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miR172 Regulates both Vegetative and Reproductive Development in the Perennial Woody Plant *Jatropha curcas*

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***Jatropha curcas* is a promising feedstock for biofuel production because its oil is highly suitable for processing bio-jet fuels and biodiesel. However, *Jatropha* exhibits a long juvenile stage in subtropical areas. miR172, a conserved small non-protein-coding RNA molecule with 21 nucleotides, regulates a wide range of developmental processes. To date, however, no studies have examined the function of miR172 in *Jatropha*. There are five miR172 precursors encoding two mature miR172s in *Jatropha*, which are expressed in all tissues, with the highest expression level in leaves, and the levels are up-regulated with age. Overexpression of *JcmiR172a* resulted in early flowering, abnormal flowers, and altered leaf morphology in transgenic *Arabidopsis* and *Jatropha*. The expression levels of miR172 target genes were down-regulated, and the flower identity genes were up-regulated in the *JcmiR172a*-overexpressing transgenic plants. Interestingly, we showed that *JcmiR172* might be involved in regulation of stem vascular development through manipulating the expression of cellulose and lignin biosynthesis genes. Overexpression of *JcmiR172a* enhanced xylem development and reduced phloem and pith development. This study helped elucidate the functions of miR172 in perennial plants, a known age-related miRNA involved in the regulation of perennial plant phase change and organ development.**

Keywords: Age • Early flowering • Flower pattern • Lignification • miR172 • Physic nut.

Abbreviations: AP1, APETALA 1; AP2, APETALA 2; AP3, APETALA 3; AG, AGAMOUS; CAL, CAULIFLOWER; CaMV, cauliflower mosaic virus; FUL, FRUITFULL; FT, FLOWERING LOCUS T; LD, long day; LFY, LEAFY; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SEP, SEPALLATA; SMZ, SCHLAFMUTZE; SNZ, SCHNARCHZAPFEN; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; SVP, SHORT VEGETATIVE PHASE; TFL1, TERMINAL FLOWER 1; TOE, TARGET OF EAT.

Introduction

With the decreasing availability of fossil fuels and the deterioration caused by environmental pollution, biodiesel resources have gained significant attention as a promising fuel (Mofijur et al. 2016). Physic nut (*Jatropha curcas* L.), a perennial woody plant belonging to the Euphorbiaceae family, is monoecious, with male and female flowers borne on the same inflorescence (Divakara et al. 2010, Wu et al. 2011, Pandey et al. 2012). The genome sequence and genetic mapping of *Jatropha* have been published (Sato et al. 2011, Hirakawa et al. 2012, Wu et al. 2015, Xia et al. 2018), and several genetic transformation methods mediated by *Agrobacterium tumefaciens* have been established (Kumar et al. 2010, Pan et al. 2010, Kajikawa et al. 2012, Misra et al. 2012, Fu et al. 2015, Gu et al. 2015). Hence, compared to other perennial woody plants, it is fully realizable to isolate *Jatropha* genes and analyse their functions. *Jatropha* has been suggested to have oil-crop potential because of its high oil content, high biomass productivity, adaptability to marginal land under a wide range of climatic conditions, and non-competitiveness with food production (Pua et al. 2011, Akashi 2012, Pandey et al. 2012, Khalil et al. 2013). The highest oil contents of *Jatropha* seeds and kernels are 40% and 50% by weight, respectively (Pan and Xu 2011, Sinha et al. 2015). Oil of *Jatropha* seed contains high concentration of polyunsaturated fatty acids which improves the crude oil flow; therefore, *Jatropha* seed oil is suitable as a feedstock for the production of bio-jet fuel and biodiesel (Pramanik 2003, Ong et al. 2011). *Jatropha* cultivation can alleviate future energy crises and reduce environment pollution. However, the potential of *Jatropha* as an energy plant is limited by its low seed yield production character (King et al. 2015). *Jatropha* exhibits an overabundance and undesirable range of vegetative leaves and branches that could develop into reproductive branches under suitable conditions and exhibits a long juvenile phase in subtropical areas (Tang et al. 2016a, b). Thus, it is necessary to reduce abundant vegetative growth (Ghosh et al. 2010, Song et al. 2013, Tjeuw et al. 2015). In addition, unreliable and poor flowering are crucial factors that contributes to low seed yield production in *Jatropha* (Divakara et al. 2010). Furthermore, soft stems make *Jatropha* highly susceptible to lodging and root rot diseases (Dhillon et al. 2009).

In animals and plants, stage transitions are necessary and vital in the developmental process (Moss 2007). In *Caenorhabditis elegans*, transitions between the stages of larval development are mediated by an increase in the expression of two sequentially

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expressed miRNAs, lineage (lin)-4 and lethal (let)-7 (Pasquinelli and Ruvkun 2002, Carrington and Ambros 2003, Moss 2007). lin-4 and let-7 were the first miRNAs identified, and they have since served as paradigms for the functions of these regulatory molecules in animals (Bagga et al. 2005). MicroRNAs function as post-transcriptional modulators of gene expression in eukaryotic cells (Inui et al. 2010). In the annual plants *Arabidopsis* and maize, vegetative phase change was controlled by the sequential activity of miR156 and miR172 (Chuck et al. 2007a, Wu et al. 2009). miR156 is highly expressed early in plant development and decreases with time, while miR172 exhibits the opposite expression pattern (Aukerman and Sakai 2003, Lauter et al. 2005, Jung et al. 2007, Wang et al. 2009, Wu et al. 2009). In woody species, such as *Acacia confusa*, *A. colei*, *Hedera helix*, *Eucalyptus globulus*, and *Quercus acutissima*, similar expression patterns were observed in the expression of miR156 and miR172 and their target genes (Wang et al. 2011).

The mature miR172 sequence is conserved in higher plants. However, the numbers of pri-miR172, mature miR172, and target genes are varied. In *Arabidopsis*, five pri-miR172s encode three mature miR172s, and these miR172s repress the expression of six members of the APETALA 2 (AP2)-like family of transcription factors—*AP2* itself, three TARGET OF EAT (TOE) proteins (TOE1, TOE2, and TOE3), and SCHLAFMUTZE (SMZ) and its paralog SCHNARCHZAPFEN (SNZ) (Fornara and Coupland 2009, Mathieu et al. 2009). In maize, five pri-miR172s encode only one mature miR172, which represses the expression of six members of the *AP2*-like family of transcription factors—ZmGL15, ZmIDS1, ZmSTD1, ZmTOE1, TS6-ref, and TS6-GN2230 (Zhu and Helliwell 2011). In rice, four pri-miR172s encode two mature miR172s and repress five members of the AP2-like family of transcription factors, including *OsSNB*, *Os03g60430*, *Os05g03040*, *Os04g55560*, and *Os6g43220* (Zeng et al. 2009, Zhu and Helliwell 2011, Lee and An 2012). In *Populus trichocarpa*, nine pri-miR172s encode four mature miR172s with six target genes (Zeng et al. 2009).

The transcription of miR172 is positively regulated by *SQUAMOSA PROMOTER* BINDING PROTEIN-LIKE 9 (*SPL9*) and *SPL10* (Wu et al. 2009), and *SPLs* are the direct targets of miR156 (Wu and Poethig 2006, Wang et al. 2008, Fornara and Coupland 2009), thus establishing a miR156-SPL-miR172 regulatory cascade (Yu et al. 2015). However, *SHORT VEGETATIVE PHASE* (*SVP*) negatively regulates miR172a transcription by direct binding to its promoter (Cho et al. 2012). *TOE1* and *TOE2* positively regulate miR172 by a negative feedback loop (Wu et al. 2009). Recent research found that miRNA172 is modulated by auxins (Diaz-Manzano et al. 2018).It has been shown that miR172 is involved in various developmental processes in plants, including stem cell fate (Zhao et al. 2007), developmental timing (Fornara and Coupland 2009, Wu et al. 2009, Jung et al. 2011, Wang et al. 2011, Lee et al. 2014, Yu et al. 2015, Fouracre and Poethig 2016), sex determination (Chuck et al. 2007b, Tang and Chu 2017), floral organ identity and flower pattern (Aukerman and Sakai 2003, Lee and An 2012), fruit growth (Xue et al. 2009, Gasser 2015, Jose Ripoll et al. 2015), spike architecture and grain threshability in bread wheat (Debernardi et al. 2017, Liu et al. 2018), tuberization in potato (Martin et al. 2009, D'Ario et al. 2017), and nodulation in soybean (Yan et al. 2013). Also miR172 was found to have a role in the abiotic response of *Arabidopsis* (Han et al. 2013) and biotic stress resistance in tomato (Luan et al. 2018). In addition, miR172 causes early flowering and defects in floral organ identity when overexpressed (Aukerman and Sakai 2003). Furthermore, miR172 promotes flowering primarily by post-transcriptionally repressing a set of *AP2-like* genes, such as *AP2*, *TOE1*, *TOE2*, *TOE3*, *SMZ*, and *SNZ* (Fornara and Coupland 2009, Mathieu et al. 2009, Yant et al. 2010). Previous studies have shown that miR172-overexpressing *Arabidopsis* plants exhibit early flowering by regulating *FLOWERING LOCUS T* (*FT*) (Lee et al. 2007, Lee et al. 2010, Diaz-Manzano et al. 2018). *FRUITFULL* (*FUL*) positively regulates *miR172c* expression in fruit valves by directly binding to CArG motifs in the *miR172c* promoter (Jose Ripoll et al. 2015). Overexpression of *miR172a* and *b* in tomato increased resistance to *Phytophthora infestans* infection by suppressing an AP2/ERF transcription factor (Luan et al. 2018).

