Identification and expression analysis of cytokinin metabolic genes *IPTs*, *CYP735A* and *CKXs* in the biofuel plant *Jatropha curcas*

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16 **ABSTRACT**

17 The seed oil of *Jatropha curcas* is considered a potential bioenergy source that could replace

18 fossil fuels. However, the seed yield of *Jatropha* is low and has yet to be improved. We

19 previously reported that exogenous cytokinin treatment increased the seed yield of *Jatropha*.

20 Cytokinin levels are directly regulated by isopentenyl transferases (IPT), cytochrome P450

21 monooxygenase (CYP735A) and cytokinin oxidase/dehydrogenase (CKX). In this study, we

22 cloned six *IPT* genes, one *JcCYP735A* gene, and seven *JcCKX* genes. The expression patterns of

23 these 14 genes in various organs were determined using real-time quantitative PCR. *JcIPT1* was

24 primarily expressed in roots and seeds, *JcIPT2* was expressed in roots, apical meristems, and

25 mature leaves, *JcIPT3* was expressed in stems and mature leaves, *JcIPT5* was expressed in roots

26 and mature leaves, *JcIPT6* was expressed in seeds at 10 days after pollination, and *JcIPT9* was

27 expressed in mature leaves. *JcCYP735A* was mainly expressed in roots, flower buds, and seeds.

28 The seven *JcCKX* genes also showed different expression patterns in different organs of

29 *Jatropha*. In addition, CK levels were detected in flower buds and seeds at different stages of

30 development. The concentration of iP, iPR and tZ increased with flower development, and the

31 concentration of iP decreased with seed development, while that of tZ increased. We further

32 analyzed the function of *JcCYP735A* using the CRISPR-Cas9 system, and found that the

33 concentrations of tZ and tZR decreased significantly in the *Jccyp735a* mutants, which showed

34 severely retarded growth. These findings will be helpful for further studies of the functions of

35 cytokinin metabolic genes and understanding the roles of cytokinins in *Jatropha* growth and

36 development.

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INTRODUCTION

Jatropha curcas is a multipurpose tree that belongs to the Euphorbiaceae family. It can endure drought and adapt to barren land in tropical and subtropical regions. *Jatropha* is considered a promising biofuel plant due to the high oil content in its seeds (Akashi 2012; Francis et al. 2005; Makkar & Becker 2009). However, the seed yield is very low, potentially because of the relatively low number of total flowers and/or the ratio of female to male flowers in *Jatropha* (Kumar & Sharma 2008; Kumar Tiwari et al. 2007; Rao et al. 2008). Recently, several studies have reported that exogenous cytokinin (CK) treatment can significantly increase the total number of flowers per inflorescence, the female-to-male flower ratio, and the seed yield (Fröschle et al. 2017; Pan & Xu 2011).

CKs are important hormones in plants and participate in many biological processes, such as apical dominance (Shimizu-Sato et al. 2009; Tanaka et al. 2006), root proliferation (Kudo et al. 2010; Werner et al. 2003), reproductive development (Ashikari et al. 2005), and senescence (Gan & Amasino 1995). Endogenous CKs containing N6-(Δ^2- isopentenyl)-adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin (DZ), and their conjugates are known as isoprenoid CKs (Mok & Mok 2001).The major derivatives are generally iP- and tZ-type CKs (Sakakibara 2006).

CK biosynthesis and degradation pathways have been well studied in the past decade (Figure 1) (Galuszka et al. 2007; Kudo et al. 2010; Sakakibara 2006). The first step of iP and tZ biosynthesis is catalysed by adenosine phosphate-isopentenyltransferases (IPTs). IPTs produce iP-ribotides from dimethylallyl diphosphate (DMAPP) and adenosine 5'-diphosphate (ADP) or adenosine 5'-triphosphate (ATP) (Ihara et al. 1984; Taya et al. 1978). iP-ribotides can then be hydroxylated to tZ-ribotides by cytochrome P450 monooxygenase (CYP735A) (Takei et al. 2004). These cytokinin ribotides are converted to free-base CKs by cytokinin-activating enzymes LONELY GUYs (LOGs) (Kurakawa et al. 2007; Kuroha et al. 2009; Tokunaga et al. 2012). In addition, cZ and tZ can be enzymatically interconverted by zeatin *cis-trans* isomerase (Bassil et al. 1993; Sakakibara 2006). In *Arabidopsis*, IPT1 and IPT3 to IPT8 are involved in iP and tZ biosynthesis (Kakimoto 2001; Sun et al. 2003; Takei et al. 2001), while IPT2 and IPT9 are involved in cZ biosynthesis (Golovko et al. 2002). *CYP735A1* is abundant in roots and flowers in

71 *Arabidopsis*, while *CYP735A2* specifically accumulates in roots (Takei et al. 2004). *CYP735As*

72 are required for shoot growth (Kiba et al. 2013). Cytokinin oxidase/dehydrogenase (CKX)

73 catalyses the irreversible degradation of CKs (Galuszka et al. 2001; Galuszka et al. 2007;

74 Schmulling et al. 2003). *CKXs* play important roles in controlling CK levels in plant tissues. In

75 *Arabidopsis*, *CKX3* and *CKX5* regulate the activity of reproductive meristems (Bartrina et al.

