three-primer method was used to verify the homozygosity of *A. thaliana lfy* mutants, and a 1/2MS KAN plate was used to screen the *A. thaliana lfy* mutants for resistance. As shown in Fig. 4, the length of the wild-type band was 900 bp and that of the *lfy* mutant *A. thaliana* was approximately 800 bp. It can be concluded that the *lfy* mutant is homozygous for *Arabidopsis*, which is in line with the expected results (Fig. 5). After screening on a 1/2 MS KAN plate, it was found that the *A. thaliana lfy* mutant had yellow leaves, could not grow roots and true leaves, and could not survive for a long time (Fig. 6), indicating that it was no KAN resistant and could be used for subsequent transformation validation experiments.

Phenotypic observation showed that the inflorescence of the *lfy* mutant was transformed into secondary branches, with absent petals and incomplete flower development (Fig. 7), while the *lfy* mutant plants were highly sterile (Zhang et al., 2008).

Screening of positive plants

Wild-type and *lfy* mutant *A. thaliana* transformed into the pBI121-*JmLFY* vector were screened for KAN plate resistance. The positive plants grew true leaves and took root on the KAN plate, while false positives did not (Fig. 8). At approximately 20 days, they were transferred to the prepared matrix soil. Five seedlings with the best growth were selected, and the selected *JmLFY* transgenic wild-type *A. thaliana* were named L-line plants while the selected *lfy* mutants *A. thaliana* were named C-line plants.

After flowering, the leaves were collected from the preliminarily screened lines, and the extracted RNA was reverse-transcribed into cDNA for positive PCR detection. The test results are shown in Fig. 9. L1, L2, L4, L5 and C1, C3, and C4 were found to be positive. The transformation rates of these two strains were not the same, which may be related to the quality of the recombinant plant expression vector and the operation method.

The positive seedlings screened above were subjected to qRT-PCR analysis, and *A. thaliana Actin* was used as the internal reference gene to analyze the expression of the *JmLFY* gene in

199 different lines (Fig. 10 and supplementary file [raw data]). The C4 expression levels were found
200 to be higher. The four harvested strains were continuously cultivated to the T3 generation, and
201 C23, C28, and C7, which had the highest *JmLFY* gene expression, were screened for further
202 verification.
203 **Regulation of *JmLFY* gene on other flowering-related genes**
204 To further explore the regulation of other flowering-related genes in *A. thaliana* by the *JmLFY*
205 gene of *J. mandshurica*, Primer-BLAST was used to design quantitative primers, and
206 fluorescence quantitative analysis was used to analyze the expression of each flower-related gene
207 in the *JmLFY* transgene, *JmLFY*-transfected *lfy* mutant, and wild type. Differences in foreign
208 gene expression were found in different transgenic lines (Fig. 11 and supplementary file [raw
209 data]).
210 Compared to the wild type, the expression levels of all the genes related to flower formation in
211 *Arabidopsis* plants transformed with the *JmLFY* gene changed, although the changes were
212 different. The expression level of the *AtAP1* gene was significantly increased in L23 and C7
213 plants, but did not change significantly in the L28 line (Fig. 11A). The expression level of
214 *AtSOC1* was significantly increased in L23 and C7 plants. The expression level in the L28 line
215 was also increased, and was much higher than in wild-type *A. thaliana* (Fig. 11B). The
216 expression of *AtFT* gene was significantly increased in the L23, L28, and C7 (Fig. 11D); the
217 expression of *AtPI* gene in L23 and L28 lines did not change significantly, although the
218 expression level was significantly increased in the C7 line (Fig. 11C).
219 The flower bud formation time, flowering time, number of rosette leaves, plant height, and
220 number of secondary branches in the T3 generation lines are shown in Table 2 and uploaded as a
221 supplementary file (raw data). The phenotypes of the four lines were then compared (Fig. 12).
222 The results showed that the flowering time of the transgenic *JmLFY* plants was significantly
223 earlier than that of the wild type; approximately eight days earlier, the rosette leaves of the L23
224 and L28 plants were approximately four times lower than those of the wild type, and the plant
225 height, number of secondary branches, and number of cauline leaves were higher than those of
226 the wild type. Compared to the *lfy* mutant, there was no obvious change, and the flowering time
227 of the transfected *lfy* mutant was still later than that of the wild-type *A. thaliana*; however, it was
228 also significantly earlier than that of the *lfy* mutant by approximately 7 days. The number of
229 rosette leaves in the C7 plant was higher than that of the *lfy* mutant. Approximately five fewer
230 leaves, four fewer secondary branches, and five fewer cauline leaves (Table 2); transgenic plants
231 have shorter leaves and longer pods than the wild type (Fig. 12H & I), and all shoots were
232 transformed into single flowers several times (Fig. 12F), indicating that *JmLFY,* as a
233 characteristic gene of floral meristem, can control the transition from vegetative growth to
234 reproductive growth. *lfy* mutants are sterile plants, and the flowers remained intact after the
235 transfer of the *JmLFY* gene (Fig. 12G). Most of the flowers regained their normal morphology,
236 produced more seeds than the *lfy* mutant, and produced fewer rosette leaves, secondary shoots,
237 and cauline leaves than the *lfy* mutant (Fig. 12J). Some secondary branches were also
238 transformed into single flowers, and *JmLFY* also transformed secondary branches into