To date, most of the known functions of miR172 were determined in annual herbaceous plants. miR172 was shown to have the lowest abundance in seedlings and increased during the juvenile-to-adult transition in several perennial trees (Wang et al. 2011). Although the microRNAs in *Euphorbiaceae* plants, including *Ricinus communis*, *Manihot esculenta*, *Hevea brasiliensis*, and *Jatropha*, were isolated and compared (Zeng et al. 2009, Wang et al. 2012), the functions of these microRNAs remain unknown. In this study, we analyzed the expression profiles of *Jatropha miR172* (*JcmiR172*) and further characterized the function of *JcmiR172a* in leaf morphology, flowering induction, floral organ specification, and fruit and seed morphologies using transgenic *Arabidopsis* and *Jatropha*. In particular, we found that miR172 plays a role in regulating xylem development in both *Arabidopsis* and *Jatropha*.

Results

Prediction of miR172 target genes and analysis of expression patterns of miR172 in *Jatropha*

According to the *Arabidopsis* miR172 sequence (http://www.mirbase.org/) and the *Jatropha* genomic sequence database (http://www.ncbi.nlm.nih.gov/genome/genomes/915), five miR172 precursors were found in *Jatropha* and named *JcmiR172a-e* (Supplementary Fig. S1). *JcmiR172a-d* encode a mature miR172, whose sequence (AGAAUCUUGAUGAUGCUGCAU) is the same as that of *Arabidopsis* miR172, whereas *JcmiR172e* encodes another mature miR172 with a sequence (GGAAUCUUGAUGAUGCUGCAG) different from that of *Arabidopsis* miR172s (Supplementary Fig. S1A).

The *Jatropha* genomic database was screened by TBLASTN using the amino acid sequence of the miR172-targeted *Arabidopsis AP2-like* family of transcription factors as a query. By this screening, four putative cDNAs with high homology to *AP2-like* were identified in the *Jatropha* genomic database. Based on this similarity, we named these four genes *JcAP2*, *JcTOE1*, *JcTOE2*, and *JcTOE3* (GenBank accession numbers are listed in Supplementary Table S1). Further analysis found the

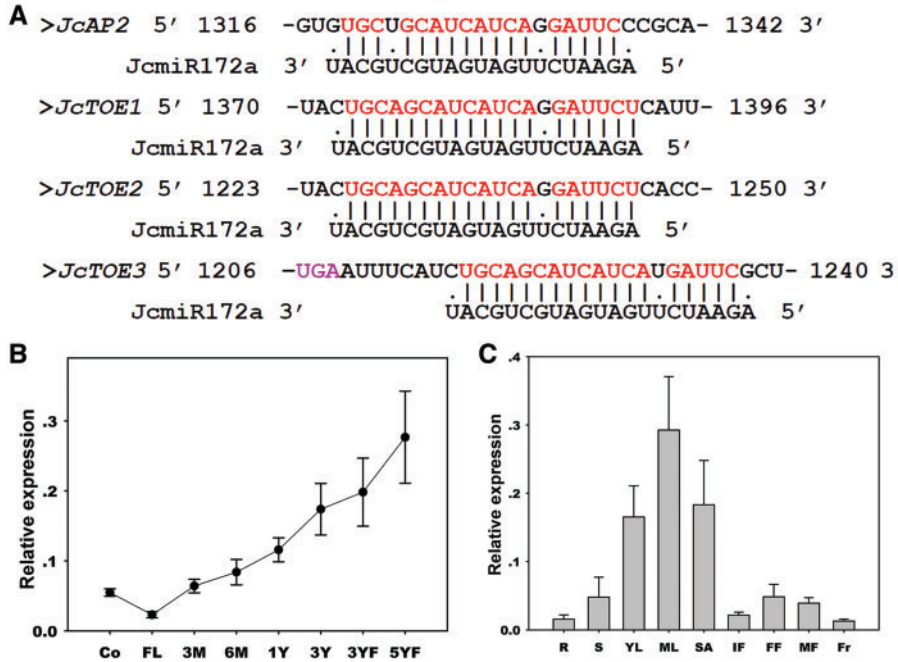

Fig. 1 Prediction of *JcmiR172* target genes and the expression patterns of *JcmiR172* in *Jatropha*. (A) Predicted *JcmiR172a* target genes and positions of *miR172a* target sites within the putative *JcmiR172* target genes are shown; pink UGA in *JcTOE3* indicates the termination codon. The cDNA sequences of *JcAP2* (sequence ID XM_012223882), *JcTOE1* (sequence ID XM_012209527), *JcTOE2* (sequence ID NW_012124616), and *JcTOE3* (sequence ID XM_012218287) were downloaded from the NCBI web site (http://www.ncbi.nlm.nih.gov/). (B) Expression levels of *JcmiR172* in cotyledons and young leaves of plants of different ages; Co, cotyledon; FL, the first leaf of *Jatropha* seedlings; 3M, young leaves of 3-month-old non-flowering *Jatropha*; 6M, young leaves of 6-month-old non-flowering *Jatropha*; 1Y, young leaves of 1-year-old non-flowering *Jatropha*; 3Y, young leaves of 3-year-old non-flowering *Jatropha*; 3YF, young leaves of 3 years old flowering *Jatropha*; 5YF, young leaves of 5-year-old flowering *Jatropha*. (C) Expression levels of JcmiR172 in different tissues of a mature *Jatropha* plant. R, roots; S, stems; YL, young leaves; ML, mature leaves; SA, shoot apex; IF, inflorescence buds; MF, male flowers; FF, female flowers; Fr, fruits. The qRT-PCR results were obtained from three independent biological replicates and three technical replicates for each sample; the error bars show the standard deviation. The levels of detected amplicons were normalised using the amplified products of *JcActin1*.

miR172 target sites are contained in the coding sequences and 3′-UTR regions of the target genes (Fig. 1A).

To determine where miR172 is expressed during *Jatropha* development, we analyzed the miR172 expression profiles in various tissues by qRT-PCR according to Varkonyi-Gasic et al. (2007). The mature miR172a–e sequences differ only in their 5′ and 3′ end bases (Supplementary Fig. S1), and therefore, a pair of miR172a primers can detect the expression of all mature miR172s; qRT-PCR was performed with total RNAs extracted from various tissues. The results indicated that there was an age-related increase in miR172 expression in *Jatropha* (Fig. 1B), which is consistent with a recent study of dynamics of miR172s during a production cycle of *Jatropha* (Sánchez-Gutiérrez et al. 2018). The lowest expression level was observed in the first leaf of the seedlings rather than cotyledons; with the age increasing, the transcription level of miR172 was increased continually. The highest level was observed in five-year-old plants (Fig. 1B). The expression pattern of miR172 in *Jatropha* plants of different ages was consistent with that in other trees (Wang et al. 2011). We also examined the miR172 expression levels in different organs of *Jatropha* plants. As shown in Fig. 1C, miR172 was expressed in all organs. The highest level was exhibited in the leaves, and high expression was observed in the shoot apex; miR172 expression varied considerably between organs and developmental stages of *Jatropha*. Similar miR172 expression patterns had also been observed in *Arabidopsis*, rice and maize (Aukerman and Sakai 2003, Lauter et al. 2005, Zhu et al. 2009).

Characterisation of the *JcmiR172a* functions in transgenic *Arabidopsis*

To test the functions of *JcmiR172a*, we transformed the 35S: *JcmiR172a* construct into *Arabidopsis* for preliminary analysis. Transgenic plants were confirmed by qRT-PCR analysis the expression level of miR172 (Supplementary Fig. S2). We selected two independent homozygous lines, L2 and L4, in the T2 generation to further examine the phenotypes. L2 and L4 generated flowers 14–15 days earlier and produced approximately nine fewer rosette leaves than that of WT plants under LD conditions (Fig. 2A–C; Table 1). Therefore, overexpression of *JcmiR172a* in *Arabidopsis* significantly reduced vegetative growth time.

Furthermore, compared with WT plants, the transgenic plants showed altered leaf sizes and morphologies (Fig. 2D and E). The sizes of rosette and cauline leaves were reduced in transgenic *Arabidopsis*. In contrast to the rosette leaves of WT, which exhibited obvious serrations, the rosette leaves of transgenic plants showed a smooth edge. Furthermore, the leaf basal angle was smaller than that of the WT plants (Fig. 2D–G), which is similar to the results reported by Jung et al. (2011).