76 2011). In rice, *OsCKX4* mediates crown root development by integrating cytokinin and auxin

77 signalling (Gao et al. 2014).

78 CKs play important roles in flower bud development and floral sex differentiation (Chandler

79 2011; Gerashchenkov & Rozhnova 2013; Yamasaki et al. 2005). However, the roles of CK

80 biosynthesis genes *IPTs* and *CYP735A* and catabolism gene *CKXs* in *Jatropha* are not clear. In

81 this study, we isolated sequences of cytokinin metabolic genes, including 6 *IPTs*, one

82 *JcCYP735A* and 7 *JcCKXs*, using the *Jatropha* Genome Database (Hirakawa et al. 2012; Sato et

83 al. 2010; Wu et al. 2015). The 14 genes showed different expression patterns in different tissues

84 of *Jatropha*. Some of them exhibited tissue-specific expression. *JcIPT6* was only expressed in

85 seeds a few days after pollination. *JcCYP735A* was highly expressed in roots and seeds. *JcCKX4*

86 was expressed mainly in seeds. In addition, CK types and contents were detected in flower buds

87 and seeds. With flower bud development iP-type CKs increased, while tZ-type CKs decreased.

88 With seed development, tZ-type CKs increased, while iP-type CKs decreased. The *Jccyp735a*

89 mutants were obtained by the clustered regularly interspaced short palindromic repeats (CRISPR)-

90 Cas9 system. Compared with the wild-type (WT) plants, the concentrations of tZ and tZR

91 decreased significantly in the *Jccyp735a* mutants, which showed severely retarded growth. These

92 results will be helpful for future studies of the functions of these genes and for improving

93 the biological characteristics of *Jatropha*.

94 **MATERIALS & METHODS**

95 **Plant materials and growth conditions**

96 Three-year-old *Jatropha* trees were grown in the field at Xishuangbanna Tropical Botanical

97 Garden of the Chinese Academy of Sciences, Mengla County, Yunnan Province, China (21°54'

98 N, 101°46' E; 580 m in altitude). The seedlings of WT and the T1 plants of *Jccyp735a* mutants

99 were grown in the greenhouse (28°C, 12 h light/ 12 h dark, 70% humidity). Flower buds, ovules,

100 and seeds at different developmental stages were collected in May to July 2015 for qRT-PCR

101 analysis of *JcIPT6* expression and quantification of cytokinin contents. All other samples used in

102 qRT-PCR expriments were collected at the same time in May 2015. Various plant tissue

103 samples, including lateral roots of 1-2 mm in diameter with fine-roots and root tips, shoot apex

104 of 0.3 cm in length from the top of shoots, stems of 1.5 cm in diameter, young leaf blades of 2

105 cm in length, mature leaf blades of 15 cm in length, flower buds of 0.3 cm in length, just-opened

106 female and male flowers, fruits of 15 days after pollination, and seeds of 30 days after

107 pollination, were harvested for qRT-PCR analysis. All tissues were immediately frozen in liquid

108 nitrogen and stored at -80°C until needed.

109 **Gene identification and isolation**

110 Sequences of orthologous *IPT*, *CYP735A* and *CKX* genes from *Arabidopsis* that were available

111 in the GenBank database were used as query sequences for BLAST analysis using GenBank, the

112 *Jatropha* Genome Database (http://www.kazusa.or.jp/*Jatropha*/index.html) and our *Jatropha*

113 transcriptome data (Chen et al. 2014; Pan et al. 2014). The full length of complementary DNA

114 (cDNA) and genomic DNA sequences of the *JcIPT*, *JcCYP735A*, and the *JcCKX* genes were

115 obtained by PCR amplification. The PCR products were subsequently cloned into the pGEM-T

116 vector (Promega Corporation, Madison, WI, United States) and sequenced. The GenBank

117 accession numbers for the nucleotide sequences of these genes are listed in Table S1. Primers

118 used in PCR are listed in Table S2.