239 inflorescences. In addition, *JmLFY* regulates the expression of other flowering-related genes in *A.*
240 *thaliana* and promotes the expression of these genes, indicating that the function of *JmLFY* may
241 also be related to the regulation of these genes. These results illustrate the important role of
242 *JmLFY* in flower formation.
243
Discussion
245 The transition from vegetative to reproductive growth is an important process in the plant life
246 cycle, and many physiological and metabolic changes and gene regulation mechanisms are
247 involved in this process (Saquib, 2020; Dong, 2021). These gene regulatory networks do not
248 exist independently but interact and regulate each other to jointly regulate the flowering process
249 of plants (Lu, 2020; Kalve, 2020). The *LFY* homologous gene regulates the formation of floral
250 meristems and organs and is critical for flowering (Bosl et al., 2004). As a transcription factor,
251 *LFY* regulates the transition of plants from vegetative growth to reproductive growth and is
252 mainly expressed in the apical meristem. Under the control of the 35S promoter, *LFY*
253 overexpression promotes early flowering and transforms secondary shoots into single flowers
254 (Zhang et al., 2008). *Lfy* mutants are defective in flowering and inflorescence formation (Mori et
255 al., 2017; Ma, 2020). When *GhLFY* is overexpressed in *A. thaliana lfy*-5 mutants, lateral shoot
256 secondary buds are transformed into individual flowers and the wild-type flower phenotype is
257 restored (Tang et al., 2016). In this study, the secondary shoots of the transgenic *A. thaliana* were
258 transformed into single flowers, the mutant plants of the transgenic *JmLFY* gene exhibited
259 normal petals, the phenotype of the mutant plants was restored to normal, and the seeds produced
260 are more and better quality than the *lfy* mutant. These results are similar to those of mutant *lfy*
261 overexpressing the *JcLFY* gene of *Jatropha curcas* (Tang et al., 2016) and the *EgLFY* gene of
262 *Eucalyptus grandis* (Dornelas et al., 2006), indicating that the *JmLFY* gene can complement the
263 late-flowering phenotype of *lfy* mutants and promote the transition from vegetative growth to
264 reproductive growth.
265 Plant flower bud differentiation is a complex physiological process regulated by multiple genes
266 (Zhang et al., 2019). In *J. regia*, expression of *FT* gene activated downstream floral meristem
267 identity genes including *SOC1*, and *LFY* which consequently led to release bud dormancy as well
268 as flower anthesis and induction (Hassankhah et al., 2020). As a master regulator of flowering
269 and floral gene networks, *LFY* activates downstream floral meristem recognition genes (*AP1* and
270 *CAL*) (Zou et al., 2014). *AP1* is a floral meristem signature gene and a direct target of *LFY* (Ma,
271 2020). *AP1* mRNA gradually accumulates after *LFY* expression (Haughn et al., 1991). In
272 addition, *LFY* also promotes the expression of floral organ-specific recognition genes, such as *PI*,
273 *AG*, and *AP3*, as well as the E functional genes *SEP1*, *SEP2*, and *SEP3*. In this study, compared
274 with the wild type, the expression of *AtAP1* gene was significantly increased in L23 and C7
275 plants, but did not significantly change in the L28 line; the expression of *AtSOC1* gene was
276 significantly increased in L23 and C7 plants, and the expression level of the L28 line was also
277 higher than that of wild-type *A. thaliana*. The expression level of the *AtFT* gene in L23 and L28
278 was significantly increased; and the expression level of *AtPI* gene in L23 and L28 lines showed

279 no significant change; however, the expression level was significantly increased in the C7 line.
280 This indicated that the three genes *AtAP1*, *AtSOC1*, and *AtPI* may contribute to the normal
281 flower morphology of C7.
282 Transferring *JcLFY* into *Jatropha curcas* caused transgenic plants to bloom seven months earlier
283 (Tang et al., 2016). Peña et al. (2001) transformed the *AtLFY* and *AtAP1* genes into *Citrus*
284 *sinense* and *Poncirus trifoliata*, and found that transformed plants flowered 3–5 years earlier than
285 untransformed plants (Pineiro et al., 1998; He et al., 2011; Ma et al., 2019). In the present study,
286 the transgenic *A. thaliana* L23 and L28 lines flowered six days earlier than the wild-type plants,
287 and the C7 line flowered eight days earlier than the corresponding mutant *lfy*. These results
288 suggest that *JmLFY* promotes early flowering in *A. thaliana*, shortens the vegetative phase, and
289 complements the delayed flowering of the mutant *lfy*. In addition, the L23 and L28 lines had
290 fewer rosette and cauline leaves than the wild type. And also, the number of clump leaves,
291 cauline leaves, and branches of the transgenic line C7 were higher than those of the wild-type
292 plants but lower than those of the *lfy* mutant. The results obtained in this study are in agreement
293 with those obtained in *A. thaliana* (Weigel et al., 1995), New Zealand radiata pine (Vazquez et
294 al., 2007), rapeseed (Roy et al., 2009) and *Jatropha curcas*. The phenotypes of plants
295 overexpressing the *LFY* gene in Tang et al. (2016) were similar. These results confirm that
296 *JmLFY* can inhibit the vegetative growth of plants, partially complement the phenotypic defects
297 of *lfy* mutants, and promote the earlier flowering of plants. And in the future research, the
298 Southern Blot method should be considered to better confirm the transgenic plants.
299

Conclusions

301 In this study, the plant expression vector pBI121-*JmLFY* was successfully constructed using
302 *JmLFY* cDNA bacterial solution. The three-primer method and KAN resistance screening
303 confirmed that the *lfy* mutant was homozygous and did not exhibit KAN resistance. Phenotypic
304 observations showed that the inflorescence of *lfy* mutant *Arabidopsis* was transformed into
305 secondary branches, with absent petals and incomplete flower development. The *lfy* mutant
306 plants were highly sterile and produced few seeds. pBI121-*JmLFY* was transformed into wild-
307 type and *lfy* mutants to obtain regenerated plants, and the *JmLFY*-transgenic *Arabidopsis* and
308 *JmLFY*-transgenic *Arabidopsis* were screened for positive seedlings using KAN plates.
309 Expression of the *JmLFY* gene was screened and plants with the highest levels of expression
310 were those from the L23, L28, and C7 lines. Validation of the T3 generation plants showed that
311 *JmLFY* also regulated the expression of other flowering-related genes in *A. thaliana* and
312 promoted the expression of these genes. The *JmLFY* gene can partially compensate for the
313 phenotypic defects of *lfy* mutants, and promote transition from vegetative to reproductive growth
314 and early flowering.
315

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317 The authors declare that they have no competing interests.
318

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439 **Figures and captions**

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465

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469 **down:L28 (I) WT(left) L28(right) (J) C7 stem leaf**
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479   **Table 1:**
480   **Primer sequences used in the construction and functional verification of *Juglans***
481   ***mandshurica***
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485