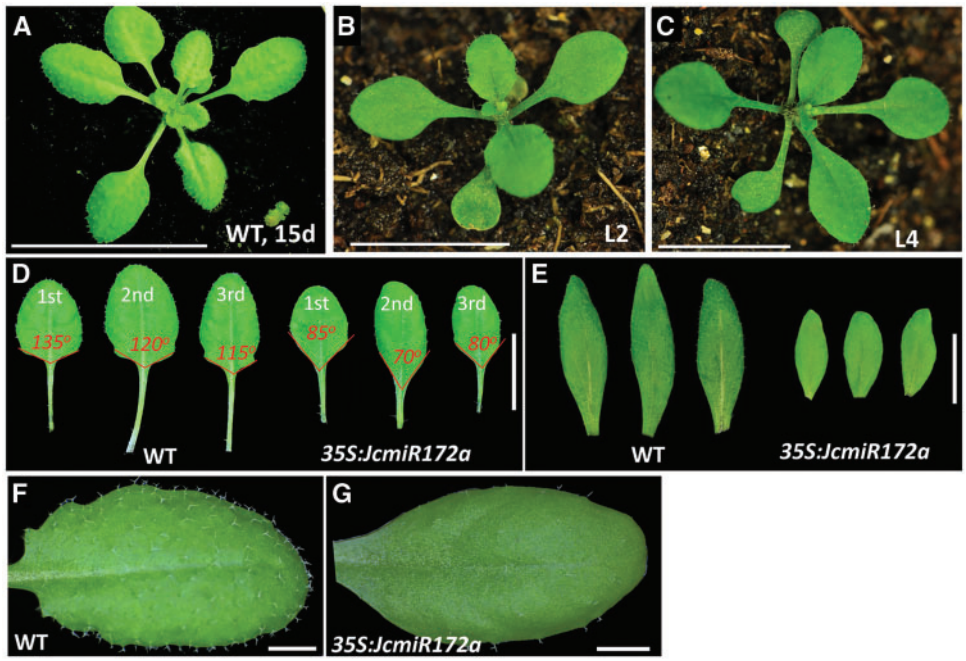

Fig. 2 *JcmiR172a* promoted vegetative to reproductive phase change in *Arabidopsis*. (A-C) Wild-type (WT, A), *35S: JcmiR172a* transgenic L2 (B) and L4 (C) *Arabidopsis* grown in long-day (LD) conditions for 15 days; flower buds appeared in the transgenic plants. (D) Rosette leaves of WT and 35S: *JcmiR172a* transgenic *Arabidopsis*; the first, second, and third rosette leaves are shown, and the leaf basal angle is labeled. (E) Cauline leaves of WT and *35S: JcmiR172a* transgenic *Arabidopsis*. Bar = 1 cm. (F) The fourth rosette leaf of WT, bar = 2 mm. (G) The fourth rosette leaf of *35S: JcmiR172a* transgenic plants; bar = 2 mm.

Table 1 Overexpression of *JcmiR172a* promotes flowering in *Arabidopsis* under LD conditions

Lines	Number of plants	Rosette leaves	Flower bud formation time/Day
WT	25	12.21 ± 1.04	24.56 ± 1.33
L2	35	3.56 ± 0.75**	9.47 ± 1.44**
L4	37	3.92 ± 0.63**	10.13 ± 1.38**

WT plants and two independent *JcmiR172a*-overexpressing lines (L2 and L4) grown under LD conditions (16 h light/8 h dark) were subjected to the analysis of rosette leaves and flowering times. The rosette leaves and flowering times are presented as the mean ± standard deviation. **Significantly different from the control at the 1% level.

The fourth rosette leaf of the 35S: *JcmiR172a* transgenic plants was ovate with few trichomes on the adaxial side (**Fig. 2G**, Supplementary Fig. S3), which represents a leaf morphology associated with the adult vegetative phase (Wu et al. 2009). More trichomes on the abaxial side, however, were found in transgenic *Arabidopsis* than that in WT plants (Supplementary Fig. S3). These observations indicate that miR172 also regulates developmental timing in addition to floral induction.

The floral pattern was also changed in transgenic *Arabidopsis*. The sepals, petals, and pistils were all smaller than that in WT (**Fig. 3B–G and J**); the petals of each flower were variable in size (**Fig. 3G**). Petals and stamens were partially absent (**Fig. 3C, I**), and the absent stamens in the third whorl were fused to the petals in the second whorl in transgenic *Arabidopsis* (**Fig. 3G**). The major phenotypes of *JcmiR172a*-overexpressing plants were similar to those of the *Arabidopsis ap2* mutants described by Kunst et al. (1989).

We further analyzed the gene expression in 35S: *JcmiR172a* transgenic *Arabidopsis* plants showing early flowering and abnormal flowers via qRT-PCR. The results showed that high miR172 levels down-regulated several *AP2*-like genes, including *AtAP2*, *AtTOE1*, *AtTOE2*, *AtSMZ*, and *AtSNZ*, in seedlings and flowers (Supplementary Fig. S2), which act as flowering repressors (Aukerman and Sakai 2003). In addition, the expression of the floral meristem identity genes *AtLEAFY* (*AtLFY*), *AtFUL*, *AtAP1* and *AtCAL* and the floral organ identity genes *AtSEP*s (*AtSEP1*, *AtSEP2*, *AtSEP3*) were significantly up-regulated in ten-day-old transgenic seedlings (Supplementary Fig. S2). However, the floral organ identity genes *AtAGAMOUS* (*AtAG*), *AtAP3* and *AtSEP2* were down-regulated in transgenic *Arabidopsis* flowers (Supplementary Fig. S2).

The phenotypes of early flowering and abnormal flowers produced by ectopic expression of *JcmiR172a* in transgenic *Arabidopsis* were similar to those produced by miR172 overexpression (Chen 2004). Gene expression analysis indicated that miR172 caused early flowering by down-regulating *AP2*-like floral repressors. The abnormal flowers appeared due to miR172 directly repressing the floral identity gene *AP2* and indirectly repressing other floral identity genes, including *AtAP3*, *AtAG*, and *AtSEP2* (Supplementary Fig. S2).

Overexpression of *JcmiR172a* in *Jatropha* changed the leaf and stem morphologies

We generated transgenic *Jatropha* plants overexpressing the *JcmiR172a* precursor driven by the 35S promoter. Elevated levels of miR172 were detected in *JcmiR172a* overexpression plants (Supplementary Fig. S4A). The leaves of the transgenic

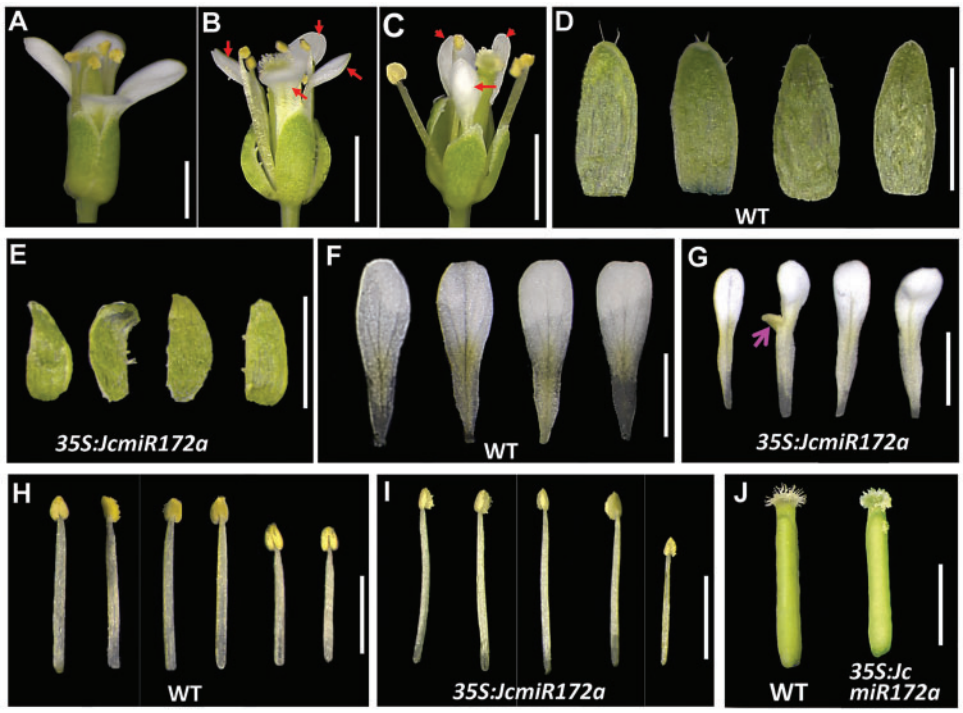

Fig. 3 Flower phenotypes of *35S: JcmiR172a* transgenic *Arabidopsis*. (A) Flower of WT. (B and C) Flowers of *35S: JcmiR172a* transgenic *Arabidopsis*; the petals are shown by red arrows. (D) Sepal anatomy of WT *Arabidopsis*. (E) Sepal anatomy of *35S: JcmiR172a* transgenic *Arabidopsis*. (F) Petal anatomy of WT *Arabidopsis*. (G) Petal anatomy of *35S: JcmiR172a* transgenic *Arabidopsis*; a stamen fused to petal is shown by a pink arrow. (H) Stamen anatomy of WT *Arabidopsis*. (I) Stamen anatomy of *35S: JcmiR172a* transgenic *Arabidopsis*; one stamen absent in *35S: JcmiR172a* transgenic *Arabidopsis*. (J) Comparison of the carpels in WT and transgenic plants; bar = 1 mm.