119 **Sequence comparison and phylogenetic analysis**

120 Sequence chromatograms were examined and edited using Chromas Version 2.23

121 (http://technelysium.com.au/). Related sequences were identified with BLAST

122 (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was generated with MEGA 7.0

123 (http://www.megasoftware.net/) using the Poisson model with gamma-distributed rates and 1000

124 bootstrap replicates.

125 **Expression pattern analysis by qRT-PCR**

126 Total RNA was extracted from each tissue, and first-strand cDNA was synthesized with a

127 PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the

128 manufacturer's instructions. qRT-PCR was performed with LightCycler® 480 SYBR Green I

129 Master (Roche) on the Roche 480 Real-Time PCR Detection System (Roche Diagnostics). qRT-

130 PCR was performed with two independent biological replicates (tissue samples were harvested

131 from different plants) and three technical replicates for each sample. Data were analysed using

132 the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (Livak & Schmittgen 2001).

133 Expression levels of specific genes were normalized to that of the *actin* gene in *Jatropha* (Zhang

134 et al. 2013). Primers used in qRT-PCR are listed in Table S3.

135 **Quantification of cytokinin**

136 Cytokinin contents were determined by the Wuhan Greensword Creation Technology Co. Ltd.,

137 using a polymer monolith microextraction coupled with hydrophilic interaction chromatography-

138 tandem mass spectrometry method as described previously (Liu et al. 2010).

139 The leaves used to quantify the CKs were the third and fourth new leaves from four-month-old

140 WT and the T1 plants of *Jccyp735a* mutants. Three independent biological replicates and three

141 technical replicates were measured for each sample. The data were analyzed using the Statistical

142 Product and Service Solutions software (SPSS Inc., Chicago, IL, USA, version 16.0).

143 Differences among the means were determined using a one-way ANOVA with Tukey's or

144 Tamhane's post hoc tests ($p < 0.05$).

145 **Construction of CRISPR/Cas9 vectors and transformation of *Jatropha***

146 The sequence of *JcCYP735A* (GenBank accession no. XM_012222581.2) was analyzed with the

147 online tool CRISPR-P (http://cbi.hzau.edu.cn/crispr/) to find the target sites of CRISPR/Cas9.

148 pYLsgRNA-AtU3d/LacZ (GenBank accession no. KR029100) as the single-guide RNA

149 (sgRNA) intermediate plasmid, and pYLCRISPR/Cas9P$_{35S}$-N (GenBank accession no.

150 KR029112) as the binary vector were used for the CRISPR-Cas9 construction of *JcCYP735A*

151 following the instruction of the CRISPR-Cas9 System (Ma et al. 2015). Transformation of

152 *Jatropha* with *Agrobacterium* strain EHA105 carrying the *JcCYP735A* CRISPR/Cas9

153 construction was performed according to the protocol described by Fu et al. (2015). The

154 *Jccyp735a* mutants in transgenic *Jatropha* plants were identified by PCR amplification and DNA

155 sequencing using a pair of primers, XB619 (5'-ATGGCCATGATATTAACAACTCTATTAG-

156 3') and XB620 (5'-GCGGTTCTATCCCATTCCAGTATAT-3').

157   **RESULTS**

158   **Cloning and identification of *JcIPTs*, *JcCYP735A*, and *JcCKXs***

159   Using all annotated *Arabidopsis* IPT, CYP735A and CKX family members in the TAIR as query

160   sequences to perform a BLAST analysis in GenBank and with our *Jatropha* transcriptome data

161   (Chen et al. 2014; Pan et al. 2014), we identified and cloned *IPT*, *CYP735A* and *CKX*

162   orthologous sequences in *Jatropha*. The *Jatropha IPT* family included only six members, while

163   there are nine members in *Arabidopsis*. These genes were named *JcIPT1*, *JcIPT2*, *JcIPT3*,

164   *JcIPT5*, *JcIPT6*, and *JcIPT9*. The BLAST analysis identified only one member of the *CYP735A*

165   family, *JcCYP735A*. The *CKX* gene family, encoding degradation enzymes, included the same

166   seven members in *Jatropha* as in *Arabidopsis*. These genes were named *JcCKX1*, *JcCKX2*,

167   *JcCKX3*, *JcCKX4*, *JcCKX5*, *JcCKX6*, and *JcCKX7*.

168   Sequence structure analysis showed that *IPT*, *CYP735A*, and *CKX* family members shared almost

169   the same numbers of exons and introns between *Jatropha* and *Arabidopsis* and had similar exon

170   lengths (Figure 2, 3). *JcIPT6* has two more exons than *IPT6* from *Arabidopsis*. However, the

171   extra two exon sequences are short and are not part of the P-loop NTPase domain (Figure 2a).