Figure 1

Genetic transformation of Arabidopsis thaliana

Genetic transformation of *Arabidopsis thaliana* (A) *Arabidopsis* sowing (B) *Arabidopsis*

flowering (C) Inflorescence infection process (D) *Arabidopsis* seed collection

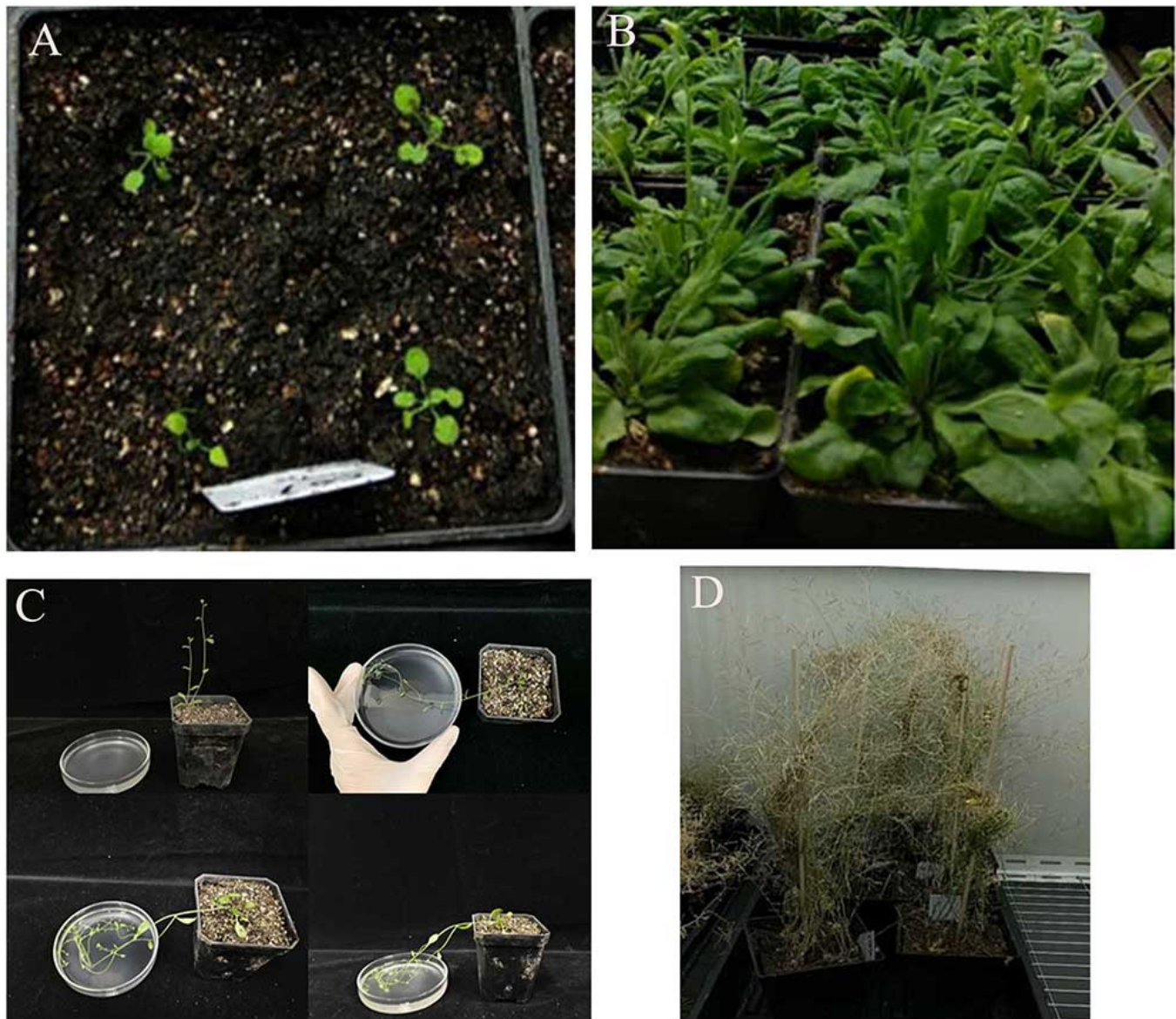



Figure 2

Amplification of the JmLFY gene related to flowering in *Juglans mandshurica*

Amplification of JmLFY gene related to flowering in *Juglans mandshurica.*

>*JmLFY* (1158bp):

ATGGATCCCGACCCCCTTTACTGCGAGTTTGTTCAAGTGGGACCCGCGTGGGGTAGTCACGCCTC
CGAATCGGCTGCTGGAAGCCGTGGCGGCACCAGCCGCAGCTGCGACGGCAGCATACTCATTAC
GGCCGAGAGAGCTTGGAGGGCTGGAGGACTTGTTCCAAGCTTACGGGATTAGATACTATACGG
CGGCAAGGATTGCAGAGCTTGGGTTTACAGTGAGTACGCTGTTAGACATGAAGGACGAGGAGC
TGGATGACATGATGAACAGCCTCTCTCAAATATTCAGGTGTGATCTTCTCGTGGGGGGAGAGGTA
CGGTATTAAAGCTGCAGTTAGAGCCGAGCGCAGACGCCTCGACGAGGAGGAGTCTCGCCGGC
GCCACCTCTCTGCCGACACCACCACCAATGCTCTCGATGCGCTCTCCCAAGAAGGGTTGTCCG
AAGAGCCGGTGCAGCAGGAAAAGGAGGTGGTGGGAAGCGGCGGAGGTGGCACGTGGGAGGT
GGTGGGCGAGAGAAGGAAACAGAGGCGGAGGAAGAGCCTGAGGAAAGTGGTGGACGTTGAT
TATGATGATAACGAGGGTGCGGAGGACGATGACAATGGTAATGGTAATGGCAATGGCGGTGGA
AGTAAGAGACAGCGGGAGCACCCTTTCATTGTAACGGAGCCCGGTGAGGTTGCACGTGGAAA
GAAGAACGGTCTCGATTACCTCTTCCATCTCTACGAGCAGTGCCGTGATTTCTTGATCCAGGTCC
AGAACATAGCGAAGGAGCGCGGCGAAAAATGCCCCACCAAGGTGACGAACCAAGTGTTTAGG
TATGCGAAGAAGGCTGGAGCTAGCTACATTAACAAGCCCAAAATGCGACACTACGTGCATTGTT
ATGCGTTGCACTGCATCGACGAGGGAGTCTCCAACACACTGAGAAGAGCATTTAAGGAGAGAG
GAGAAAATGTTGGGGCGTGGAGGCAGGCATGCTACAAGCCTTTGGTCGCCATAGCAGCACGCC
AAGGATGGTACATTGATACCATTTTCAACGCACACCCGCGACTATCCATTTGGTATGTACCCACC
AAACTTCGTCAACTTTGTCACGCTGAGAGAAACAATGCCACAGCTTCCAGCTCTGTTTCTGGA
GGGGGTGATCACATGCCCCTCTAA