Jatropha were smaller, the leaf shape and leaf margin were altered, and the petiole length, leaf length and width were all significantly reduced compared to those of the WT plants (**Fig. 4A, B**, **Table 2**). The leaf lobes disappeared in mature leaves of transgenic *Jatropha* plants (**Fig. 4A, B**). The stem morphologies also changed in transgenic *Jatropha*. We compared 20 progeny seedlings of L32 and L47 transgenic plants with WT plants and found that the transgenic progeny seedings exhibited a thinner but harder stem phenotype (**Fig. 4C–F**). A comparison of stems of miR172 transgenic *Jatropha* and WT via cross-sections and longitudinal sections revealed that the thickness of transgenic *Jatropha* xylem was 0.36–0.75 mm thicker than that of WT in two-month-old seedlings (**Fig. 4C, E)** and 1.5 mm thicker in five-month-old seedlings (**Fig. 4D**). The percentage of xylems of transgenic *Jatropha* increased by approximately 10% compared to that of WT, whereas the percentages of phloem and piths were significantly reduced (**Fig. 4C–F**).

Overexpression of *JcmiR172a* enhanced xylem development via regulation of cell size and number in vascular tissues of *Arabidopsis* and *Jatropha*

In this study, we first found the miR172 regulates xylem development. To confirm this function of miR172, we further surveyed the stem traits in *JcmiR172a* transgenic *Arabidopsis* using 40-day-old T2 seedings. Notably, we also found that the stem diameter of miR172-overexpressing *Arabidopsis* was reduced by approximately 0.4–0.6 mm (Supplementary Fig. S5D); the results of transgenic *Arabidopsis* stem were similar to those of transgenic *Jatropha*. A comparison of the stem structure from pictures of shoot cross-sections of stems from different genotype plants showed that the number of vascular bundles was reduced in transgenic *Arabidopsis*. Generally, there are eight vascular bundles in WT *Arabidopsis*, but there were only 5 or 6 vascular bundles in *35S: JcmiR172a* transgenic plants (**Fig. 5A–C**). To further examine xylem-related traits, we prepared paraffin cross-sections of basal stems, and different vascular bundle attributes, including area and number of cell rows and lines, were evaluated (**Fig. 5D**, Supplementary Fig. S5). Both numbers of xylem cell rows and lines were significantly higher in *35S: JcmiR172a* transgenic plants than WT plants (Supplementary Fig. S5E, F). Furthermore, we recorded the ratio of the xylem/stem area calculated as the fraction of total xylem area with respect to the total stem area ratio. This parameter reflected the proportion of xylem in the total stem area. Notably, we observed a significant increase in the xylem ratio among the *35S: JcmiR172a* transgenic plants and WT plants (**Fig. 5D**).

The increased xylem may result in an increase of xylem cell volume. To verify this hypothesis, we analyzed the paraffin cross-sections with an oil immersion lens. Unexpectedly, a comparison of the xylem cell morphologies showed that the xylem cell size in 35S: *JcmiR172a* plants was smaller than that in

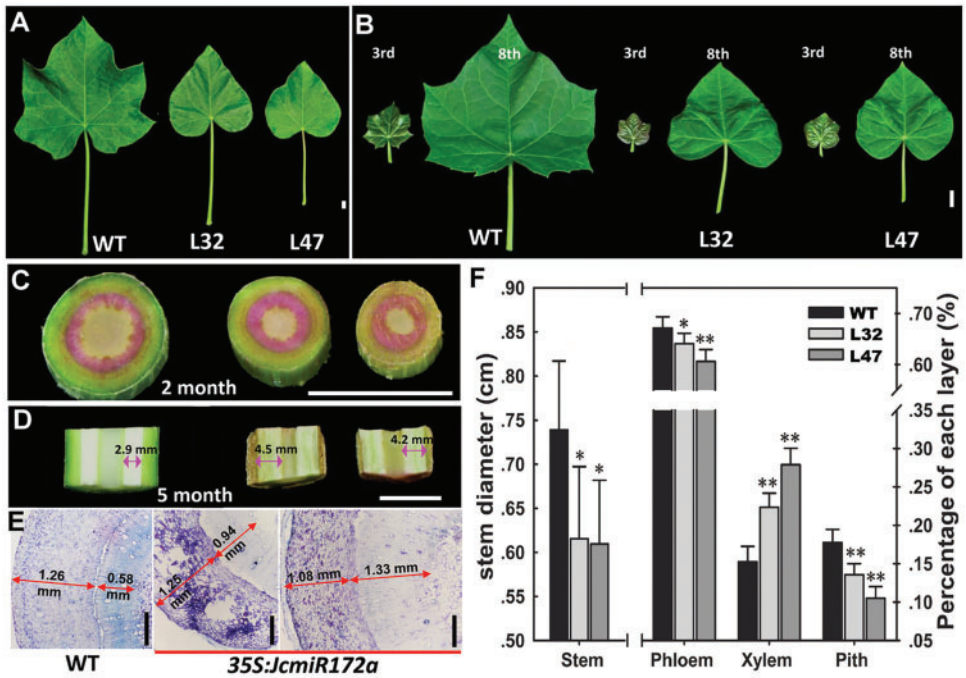

Fig. 4 Leaf and stem phenotypes of 35S: *JcmiR172a* transgenic *Jatropha*. (A) The mature leaves of WT and 35S: *JcmiR172a* transgenic T0 plants L32 and L47. (B) The mature and young leaves of WT and 35S: *JcmiR172a* transgenic T1 plants L32 and L47; mature leaves are the eighth leaf from the shoot apex, young leaves are the third leaf from the shoot apex. (C) Cross sections of the stem of 2-month-old seedlings of WT and transgenic T1 plants L32 and L47. (D) Longitudinal sections of the stem of 4-month-old seedlings of WT and transgenic T1 plants L32 and L47, bar = 1 cm. (E) The thickness of phloem and xylem was determined with paraffin sections using toluidine blue O, bar = 0.5 mm. (F) Analysis of stem diameter and percentages of phloem, xylem and pith. The areas of phloem, xylem and pith were calculated according to each diameter. Values are the mean ± standard deviation. *Statistically different from the control at the 5% level. **Statistically different from the control at the 1% level; error bars indicate standard deviations for 50 seedlings.

Table 2 Characteristics of mature leaves and flowering time of *JcmiR172a* transgenic plants

	Sample	*N*	Petiole length (cm)	Leaf length (cm)	Leaf width (cm)	Flower bud formation time/Day
T0	WT	24	$17.67. \pm 1.65^{c}$	15.00 ± 1.05^{c}	16.07 ± 1.71^{b}	-
	L32	19	12.72 ± 1.13^{b}	12.07 ± 1.07^{b}	11.13 ± 1.00^{a}	-
	L47	21	10.96 ± 1.42^{a}	10.93 ± 0.92^{a}	10.47 ± 0.99^{a}	-
T1	WT	15	6.95 ± 1.58^{c}	10.42 ± 0.95^{c}	11.53 ± 0.81^{c}	324.56 ± 53.61^{B}
	L32	15	5.47 ± 0.74^{b}	8.17 ± 0.85^{b}	8.97 ± 0.91^{b}	139.47 ± 15.42^{A}
	L47	15	4.48 ± 0.43^{a}	6.49 ± 0.73^{a}	7.15 ± 0.51^{a}	120.13 ± 13.24^{A}

Leaves of WT plants and two independent *JcmiR172a*-overexpressing lines (L32 and L47) grown in the field were subjected to an analysis of leaf size. *N* = sample number. The petiole length, leaf length, leaf width, and flower bud formation time are presented as the mean ± standard deviation. Values with different lowercase letters are significantly different at $P < 0.05$ by Tukey's test. Values with different capital letters are significantly different at $P < 0.01$ by Tukey's test.

WT plants (**Fig. 6**). However, the xylem cell density was higher than that in WT. Analysis of the microphotographs showed that in WT plants there were only 150 xylem cells in a 200 μm × 200 μm zone, but in miR172 overexpression plants, there were 220–230 xylem cells in a 200 μm × 200 μm zone (**Fig. 6D–F**). In transgenic *Arabidopsis*, the xylem cell size was also smaller than that in WT (**Fig. 5E–G**), and the smallest cell size was observed in plant line 2, which had the highest miR172 expression level (Supplementary Fig. S2A). These results were consistent with the findings in transgenic *Jatropha*. The changes in xylem traits were mostly explained by an increased number of xylem cell rows, which caused increased total xylem thickness and ratio (**Figs. 5, 6**, Supplementary Fig. S5).