172   **Phylogenetic analysis of JcIPTs, JcCYP735A, and JcCKXs**

173   To analyse the phylogenetic relationships between orthologous genes, phylogenetic analysis

174   were performed. IPT, CYP735A and CKX family members from *Arabidopsis thaliana*, *Ricinus*

175   *communis*, and *Oryza sativa* were compared with those from *Jatropha*. Orthologues of IPT1, 2,

176   3, 5, and 7 formed a clade, while IPT9 formed a single clade (Figure 4a). JcCYP735A along with

177   other dicotyledon CYP735As formed a clade, while CYP735A3 and 4 of *Oryza sativa* formed

178   another clade (Figure 4b). Orthologues of CKX1, 5, 6, and 7 formed a clade, while those of

179   CKX2, 3, and 4 formed a separate clade (Figure 5). These results showed that JcIPTs,

180   JcCYP735A, and JcCKXs were most closely related to genes from *Ricinus communis*, which

181   also belongs to the Euphorbiaceae family.

182   **Expression patterns of *JcIPTs*, *JcCYP735A*, and *JcCKXs* in different tissues**

183   In order to gain more information of these gene family members in *Jatropha*, the temporal and

184   spatial expression patterns of these genes were analysed using qRT-PCR. *JcIPT1* was mainly

185   expressed in roots (Figure 6a). *JcIPT2* was mainly expressed in roots, shoot apical meristems,

186 mature leaves, and flower buds (Figure 6b). *JcIPT3* showed much higher expression levels in

187 stems and mature leaves than other tissues (Figure 6c). *JcIPT5* exhibited high expression levels

188 in roots and mature leaves (Figure 6d). *JcIPT9* only showed high expression levels in mature

189 leaves (Figure 6f). The expression of *JcIPT6* was not detected in most of the plant tissues

190 indicated above. After analysing more tissues (Figure S1), we found that *JcIPT6* began to be

191 expressed in seeds a few days after fertilization, with the strongest expression observed in seeds

192 at 10 days after fertilization; the expression levels then decreased rapidly. In seeds at 20 days

193 after fertilization, *JcIPT6* expression decreased by a factor of 5 compared with seeds at 10 days

194 after fertilization (Figure 6e). The expression levels of *JcCYP735A* were higher in roots, flower

195 buds, and seeds than other tissues (Figure7a). *JcCKX1* was mainly expressed in flower buds,

196 roots, and female flowers (Figure 7b). *JcCKX2* showed very strong expression in female flowers

197 and seeds (Figure 7c). *JcCKX3* was highly expressed in male flowers (Figure 7d). *JcCKX4*

198 exhibited high expression levels in mature leaves and extremely high expression in seeds (Figure

199 7e). *JcCKX5* was mainly expressed in stems, young leaves, and fruit (Figure 7f). *JcCKX6* was

200 expressed in all tissues (Figure 7g). *JcCKX7* was mainly expressed in roots (Figure 7h).

201 **Endogenous CK contents in flower buds and seeds**

202 In order to learn more about the distribution of endogenous cytokinins, we measured the contents

203 of endogenous CKs in *Jatropha* flower buds and seeds at different developmental stages (Figure

204 S1). Different profiles were observed for each CK variant with flower bud and seed development

205 in *Jatropha* (Table 1). The contents of iP and its variant iPR increased approximately 177-fold

206 and 9-fold, respectively, from the flower bud 1 (FB1) stage to the flower bud 2 (FB2) stage.

207 Compared with the FB1 stage, tZ content was approximately doubled in the FB2 stage, while

208 tZR content was reduced by 93%, resulting in a decrease in the amount of total active tZ

209 variants. Conversely, the contents of tZ variants increased remarkably during seed development;

210 compared with ovules, the tZ content increased 187-fold and the tZR content increased 496-fold

211 in seeds at 20 DAP.

212 ***Jccyp735a* mutants generated by CRISPR-Cas9 system showed retarded growth**

213 To explore the biological function of *JcCYP735A* in *Jatropha*, we generated *Jatropha*

214 transformants with *JcCYP735A* knocked out using the CRISPR-Cas9 system (Figure 8). Three

215 homozygous mutant lines, L1, L2, and L3, were obtained by DNA sequencing (Figure 8a).

216   Endogenous contents of CKs in the leaves of two lines of *Jccyp735a* mutants (L2 and L3) and

217   WT plants were examined. The results showed that the concentrations of tZ and tZR, and cZ and

218   cZR significantly decreased, whereas the concentrations of iP and iPR significantly increased in

219   *Jccyp735a* mutants compared with that of the WT (Figure 8b). *Jccyp735a* mutants showed

220   severely retarded growth, and the mutant plants were only approximately a quarter the height of

221   the WT plants (Figure 8c and d).