Figure 3

Construction of plant expression vector pBI121-*JmLFY*

Construction of plant expression vector pBI121-*JmLFY*

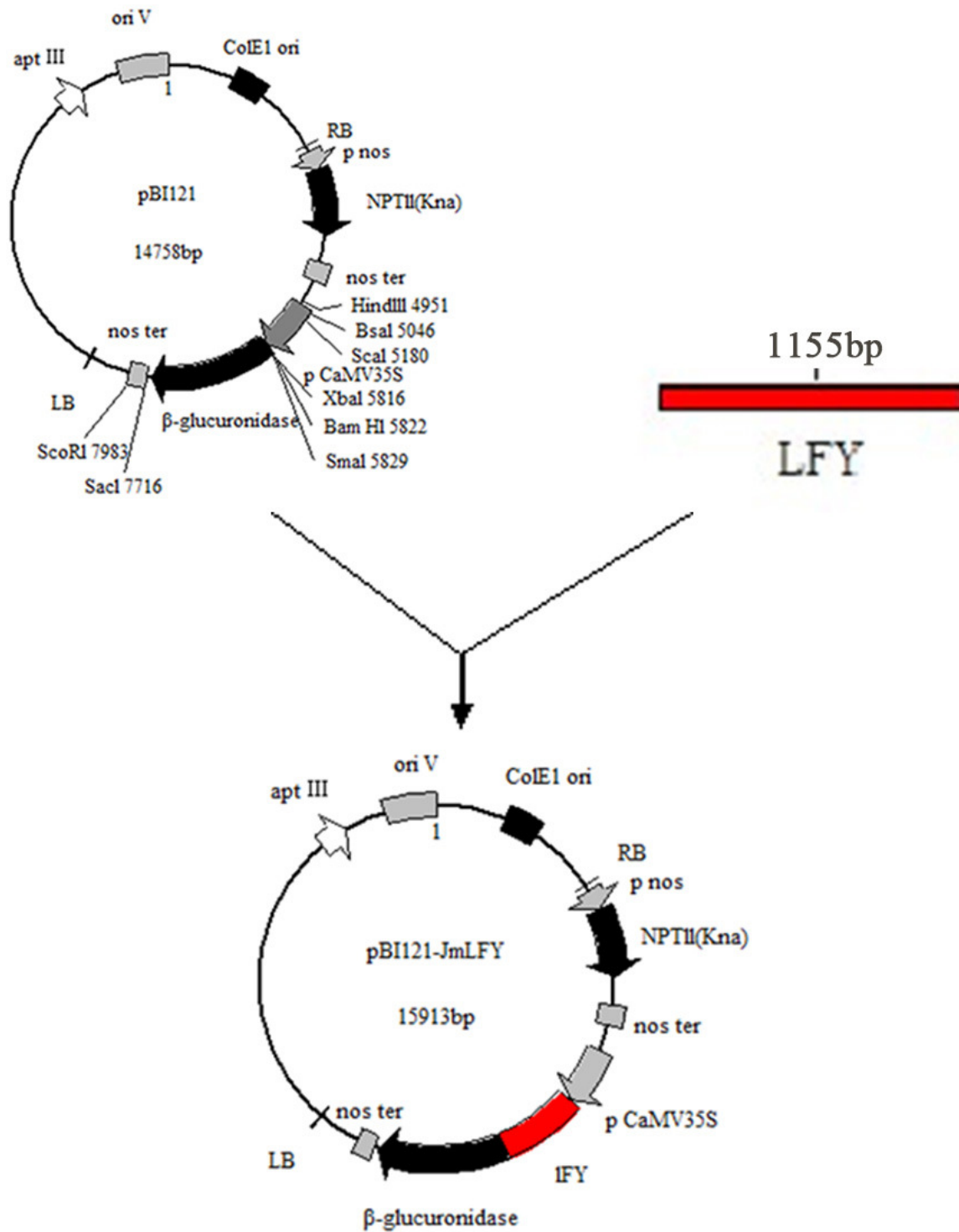



Figure 4

Homozygous verification of *Arabidopsis*

Homozygous verification of *Arabidopsis*

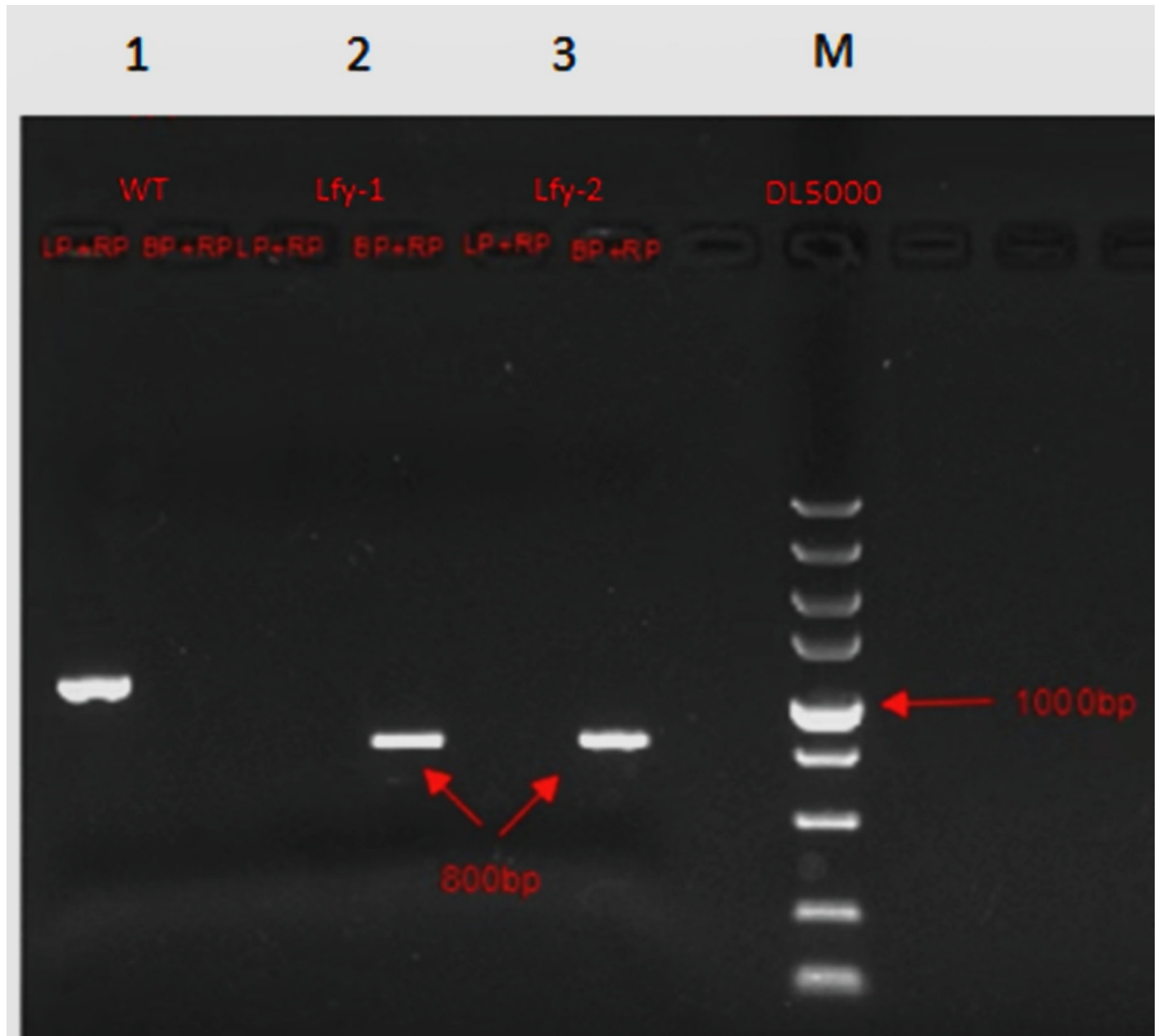



Figure 5

Expected results for *Arabidopsis* 1-3: PCR products; M: DL5000 Marker; WT: Wild *Arabidosis*; HZ: Heterozygote; and HM Homozygous verification of *Arabidopsis*