We further analyzed the lignification-related genes in 35S: *JcmiR172a* transgenic *Jatropha* via qRT-PCR. Total RNA samples were extracted from stems of two-month-old T1 transgenic and WT seedings. The results showed that the expression levels of the lignin biosynthesis genes *Cinnamyl alcohol dehydrogenase 6* (*CAD6*) and caffeoyl CoA O-methyltransferase (*CCoAOMT*) were strongly up-regulated (**Fig. 7A, B**), and the cellulose synthase A (*CesA*) genes *JcCesA1*, *JcCesA3*, *JcCesA4*, *JcCesA7*, and *JcCesA8* were also substantially up-regulated (**Fig. 7C, F, H–J**). However, the expression levels of *JcCesA2*, *JcCesA2-L*, and *JcCesA3-L* showed no significant increases (**Fig. 7D, E, G**). These results indicated that JcmiR172 accelerates transgenic *Jatropha* xylem development by indirectly

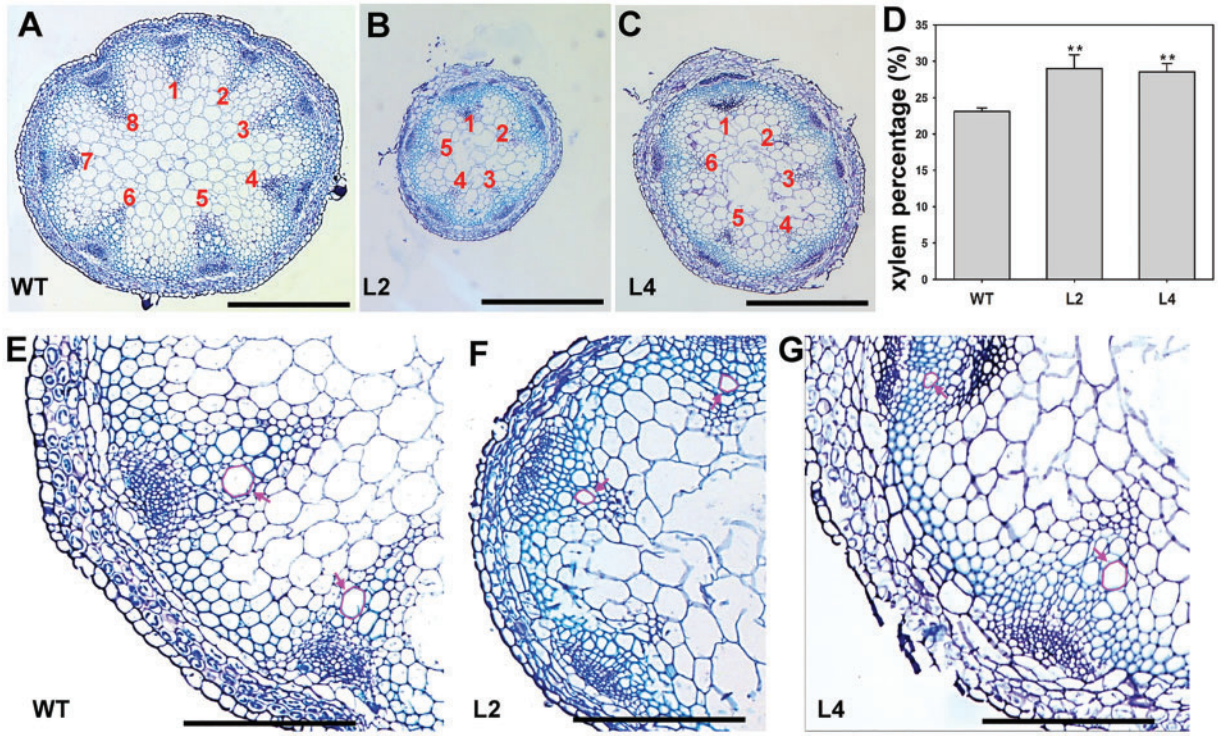

Fig. 5 Comparison of stem characteristics between transgenic *Arabidopsis* and WT. (A-C) Cross-section of the first internode of the main stem, which was stained by toluidine blue O, bar = 500 μm. (D) Analysis of the xylem/stem area ratio ($N = 15$), **indicates $P < 0.01$. (E–G) Comparison of phloem, xylem, and pith cell morphologies; xylem cells were stained light blue, bar = 200 μm.

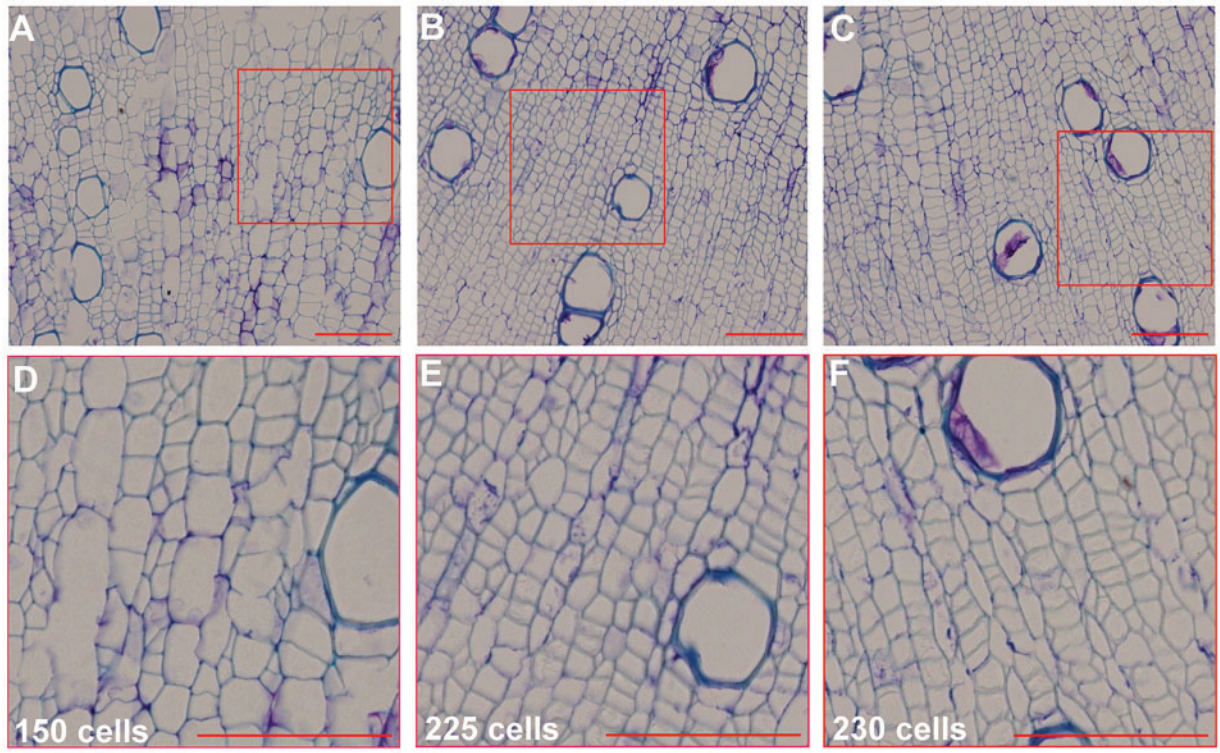

Fig. 6 Comparison of the xylem cell morphology and density between WT and *35S: JcmiR172a* transgenic *Jatropha*. (A–C) Paraffin sections of WT (A), *35S: JcmiR172a* L32 (B) and L47 (C), 200 μm × 200 μm zones are marked by red squares. (D–F) Magnifying marked zone of (A–C) respectively, the number of cells in this zone were counted; bar = 100 μm.

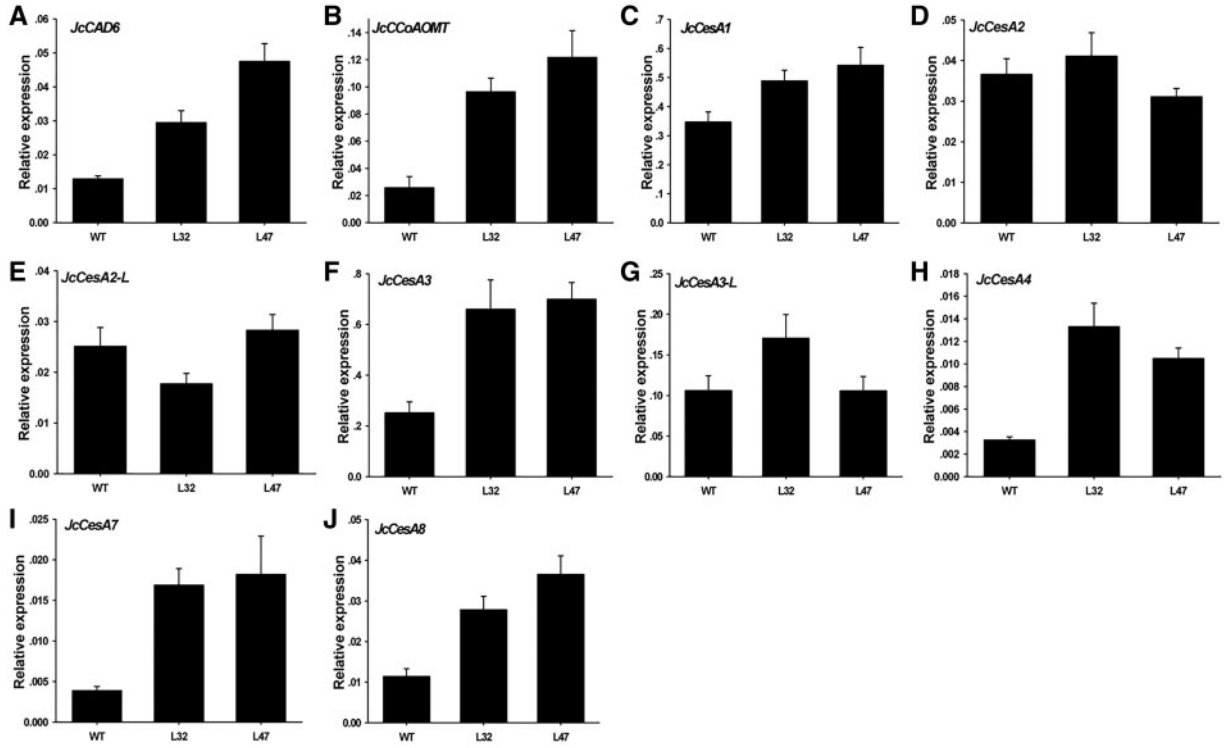

Fig. 7 qPCR analysis of lignification-related genes in the transgenic *Jatropha* overexpressing *miR172*. The expression levels of the lignin biosynthesis genes Cinnamyl alcohol dehydrogenase 6 (*JcCAD6*) and caffeoyl CoA O-methyltransferase (*JcCCoAOMT*) and cellulose synthase A (*JcCesA*) genes. RNA samples were extracted from 2-month-old T1 seedling shoots; error bars indicate standard deviations for three replicates.

promoting the expression of lignin biosynthesis and cellulose synthase genes.