222   **DISCUSSION**

223   In our study, six *IPT* family members were identified in *Jatropha*. The number of *IPT* genes

224   differs among plant species; for example, there are six *IPTs* in *Ricinus communis*, while there are

225   nine *IPTs* in both *Arabidopsis* (Kakimoto 2001; Takei et al. 2001) and rice (Figure 4a). *JcIPTs*

226   have the same number of exons as those in *Arabidopsis*, except *JcIPT6*, which has two more

227   exons than *AtIPT6*. The third exon of *JcIPT6* has almost the same number of base pairs as the

228   only exon in *AtIPT6* (Figure 2a). It appears that the other two small exons were lost during

229   evolution. Expression pattern analysis revealed that different *JcIPT* members are expressed in

230   different tissues in *Jatropha* (Figure 6). *JcIPT1* had an expression pattern similar to that of

231   *AtIPT1* and was mostly expressed in roots, shoot apical meristems, and seeds (Miyawaki et al.

232   2004). JcIPT2 and JcIPT9 were assigned to the same cluster as their orthologues (Figure 4a). In

233   *Arabidopsis, AtIPT2* and *AtIPT9* are expressed ubiquitously, with stronger expression in

234   proliferating tissues, including the root and shoot apical meristems and leaf primordia (Miyawaki

235   et al. 2004). Similarly, *JcIPT2* and *JcIPT9* were expressed ubiquitously in *Jatropha*. However,

236   the strongest expression of both was in mature leaves instead of meristems and young leaves

237   (Figure 6b, f). Considering that AtIPT2 and AtIPT9 are tRNA-IPTs, which only catalyse cZ

238   biosynthesis (Golovko et al. 2002), cZ might be produced mainly in mature leaves of *Jatropha*.

239   *AtIPT3* was found to be responsible for nitrate-dependent cytokinin biosynthesis and is

240   predominantly expressed in the phloem (Miyawaki et al. 2004; Takei et al. 2002). In our study,

241   *JcIPT3* was strongly expressed in stems and mature leaves, which contain abundant phloem

242   (Figure 6c). A study by Miyawaki et al. (2004) showed that GUS activity is not detected in

243   *Arabidopsis* transformants carrying *AtIPT6::GUS*. RT-PCR analysis indicated that it was

244   abundant in siliques. Analogously, we did not detect *JcIPT6* in most tissues of *Jatropha*,

245   including fruits. We found that *JcIPT6* was mainly expressed in seeds at 10 days after

246 pollination, and its expression decreased rapidly thereafter (Figure 6e). It is possible that the

247 previous study missed this critical phase in the seeds chosen for GUS staining (Miyawaki et al.

248 2004). Our results suggested that different *JcIPT* family members play different roles in the

249 development of *Jatropha* and that some *JcIPT* members could be used to cultivate high-yield

250 varieties of *Jatropha* using transgenic technology.

251 Although none of the ATP/ADP *JcIPTs* were found to be highly expressed in flowers, iP, iPR,

252 and tZ contents increased with the development of flower buds (Table 1). It is known that CKs

253 can be transported through the plant vascular system (Hirose et al. 2007). *JcCYP735A* was found

254 to be highly expressed in flower buds (Figure 7a). iP-type CKs may be transported into flowers,

255 and some may then be used to generate tZ-type CKs via JcCYP735A. In addition, it has been

256 reported that the tZ concentration is up-regulated in the early development of tomato fruits

257 (Matsuo et al. 2012). Our study showed that *JcCYP735A* was highly expressed and that the tZ

258 concentration increased with seed development in *Jatropha* (Table 1). Thus, *JcCYP735A* might

259 play important roles in seed development by controlling tZ biosynthesis. Similar to *Ricinus*

260 *communis* (Chan et al. 2010), only one *CYP735A* gene was found in *Jatropha*, although there are

261 two *CYP735A* members in *Arabidopsis* and rice (Takei et al. 2004; Tsai et al. 2012).

262 Unlike *JcIPT* or *JcCYP735A* family, the *JcCKX* family in *Jatropha* has the same number of

263 members as that in *Arabidopsis* (Figure 3). In addition, the *JcCKX* and *AtCKX* orthologues

264 contain the same number of exons, with 5 exons in *CKX1-6* and 4 exons in *CKX7* (Figure 3).