Expected results of *Arabidopsis* 1-3: PCR products M: DL5000 Marker WT: Wild *Arabidosis* HZ: Heterozygote HM Homozygous verification of *Arabidopsis*

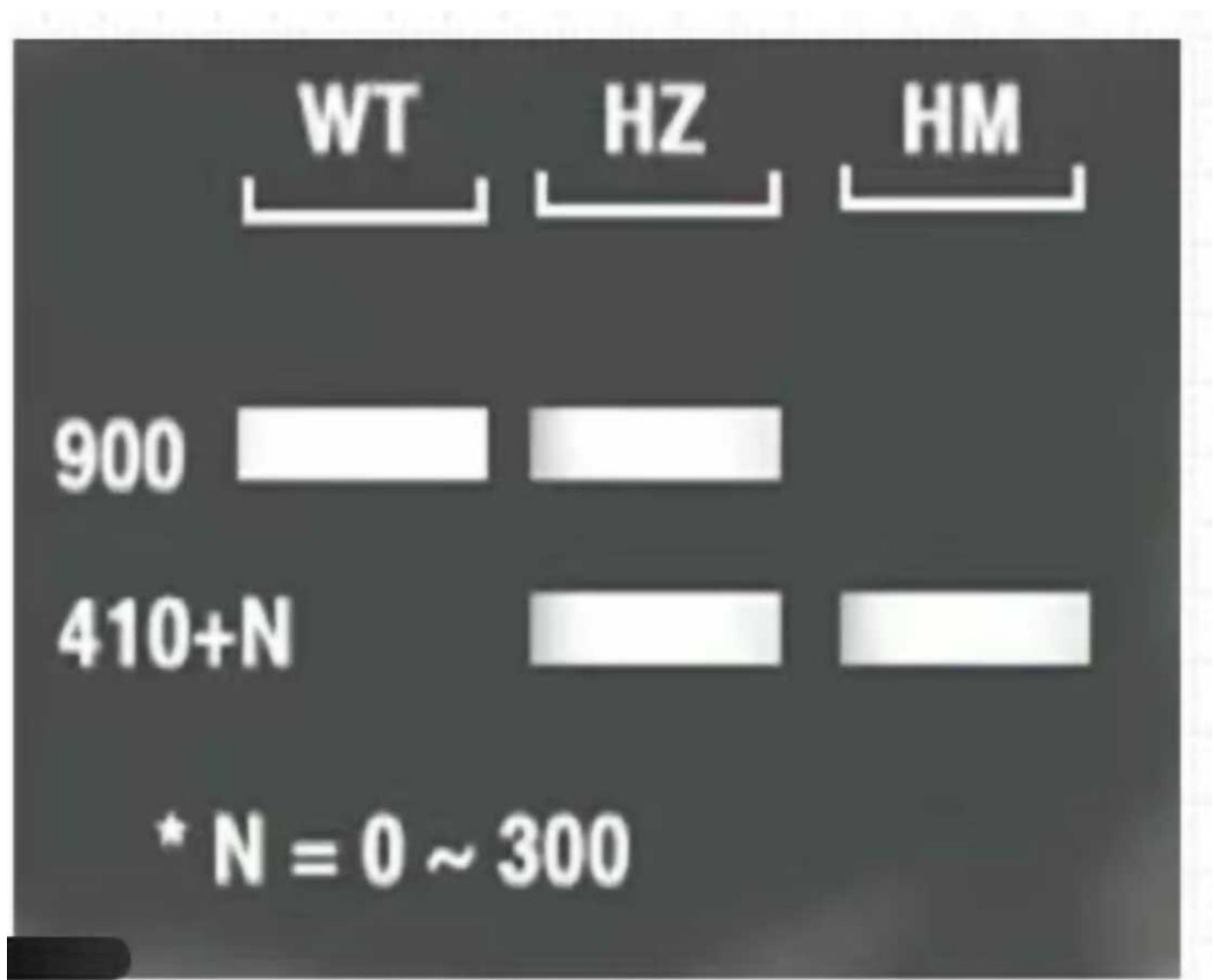



Figure 6

Verification of kana resistance of *lfy* mutant

Figure 7

lfy mutant phenotype with secondary branch A/B (White arrow)

lfy mutant phenotypic observatiosecondary Branch A/B (White arrow)