Overexpression of *JcmiR172a* in *Jatropha* caused early flowering and abnormal development of reproductive organs

Transgenic analysis performed in *Arabidopsis* suggested that miR172 might act as a flowering accelerator in *Jatropha*. To test this hypothesis, we generated transgenic *Jatropha* with the *35S: JcmiR172a* construct as previously described. Non-transgenic/WT plants were used as control. Fifty independent transgenic lines were confirmed via PCR. To our surprise, all the transgenic *Jatropha* lines lacked the early-flowering phenotype in a tropical area (Xishuangbanna). When regenerated plantlets (Supplementary Fig. S6) were grown in the field for 5 months, flower buds emerged in both transgenic and control plants (Supplementary Fig. S6D–G). In contrast, two (L8 and L21) of the 15 transgenic lines of the *35S: JcmiR172a*-overexpressing *Jatropha* grown in a subtropical area (Kunming) produced flowers in the second year (Supplementary Fig. S7B–C, E–F). The transgenic plants produced flowers only once and only in spring every year, while the WT plants did not produce flowers until 5 years after they were planted in the same conditions (Supplementary Fig. S7A, D). We further analyzed the T1 seedling phenotypes and several floral identity-related genes in a greenhouse in a tropical area. We found that the T1 transgenic plants generated flower buds at least 5 to 6 months earlier than the WT plants (**Table 2**). The expression results showed that the transcript levels of the miR172 target genes *JcAP2*, *JcTOE1*, and *JcTOE2* were down-regulated (Supplementary Fig. S4B–D), and the transcript levels of *JcFT*, *JcLFY*, and *JcAP1* were slightly altered in the transgenic seedlings (Supplementary Fig. S4F–H). However, the expression levels of *JcSOC1* and *JcSEP2* were increased more than 10-fold (Supplementary Fig. S4I, M). These results indicated that miR172 is involved in floral meristem determination in *Jatropha*.

Comparing the flowers, we found that the floral organ patterns were obviously different (**Fig. 8A, B**). We dissected the flowers and found that the flower organs were partially absent in transgenic plants; in the first and second whorls, there were 2–4 sepals and 2–3 petals. In addition, 2–3 nectaries were observed. In WT flowers, there were five sepals, petals and nectaries (**Fig. 8A–F; Table 3**). In male flowers, 5–6 stamens were observed, while WT flowers had 10 stamens (**Fig. 8G, H; Table 3**), and similar to transgenic *Arabidopsis* (**Fig. 3G**), there were two stamens fused to the petals (**Fig. 8F**). The carpels were present, but the ovules are partially absent in female flowers (**Fig. 8I, J; Table 3**). The expression profiles showed that the transcript levels of floral organ identity genes, including *JcAP2*, *JcAP3*, *JcAG*, *JcSEP1*, and *JcSEP3* (Supplementary Fig. S4B and J–L, N), were down-regulated in transgenic flowers. Because the abundance of miR172 in wild-type plants is low in young seedlings and high in inflorescences, the increase in miR172 abundance in the *35S: JcmiR172a* lines was more evident in young seedlings than in inflorescences. In addition, in *35S: JcmiR172a* transgenic plants, the miR172 levels were not significantly different at different ages (Supplementary Fig. S4A).

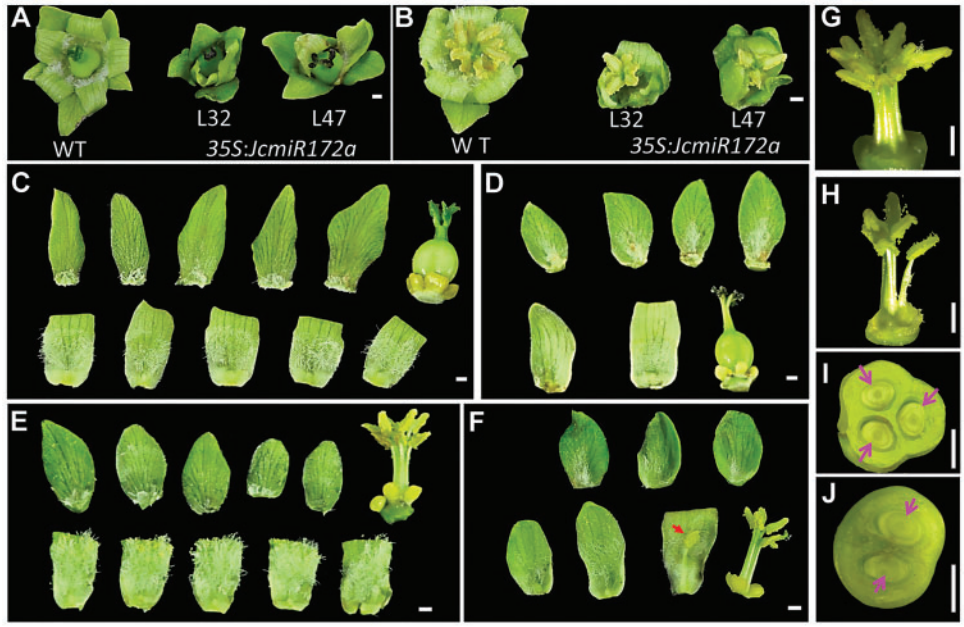

Fig. 8 Changes in the floral organ number in *JcmiR172a* transgenic *Jatropha*. (A, B) Female (A) and male (B) flowers of WT and *35S: JcmiR172a* transgenic *Jatropha*. (C, D) Female flower anatomy of WT (C) and *35S: JcmiR172a* transgenic plants (D). (E, F) Male flower anatomy of WT (E) and *35S: JcmiR172a* transgenic plants (F); a stamen fused to petal is shown by a red arrow. (G, H) Stamen of WT (G) and *35S: JcmiR172a* transgenic (H) male flowers. (I, J) Cross-Sections of WT (I) and *35S: JcmiR172a* transgenic (J) pistils; ovules are indicated by pink arrows; bar = 1 mm.

Table 3 Number of floral organs in *JcmiR172a* transgenic plants

Sample	*N*	sepals	petals	nectaries	stamens	carpels	ovules
WT male	25	5.00 ± 0.00^{a}	5.00 ± 0.00^{a}	5.00 ± 0.00^{a}	9.92 ± 0.28^{a}	–	–
WT female	25	5.00 ± 0.00^{a}	5.00 ± 0.00^{a}	5.00 ± 0.00^{a}	–	1.00 ± 0.00	3.00 ± 0.00^{a}
L32 male	18	4.33 ± 0.59^{b}	3.28 ± 0.46^{b}	3.72 ± 0.46^{b}	6.28 ± 0.46^{b}	–	–
L32 female	15	4.00 ± 0.53^{bc}	3.13 ± 0.64^{bc}	3.40 ± 0.51^{bc}	–	1.00 ± 0.00	2.80 ± 0.41^{b}
L47 male	33	3.21 ± 0.65^{d}	2.88 ± 0.60^{cd}	3.42 ± 0.56^{bc}	5.15 ± 0.57^{b}	–	–
L47 female	25	3.64 ± 0.70^{cd}	2.72 ± 0.46^{d}	3.24 ± 0.51^{c}	–	1.00 ± 0.00	2.84 ± 0.47^{b}

The male and female flowers of WT plants and two independent *JcmiR172a*-overexpressing lines (L32 and L47) were subjected to the analysis of flower organ numbers. *N* = flower number. The flower organ numbers are presented as the mean ± standard deviation. Values with different letters are significantly different ($P < 0.05$, Tukey's test).

The results obtained from the transgenic *Jatropha* indicate that *miR172* is important in regulating flower organ development and sustaining normal patterns through regulation of the expression of floral organ identity genes.

Jatropha plants overexpressing *JcmiR172a* showed significant floral organ defects, altered fruit shape, and reduced seed yield compared to WT plants. In transgenic plants, the length of the fruits was longer than that of WT plants, the width of the fruits was narrower than that of WT, and the ratio of length to width (L/W) was increased (**Fig. 9A, B**). Further analysis of the transgenic fruits showed that some seeds in the fruits were aborted (**Fig. 9C**). One or two abortive seeds were observed in each fruit; on average, the normal seed number in each fruit was 1.3—1.6 (**Fig. 9D**), but in WT, the seed number of each fruit was three, indicating a significant reduction in transgenic fruits. We compared the transgenic seeds to WT seeds and found that the transgenic seeds were bigger, but the weight and oil contents of these seeds were decreased compared to those of WT seeds (**Fig. 9E, F**, **Table 4**). The changes in size, number, weight, and oil contents of seeds in *JcmiR172a*-overexpressing transgenic plants suggest that *miR172* may be involved in seed development.