265 Further expression analysis showed that *JcCKX2* was mostly expressed in female flowers,

266 whereas *JcCKX3* was mostly expressed in male flowers (Figure 7c, d). Both *JcCKX2* and

267 *JcCKX4* were expressed strongly in seeds (Figure 7c, e). These tissue-specific expression genes

268 may be chosen to adjust the CK content in these tissues using transgenic methods. It has been

269 reported that reduced expression of *OsCKX2* causes cytokinin accumulation in inflorescence

270 meristems and increases the number of reproductive organs, resulting in enhanced grain yield

271 (Ashikari et al. 2005). Decreased expression of *CKX* orthologues may also lead to increased

272 yield in *Jatropha*. Moreover, overexpression of some *CKX* members can also improve resistance.

273 Overexpression of *CKX1* or *CKX2* in *Arabidopsis* and other species causes elongation of the

274 primary root and increases root branching (Galuszka et al. 2004; Mrízová et al. 2013; Pospisilova

275 et al. 2016; Werner et al. 2001; Yang et al. 2003), while overexpression of *AtCKX7* results in an

276 opposite phenotype (Kollmer et al. 2014). Specific expression of *JcCKX1* or *JcCKX2* in roots

277 might be used to transform a shallow root system into a deep root system to improve the growth

278 and lodging resistance of *Jatropha*. Furthermore, root system development might enhance

279 tolerance to drought stress. Remarkably, *JcCKX4* expression was much higher in seeds than

280 other tissues (Figure 7e), suggesting that JcCKX4 may be a key enzyme regulating cytokinin

281 levels to affect seed development.

282 In early flower bud development, the content of iP-type CKs increased significantly, while that

283 of tZ-type CKs decreased. This result indicated that iP-type CKs participate more in early flower

284 bud development than tZ-type CKs. In tomato, iP-type CK contents decrease during fruit

285 ripening (Matsuo et al. 2012). By contrast, in early seed development, the content of tZ-type CKs

286 increased substantially, while that of iP-type CKs decreased. This result, which is in accordance

287 with high expression of *JcCYP735A* in seeds, suggested that tZ-type CKs are dominant in early

288 seed development. Many differences were observed in CK contents in different periods of

289 *Jatropha* flower bud and seed development. Our results indicate that iP-type CKs can be used to

290 improve the number of flowers in *Jatropha*, while tZ-type CKs can be used to enlarge seeds.

291 The single *cyp735a1* or *cyp735a2* mutant *Arabidopsis* showed slight decreases in tZ and tZR

292 concentration compared with that of WT, while the double mutants showed great decreases

293 (Kiba et al. 2013). In addition, *cyp735a1 cyp735a2* double mutants exhibited retarded shoot

294 growth (Kiba et al. 2013). Similarly, in this study, *Jccyp735a* mutants showed substantial

295 decreases in tZ and tZR concentrations (Figure 8b), which is consistent with that only a single

296 member of *JcCYP735A* was found in *Jatropha* genome (Wu et al. 2015). We noticed, however,

297 tZ and tZR did not completely disappear in *Jccyp735a* mutants (Figure 8b), which may result

298 from conversion of cZ and cZR. This notion is supported by the fact that the concentrations of cZ

299 and cZR also significantly decreased in *Jccyp735a* mutants (Figure 8b), and that *cis-trans*

300 isomerase activity for interconversion between cZ-type and tZ-type CKs has been reported in

301 several plant species (Bassil et al. 1993; Kudo et al. 2012; Suttle & Banowetz 2000).

302 **CONCLUSIONS**

303 In this study, we isolated the members of the *JcIPT*, *JcCYP735A* and *JcCKX* gene families and

304 analysed their temporal and spatial expression patterns. Different family members exhibited

305 different expression patterns. Different types of CKs seemed to influence the development of

306 flower buds and seeds, respectively. The analysis of the *Jccyp735a* mutants revealed that

307 *JcCYP735A* plays an important role in tZ biosynthesis in *Jatropha*. These results will be helpful

308 for further function studies of cytokinin metabolic genes and improving agronomic

309 characteristics of *Jatropha* by genetic engineering of cytokinin metabolism.