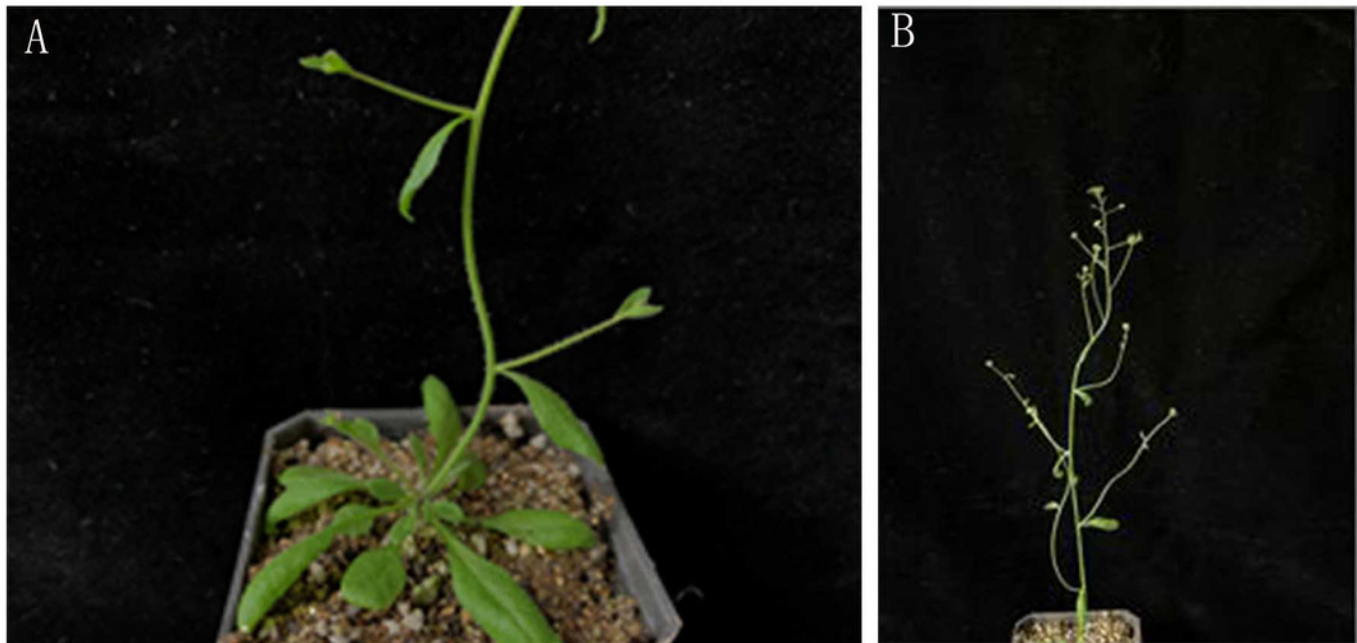



Figure 8

Positive and false positive plants screened from the T0 generation

Positive and false positive plants screened from T0 generation

Figure 9

Expression of *JmLFY* transgenic plants

Expression of *JmLFY* transgenic plants

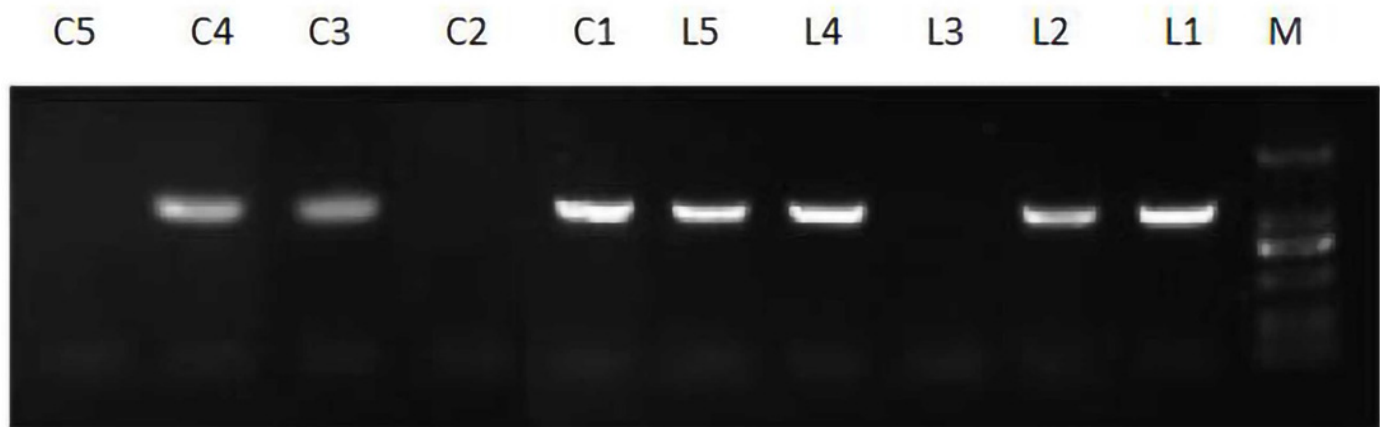



Figure 10

Expression of *JmLFY* gene in different parts

Expression of *JmLFY* gene in different parts (A) *JmLFY* transgenic plant (B) *JmLFY* transgenic plants with *lfy* mutant)