Discussion

Functional conservation and divergence of miR172 in *Arabidopsis* and *Jatropha*

There are five pri-miR172s in both *Arabidopsis* and *Jatropha*. The sequences of the mature *JcmiR172a-d* and *JcmiR172e*, which have been isolated in *Jatropha* seeds (Galli et al. 2014), differ only in their 5′ and 3′ end bases (Supplementary Fig. S1A). *JcmiR172e*, which is different from that of *Arabidopsis* miR172, shows higher similarity to that of some woody plants, such as

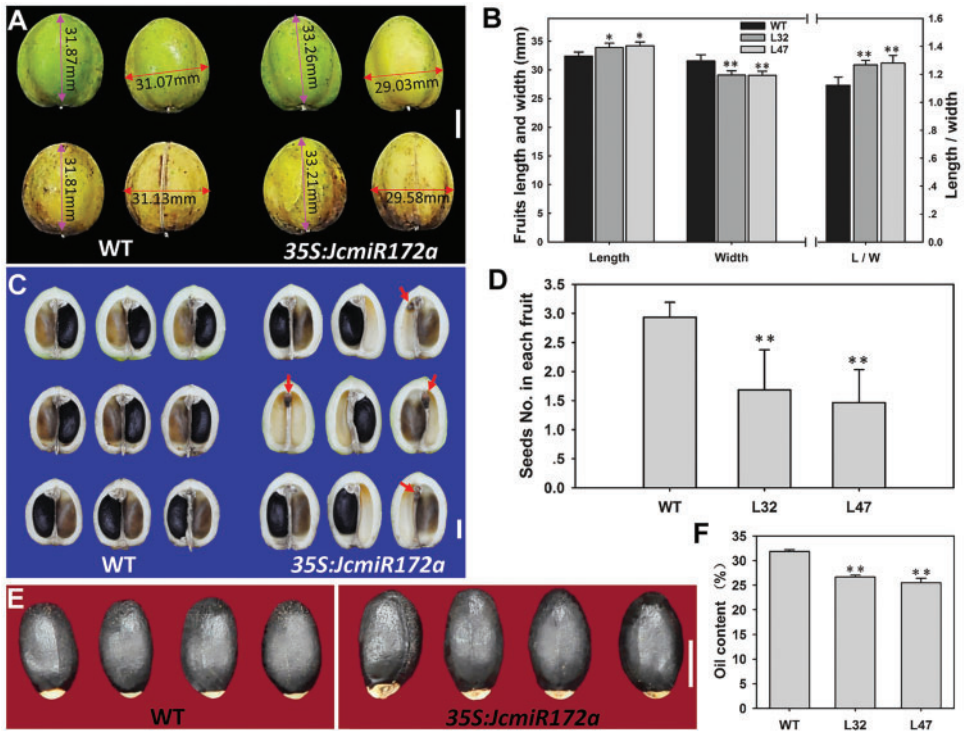

Fig. 9 Fruit and seed phenotypes of *35S: JcmiR172a* transgenic *Jatropha*. (A) Mature fruits of WT and *35S: JcmiR172a* transgenic *Jatropha*. (B) Analysis of the fruit size of WT and *35S: JcmiR172a* transgenic *Jatropha*; error bars indicate standard deviations for 30 fruits. (C) Some seeds were aborted in *35S: JcmiR172a* transgenic *Jatropha*; aborted seeds are shown by red arrows. (D) Analysis of seed numbers in each fruit; error bars indicate standard deviations for 30 seeds; (E) Mature seeds of WT and *35S: JcmiR172a* transgenic *Jatropha*. (F) Comparison of the oil contents in WT and *35S: JcmiR172a* transgenic seeds. Values are the mean ± standard deviation; *statistically different from the control at the 5% level; **statistically different from the control at the 1% level; error bars indicate standard deviations for 50 seedlings; bar = 1 cm.

Table 4 Seed characteristics of *JcmiR172a* transgenic *Jatropha*

Sample	*N*	Length (mm)	Width (mm)	Height (mm)	Weight/seed (g)
WT	30	18.36 ± 0.48	10.85 ± 0.34	8.55 ± 0.23	0.72 ± 0.04
L32	30	19.36 ± 0.45*	11.50 ± 0.48*	9.28 ± 0.59*	0.70 ± 0.05
L47	30	19.23 ± 0.50*	11.42 ± 0.53*	9.59 ± 0.72*	0.68 ± 0.04*

Values are the mean ± standard deviation ($N = 30$ seeds).
*Statistically different from the control at the 5% level.
**Statistically different from the control at the 1% level.

apple (*Malus* × *domestica*), *P. trichocarpa*, *Vitis vinifera*, *R. communis*, and *Citrus sinensis* (http://www.mirbase.org/). According to the alignment of nucleotide sequences, we found *JcmiR172e* showed higher matching scores with target genes than that of *JcmiR172a-d* (**Fig.1A**, Supplementary Fig. S1B). And the apple *Md-miR172e*, a homolog of *JcmiR172e*, has been shown to alter flowering time and floral organ identity when ectopically expressed in *Arabidopsis* (Zhao et al. 2015).

In this study, we showed the results of *JcmiR172a* overexpression in both *Arabidopsis* and *Jatropha*. In *Arabidopsis*, miR172 overexpression plants showed extremely early flowering (**Fig. 2**). This result indicates that miR172 promotes the transition from the vegetative phase to the adult phase. In *Jatropha*, *JcmiR172a* expression level continuously increased with age (**Fig. 1B**), and *JcmiR172a* overexpression plants showed an increase in xylem thickness (**Fig. 4C–F**); furthermore, transgenic *Jatropha* exhibited early flowering in a subtropical area (Supplementary Fig. S7). These results indicate miR172 is an age marker gene in *Jatropha*, which is similar to its role in other plants (Wang et al. 2011, Zhu and Helliwell 2011, Lee et al. 2014). In transgenic *Arabidopsis* and *Jatropha*, the leaf morphologies were changed, the leaves were smaller, and the leaf margins were smoother than those in WT (**Fig. 2E, F;** and **Fig. 4A, B**). The flower organs were partially defective in transgenic *Arabidopsis* and *Jatropha* (**Figs. 3, 8**). Comparing the results in these two species, and together with characterization of miR172 in other non-model plants (Glazińska et al. 2009, Nair et al. 2010, Debernardi et al. 2017, Anwar et al. 2018, Shivaraj et al. 2018), we concluded that the miR172 has conservative functions in regulating phase change, controlling leaf morphologies, and sustaining normal flower development.

In *Arabidopsis* three mature miR172s and six target genes, including *AP2*, *SMZ*, *SNZ*, *TOE1*, *TOE2*, and *TOE3*, were identified (Fornara and Coupland 2009, Mathieu et al. 2009). However, there are only two mature miR172s in *Jatropha* (Supplementary Fig. S1) and only four target genes of miR172, including *JcAP2*, *JcTOE1*, *JcTOE2*, and *JcTOE3* (**Fig. 1**). Different target gene numbers may lead to divergent functions.

In transgenic *Arabidopsis*, the flower bud appeared 14–15 days earlier than that of WT, and it produced only 3–4 rosette leaves. This phenotype was as strong as that shown by Aukerman and Sakai (2003) and Lee et al. (2010). However, we did not obtain transgenic *Arabidopsis* plants that completely lacked flower organs, but the absent flower organ phenotypes in transgenic *Jatropha* plants are much more serious than that in transgenic *Arabidopsis* (**Figs. 3** and **8**). Thus, because the pri-*miR172a* is from *Jatropha*, the sequence is distinctive from that of *Arabidopsis*.

In *Arabidopsis*, the role of miR172 in flowering time and floral organ identity gene was characterized (Aukerman and Sakai 2003). In *Arabidopsis*, *35S: miR172a* plants showed early flowering, and the late-flowering phenotype of 35S: *AP2* plants rescued. The flower closely resembles *ap2* flowers, which had sepal and petal identity defects (Chen 2004). In *Jatropha 35S: JcmiR172a*, there were flower organ defects exhibited in all flower organs, including the sepals, petals, stamens, pistils, and nectaries (**Fig. 8**, **Table 3**). There were bundles of stigmatic papillae projecting from the sepal margins of miR172-overexpressing *Arabidopsis* (**Fig. 3E**) (Aukerman and Sakai 2003). However, the stigmatic papillae structures were not found in transgene *Jatropha*. In *Arabidopsis*, miR172 is critical for fruit growth, as fruit growth was blocked when miR172 activity is compromised (Jose Ripoll et al. 2015). In this study, we found that high miR172 expression level influenced fruit morphogenesis (**Fig. 9A, B**), seed number, size, morphogenesis, and oil content, and led to seed abortion in transgenic *Jatropha* (**Fig. 9**).

These results indicated that the functions of *miR172* are different between annual herbaceous plants and perennial woody plants. A former study reported the divergence of microRNAs and their functions in Euphorbiaceous plants during plant growth and in response to abiotic stresses (Zeng et al. 2009).

Overexpression of miR172 shortened the juvenile stage in *Jatropha*

In contrast to *JcmiR172a*-overexpressing *Arabidopsis*, *JcmiR172a*-overexpressing *Jatropha* did not exhibit early flowering in the field in a tropical area (Supplementary Fig. S6). Both the WT and *JcmiR172a* transgenic plants can produce flowers in the first year in the tropical area. However, the *JcmiR172a* transgenic *Jatropha* plants produced flowers in the second year when they were planted in a subtropical area, whereas the WT plants did not produce flowers (Supplementary Fig. S7). Under normal conditions, WT *Jatropha* plants have a juvenile stage of at least 3–5 years before producing flowers in subtropical areas. The transgenic plants showed a shortened juvenile stage and accelerated flowering time, but these plants did not produce flowers continuously and did not produce flowers at other seasons. The results indicated that miR172 cannot promote flowering independently; it also depends on suitable environmental conditions.