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312 pYLCRISPR/Cas9P$_{35S}$-N and pYLsgRNA-AtU3d. The authors gratefully acknowledge the Central Laboratory of

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314 **Abbreviations**

315 BLAST   basic local alignment search tool
316 CK   cytokinin
317 CKX   cytokinin oxidase/dehydrogenase
318 CRISPR   clustered regularly interspaced short palindromic repeats
319 CYP735A   cytochrome P450 monooxygenase, family 735, subfamily A
320 cZ   *cis*-zeatin
321 DZ   dihydrozeatin
322 DZR   dihydrozeatin-riboside
323 iP   N6-(Δ2- isopentenyl)-adenine
324 IP9G   N6-(Δ2- isopentenyl)-adenine-9-glucoside
325 iPR   iP-riboside
326 IPT   adenosine phosphate-isopentenyltransferases
327 LOG   LONELY GUY
328 qRT-PCR   quantitative reverse transcriptase-polymerase chain reaction
329 tZ   *trans*-zeatin
330 tZ9G   *trans*-zeatin-9-glucoside
331 tZR   tZ-riboside

332

333 **REFERENCES**

334 Akashi K. 2012. Jatropha research: A new frontier for biofuel development. *Plant Biotechnol* 29:121.
335   10.5511/plantbiotechnology.12.0003p
336 Ashikari M, Sakakibara H, Lin S, Yamamoto T, Takashi T, Nishimura A, Angeles ER, Qian Q, Kitano H, and
337   Matsuoka M. 2005. Cytokinin oxidase regulates rice grain production. *Science* 309:741-745.
338 Bartrina I, Otto E, Strnad M, Werner T, and Schmulling T. 2011. Cytokinin regulates the activity of reproductive
339   meristems, flower organ size, ovule formation, and thus seed yield in *Arabidopsis thaliana*. *Plant Cell*

340   23:69-80. 10.1105/tpc.110.079079

341   Bassil NV, Mok DW, and Mok MC. 1993. Partial purification of a cis-trans-isomerase of zeatin from immature seed

342   of Phaseolus vulgaris L. *Plant Physiol* 102:867-872.

343   Chan AP, Crabtree J, Zhao Q, Lorenzi H, Orvis J, Puiu D, Melake-Berhan A, Jones KM, Redman J, and Chen G.

344   2010. Draft genome sequence of the oilseed species Ricinus communis. *Nat Biotechnol* 28:951.

345   Chandler J. 2011. The hormonal regulation of flower development. *J Plant Growth Regul* 30:242-254.

346   Chen M-S, Pan B-Z, Wang G-J, Ni J, Niu L, and Xu Z-F. 2014. Analysis of the transcriptional responses in

347   inflorescence buds of *Jatropha curcas* exposed to cytokinin treatment. *BMC Plant Biol* 14:318.

348   Francis G, Edinger R, and Becker K. 2005. A concept for simultaneous wasteland reclamation, fuel production, and

349   socio‐economic development in degraded areas in India: Need, potential and perspectives of *Jatropha*

350   plantations. Natural Resources Forum: Wiley Online Library. p 12-24.

351   Fröschle M, Horn H, and Spring O. 2017. Effects of the cytokinins 6-benzyladenine and forchlorfenuron on fruit-,

352   seed-and yield parameters according to developmental stages of flowers of the biofuel plant *Jatropha*

353   *curcas* L.(Euphorbiaceae). *Plant Growth Regul* 81:293-303.

354   Fu Q, Li C, Tang M, Tao Y-B, Pan B-Z, Zhang L, Niu L, He H, Wang X, and Xu Z-F. 2015. An efficient protocol

355   for Agrobacterium-mediated transformation of the biofuel plant Jatropha curcas by optimizing kanamycin

356   concentration and duration of delayed selection. *Plant Biotechnol Rep* 9:405-416.

357   Galuszka P, Frébort I, Šebela M, Sauer P, Jacobsen S, and Peč P. 2001. Cytokinin oxidase or dehydrogenase? *FEBS*

358   *J* 268:450-461.

359   Galuszka P, Frebortova J, Werner T, Yamada M, Strnad M, Schmulling T, and Frebort I. 2004. Cytokinin

360   oxidase/dehydrogenase genes in barley and wheat: cloning and heterologous expression. *FEBS J* 271:3990-

361   4002. 10.1111/j.1432-1033.2004.04334.x

362   Galuszka P, Popelková H, Werner T, Frébortová J, Pospíšilová H, Mik V, Köllmer I, Schmülling T, and Frébort I.

363   2007. Biochemical characterization of cytokinin oxidases/dehydrogenases from Arabidopsis thaliana

364   expressed in Nicotiana tabacum L. *Journal of Plant Growth Regulation* 26:255-267.

365   Gan S, and Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. *Science*

366   270:1986-1988.

367   Gao S, Fang J, Xu F, Wang W, Sun X, Chu J, Cai B, Feng Y, and Chu C. 2014. *CYTOKININ*

368   *OXIDASE/DEHYDROGENASE4* Integrates Cytokinin and Auxin Signaling to Control Rice Crown Root

369   Formation. *Plant Physiol* 165:1035-1046. 10.1104/pp.114.238584

370   Gerashchenkov G, and Rozhnova N. 2013. The involvement of phytohormones in the plant sex regulation. *Russ J*

371   *Plant Physiol* 60:597-610.