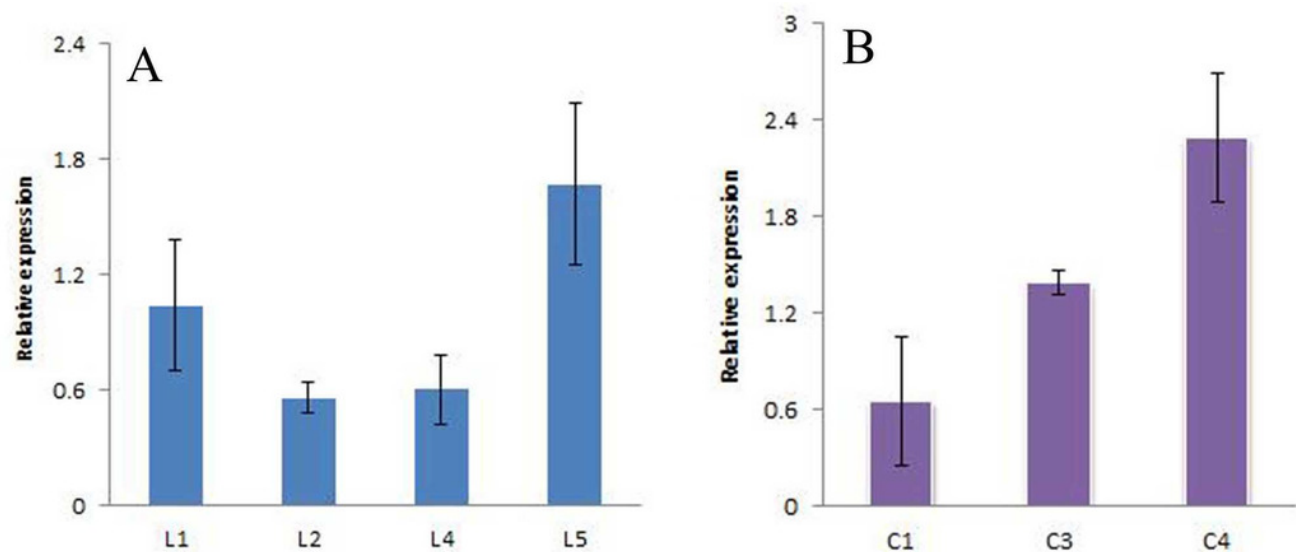



Figure 11

Expression of different flowering genes in *JmLFY* transgenic plants

Expression of different flowering genes in *JmLFY* transgenic plants (A) AtAP1gene (B) AtSOC1 gene (C) AtPI gene (D) AtFT gene

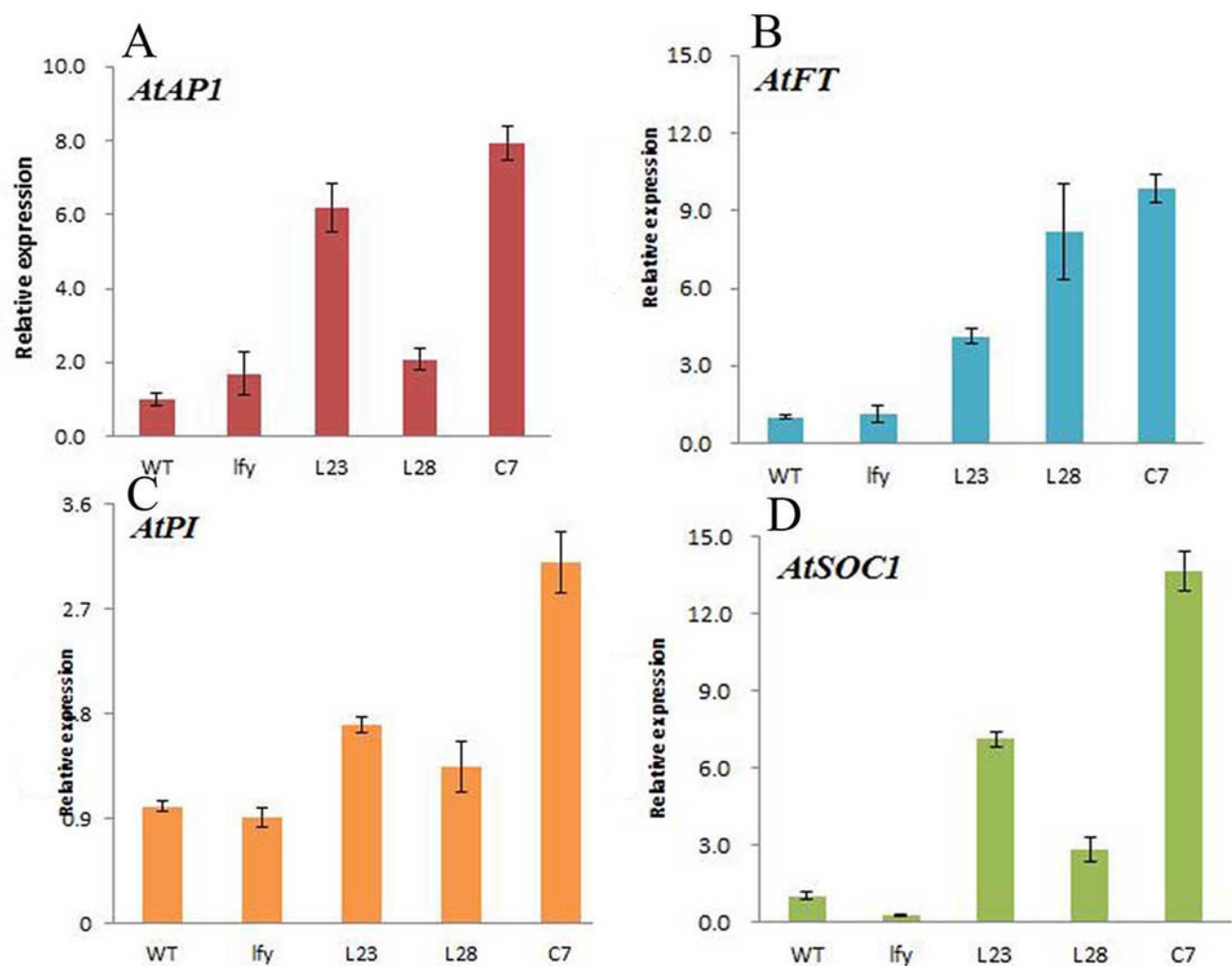
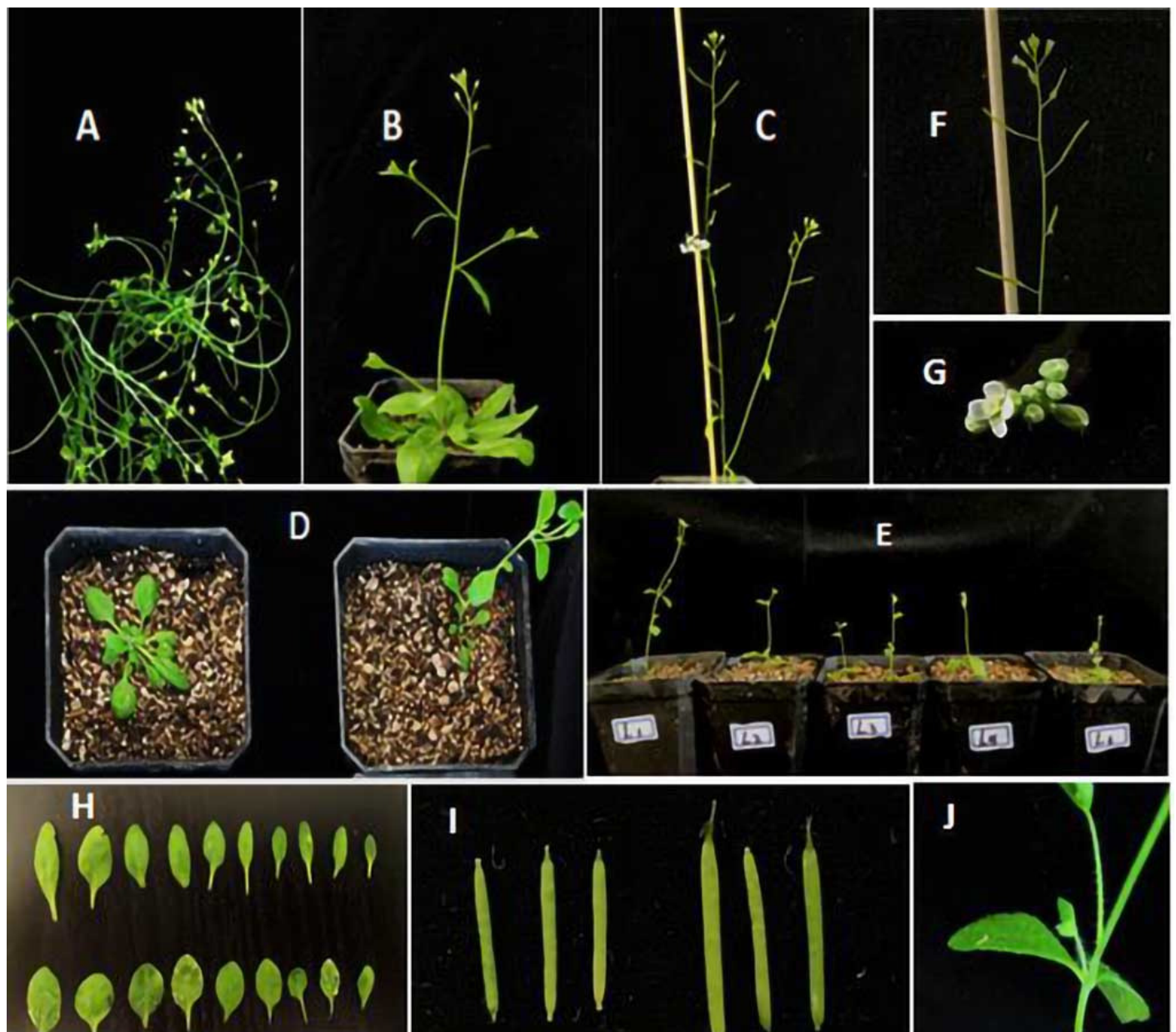