Similar to our findings, overexpression of the rice *miR172b* in rice did not lead to early flowering (Zhu et al. 2009). Recent studies in *Cardamine flexuosa* and *Arabis alpine* demonstrated that age is necessary but not sufficient to promote flowering in perennial herbaceous plants, and other factors, such as low temperature, are also necessary (Bergonzi et al. 2013, Zhou et al. 2013). *Jatropha* is a perennial woody plant. The molecular mechanisms controlling flowering in perennial woody plants have not been studied as extensively as those of herbaceous plants (Albani and Coupland 2010). In this study, miR172-overexpressing *Arabidopsis* showed extremely early flowering, and all floral meristem identity genes were up-regulated in 10-day-old seedlings (Supplementary Fig. S2); miR172-overexpressing *Jatropha* did not show extremely early flowering, and only *JcSOC1*and *JcSEP2* were up-regulated in 2-month-old seedlings (Supplementary Fig. S4). The molecular mechanism of flowering control in the woody plant *Jatropha* is likely very complex; thus, a single floral identity factor, such as age, is not sufficient to promote early flowering. Suitable environmental conditions are necessary to regulate flowering in *Jatropha*. Various environmental factors affecting flowering need to be characterized in *Jatropha* in future studies.

JcmiR172 might be involved in regulation of xylem development

In perennial woody plants, xylem cell formation is age dependent (Rossi et al. 2008). In this study, the miR172 expression profile indicates that miR172 is an age marker gene. High miR172 expression is closely correlated with the adult phases of several woody species (Wang et al. 2011). In this study, we found elevated age markers of *Jatropha* by constitutive overexpression of *JcmiR172a*, which accelerated the lignification of the secondary xylem by indirectly promoting lignin biosynthesis and the expression of cellulose synthase genes (**Fig. 7**). The transgenic *Jatropha* stem xylems were thicker than those of WT plants (**Fig. 4C–F**), and the xylem cell density was higher in transgenic *Jatropha* than in WT (**Fig. 6**). However, the size of xylem cell was smaller than that of WT (**Fig. 6**), and these results were similar to those in transgenic *Arabidopsis* (**Fig. 5E and F**). The expression levels of lignin biosynthesis genes and cellulose synthase genes were also strongly up-regulated (**Fig. 7C, F, H–J**). These results indicated that *JcmiR172* accelerates the expansion of transgenic *Jatropha* xylems by promoting lignin biosynthesis and cellulose synthase gene expressing. However, this phenotype was not found in previous studies examining rice (Zhu et al. 2009, Lee et al. 2014), *Arabidopsis* (Aukerman and Sakai 2003, Mathieu et al. 2009), *Cardamine flexuosa* (Zhou et al. 2013), barley (Houston et al. 2013), soybean (Zhao et al. 2007), and maize (Lauter et al. 2005).

Many genes participating in lignin biosynthesis and secondary cell wall formation in *Acacia* hybrids were identified by transcriptome sequencing; target genes of three putative miRNAs, e.g. miR160, miR172, and miR396, were predicted as

wood-related genes (Wong et al. 2011). However, a functional analysis of these putative miRNAs with potential roles in wood formation has not been carried out. In this study, we demonstrated the function of miR172 in regulating wood formation in *Jatropha* and *Arabidopsis*.

Several factors, such as the plant hormones gibberellin (Wang et al. 2017), auxin (Moreno-Piovano et al. 2017), jasmonic acid and cytokinin (Jang et al. 2017), and some transcription factors, e.g. bHLH complexes (Ohashi-Ito and Fukuda 2016), LAX2 (Moreno-Piovano et al. 2017), *VND6*, *VND7*, *NST3*, and *WOX4* (Stein et al. 2016), are involved in these processes. However, the secondary xylem thickening phenotype was never reported in miR172-overexpressing plants because the previous studies on overexpression of miR172 were performed in annual herbaceous plants. The miR172 transgenic *Jatropha* plants referred to in this study can be used in agriculture to improve lodging resistance due to the thickened xylems (Zheng et al. 2017). The specific functions of *miR172* were also found in other plants; for example, *miR172* is essential for nodulation in soybean and *Lotus japonicus* (Yan et al. 2013, Holt et al. 2015).

Materials and Methods

Plant materials and growth conditions

The roots, stems, young and mature leaves, inflorescence buds, female and male flowers, and fruits in one mature tree of *Jatropha* were collected as previously described Tang et al. (2016b) to compare the expression levels of miR172 in different tissues. The cotyledons and young leaves from *Jatropha* plants of various ages were collected to analyse the miR172 expression levels in plants of different ages from Kunming, Yunnan province, China. All tissues prepared for qRT-PCR were immediately frozen in liquid nitrogen and stored at −80°C until use. The *Arabidopsis* seeds were germinated on 1/2 MS medium over a one-week period, after which seedlings were transferred to peat soil in plant growth chambers at 22 ± 2°C under long-day (LD) (16 h light/8 h dark) conditions. Phenotypic analysis was performed on homozygous (T2) *Arabidopsis* plants as well as T0 and T1 *Jatropha* plants. For each *Arabidopsis* genotype, more than 20 plants were used for characterisation. The number of rosette leaves was counted along with the number of days between transfer to soil and when the first flower bud was visible. Ten-day-old *Arabidopsis* seedlings and inflorescences from 40-day-old plants were harvested to analyse mRNA transcription levels. The shoot apexes of two-month-old T1 transgenic *Jatropha* and flower buds of two-year-old T0 transgenic *Jatropha* were harvested to analyse mRNA transcription levels.

Cloning of *JcmiR172a* precursor

Total RNA was extracted from the young leaves of *Jatropha* using the protocol described by Ding et al. (2008). First-strand cDNA was synthesized using M-MLV-reverse transcriptase according to the manufacturer's instructions (TaKaRa, Dalian, China). The full-length *JcmiR172a* precursor cDNA (GenBank accession number XR_002283652) (Sato et al. 2011) (http://www.kazusa.or.jp/jatropha/) was amplified by PCR using the primers XA401 and XA402 (all primers used in this study are listed in Supplementary Table S1), which had *Kpn*I and *Sal*I recognition sites, respectively. The PCR product (422 bp) was subsequently cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA). The resultant plasmid pTMY004 was used as a template for sequencing.

Construction of the overexpression binary vector and plant transformation

For construction of the plant overexpression binary vector *35S: JcmiR172a*, the *JcmiR172a* precursor cDNA (422 bp) was excised from pTMY004 using the restriction enzymes *Kpn*I and *Sal*I and then cloned into the pOCA30 vector containing the CaMV 35S promoter, resulting in the binary vector pMYT34. Transformation of WT *Arabidopsis* with *Agrobacterium* strain EHA105 carrying the pMYT34 (*35S: JcmiR172a*) was performed using the floral dip method (Clough and Bent 1998). Transformation of *Jatropha* with *Agrobacterium* strain EHA105 carrying the same construct was performed according to the protocol described by Pan et al. (2010) and Fu et al. (2015). All transgenic plants were confirmed by genomic PCR and RT-PCR.

qRT-PCR analysis

Jatropha total RNA was extracted from frozen tissues according to the methods described by Ding et al. (2008). *Arabidopsis* total RNA was extracted from frozen tissues using TRIzol reagent (Transgene, China). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China) according to the instruction manual and Tang et al. (2016a). The miR172-specific reverse transcription (RT) primer XA822 was used for miR172. The cDNA templates were diluted 5–10 times using sterilized double distilled water for first-strand cDNA according to Tang et al. (2016b); qRT-PCR experiments were performed using SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) on a Roche LightCycler480 II Real-Time PCR Detection System (Roche Diagnostics) as previously described Tang et al. (2016b). All primers used for qRT-PCR are listed in Supplementary Table S1. qRT-PCR was performed as previously described Tang et al. (2016b), precisely using three independent biological replicates and three technical replicates for each sample. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The transcript levels of specific genes were normalized using *Jatropha curcas actin1*(*JcActin1*) (Zhang et al. 2013) or *Arabidopsis actin2* (Soni and Mondal 2018). For determination of the mature miR172 abundance, qRT-PCR was performed according to Varkonyi-Gasic et al. (2007).

Analysis of flower and fruit phenotypes

The flower and fruit anatomy was examined and photographed with a light Leica DM IRB anatomical lens (Leica, Heerbrugg, Switzerland) equipped with a Leica DFC425 C camera. The length and width of *Jatropha* fruits and the length, width, and thickness of seeds were measured with an electronic vernier caliper (to 0.1 mm).

Analysis of stem components

Two- and five-month-old T1 transgenic *Jatropha* seedlings grown in a greenhouse were harvested to measure the middle stem diameter. Then, the stems were used for paraffin sections (Sakai 1973) to observe the cell morphologies and measure and calculate the thickness and area of phloem, xylem, and pith.

Supplementary Data

Supplementary data are available at PCP online.

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Author contributions

Mingyong Tang designed and performed the experiments, analyzed the data, and wrote the paper. Xue Bai, analyzed data and revised the paper. Long-Jian Niu, Xia Chai, and Mao-Sheng Chen helped to collect data. Zeng-Fu Xu conceived the study and revised the paper.

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