Figure 12

Phenotypic study of overexpression of *JmLFY* gene in Arabidopsis

Phenotypic study of overexpression of *JmLFY* gene in *Arabidopsis* (A) *lfy* mutant (B) WT (C) L23 (D) left:WT, right:L23 (E) Transgenic lines flowering ahead of time (F) Transformation from secondary branch to single flower L28 (G) C7 flower (H) up:WT down:L28 (I) WT(left) L28(right) (J) C7 stem leaf

Tables

Tables

1 **Table 1:**

2 **Primer sequences used in the construction and functional verification of *Juglans***

3 ***mandshurica***

primer	sequence(5'-3')	use
LFY-F2	CACGGGGGACTCTAGAATGGATCCCGACCCCTTTACTG	*JmLFY vector construction*
LFY-R2	AGGGACTGACCACCCGGGTAGAGGGGCATGTGATCACCC	*JmLFY vector construction*
LP	GAGGAGGAAGTGGTTACTGGG	*lfy mutant validation*
RP	AAATGCCTACCAAAATTATAACCG'	*lfy mutant validation*
LBb1.3	ATTTTGCCGATTTCGGAAC	*lfy mutant validation*
JmLFY-F2	AGCACCCTTTCATTGTAACGGA	*JmLFY expression analysis*
JmLFY-R2	GTGCTGCTATGGCGACCAAAG	*JmLFY expression analysis*
Actin-F	ATGCCCAGAAGTCTTGTTCC	*JmLFY expression analysis*
Actin-R	TGCTCATACGGTCAGCGATA	*JmLFY expression analysis*
AtAP1-F	GCTCTTAAGGCACATCCGCAC	*JmLFY functional verification*
AtAP1-R	GCAGAGGGGGAGGCATATTG	*JmLFY functional verification*
AtFT-F	ATGCCCAGAAGTCTTGTTCC	*JmLFY functional verification*
AtFT-R	TGCTCATACGGTCAGCGATA	*JmLFY functional verification*
AtSOC1-F	AGCAGAGTTGTTGGAGACGTT	*JmLFY functional verification*
AtSOC1-R	AGGTGAGGGTTGCTAGGACT	*JmLFY functional verification*
AtPI-F	TACAACTGGAGCTCAGGCAT	*JmLFY functional verification*
AtPI-R	ATTCCTCTTGCGTTGCTTG	*JmLFY functional verification*

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28 **Table 2:**
29 **Phenotypic data of different strains**

strain	Number of rosette leaves	Flower bud formation time	Flowering time	Plant height	Number of secondary branches	Number of stem leaves
WT	13.8±0.84^c	31.4±1.14^c	37.4±0.55^c	25.92±1.12^b	4.2±0.84^c	5.4±1.14^c
lfy	23.4±1.14^a	41.6±1.14^a	49.8±0.84^a	29.5±0.46^a	12.4±1.14^a	16.8±0.84^a
L23	10.2±0.44^d	22.8±0.84^d	28.8±0.84^d	25.98±1.33^b	4±0.71^c	4.4±1.14^{cd}
L28	9.6±0.89^d	23.2±0.44^d	29.2±0.84^d	26.46±0.48^b	4.42±0.84^c	4±0.71^d
C7	18.8±0.84^b	37.4±0.55^b	41.2±0.84^b	25.7±0.64^b	7.6±0.89^b	11.4±0.55^b

30 The values are the means ± standard errors. Different letters in the same column indicate significant
31 differences at P = 0.05 level.
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