372   Golovko A, Sitbon F, Tillberg E, and Nicander B. 2002. Identification of a tRNA isopentenyltransferase gene from

373   *Arabidopsis thaliana*. *Plant Mol Biol* 49:161-169.

374   Hirakawa H, Tsuchimoto S, Sakai H, Nakayama S, Fujishiro T, Kishida Y, Kohara M, Watanabe A, Yamada M, and

375   Aizu T. 2012. Upgraded genomic information of Jatropha curcas L. *Plant Biotechnol* 29:123-130.

376   Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, and Sakakibara H. 2007. Regulation of cytokinin

377   biosynthesis, compartmentalization and translocation. *J Exper Bot* 59:75-83.

378   Ihara M, Taya Y, Nishimura S, and Tanaka Y. 1984. Purification and some properties of Δ^2-

379   isopentenylpyrophosphate: 5′ Amp Δ^2-isopentenyltransferase from the cellular slime mold *Dictyostelium*

380   *discoideum*. *Arch Biochem Biophys* 230:652-660.

Kakimoto T. 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol* 42:677-685.

Kiba T, Takei K, Kojima M, and Sakakibara H. 2013. Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. *Dev Cell* 27:452-461. 10.1016/j.devcel.2013.10.004

Kollmer I, Novak O, Strnad M, Schmulling T, and Werner T. 2014. Overexpression of the cytosolic cytokinin oxidase/dehydrogenase (CKX7) from Arabidopsis causes specific changes in root growth and xylem differentiation. *Plant J: for Cell and Molecular Biology* 78:359-371. 10.1111/tpj.12477

Kudo T, Kiba T, and Sakakibara H. 2010. Metabolism and long-distance translocation of cytokinins. *J Integr Plant Biol* 52:53-60. 10.1111/j.1744-7909.2010.00898.x

Kudo T, Makita N, Kojima M, Tokunaga H, and Sakakibara H. 2012. Cytokinin activity of *cis*-zeatin and phenotypic alterations induced by overexpression of putative *cis*-zeatin-*O*-glucosyltransferase in rice. *Plant Physiol* 160:319-331. 10.1104/pp.112.196733

Kumar A, and Sharma S. 2008. An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. *Ind Crops Prod* 28:1-10. 10.1016/j.indcrop.2008.01.001

Kumar Tiwari A, Kumar A, and Raheman H. 2007. Biodiesel production from jatropha oil (*Jatropha curcas*) with high free fatty acids: An optimized process. *Biomass and Bioenerg* 31:569-575. 10.1016/j.biombioe.2007.03.003

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, and Kyozuka J. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445:652-655. 10.1038/nature05504

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, and Sakakibara H. 2009. Functional analyses of *LONELY GUY* cytokinin-activating enzymes reveal the importance of the direct activation pathway in *Arabidopsis*. *Plant Cell* 21:3152-3169. 10.1105/tpc.109.068676

Liu Z, Wei F, and Feng Y-Q. 2010. Determination of cytokinins in plant samples by polymer monolith microextraction coupled with hydrophilic interaction chromatography-tandem mass spectrometry. *Anal Methods* 2:1676-1685.

Livak KJ, and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402-408.

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, and Lin Y. 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol Plant* 8:1274-1284.

Makkar HP, and Becker K. 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value‐added coproducts. *Eur J Lipid Sci Technol* 111:773-787.

Matsuo S, Kikuchi K, Fukuda M, Honda I, and Imanishi S. 2012. Roles and regulation of cytokinins in tomato fruit development. *J Exper Bot* 63:5569-5579.

Miyawaki K, Matsumoto‐Kitano M, and Kakimoto T. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J* 37:128-138.

Mok DW, and Mok MC. 2001. Cytokinin metabolism and action. *Annu Rev Plant Biol* 52:89-118.

Mrízová K, Jiskrová E, Vyroubalová Š, Novák O, Ohnoutková L, Pospíšilová H, Frébort I, Harwood WA, and Galuszka P. 2013. Overexpression of cytokinin dehydrogenase genes in barley (Hordeum vulgare cv.

422    Golden Promise) fundamentally affects morphology and fertility. *PLoS One* 8:e79029.

423 Pan B-Z, Chen M-S, Ni J, and Xu Z-F. 2014. Transcriptome of the inflorescence meristems of the biofuel plant

424    *Jatropha curcas* treated with cytokinin. *BMC genomics* 15:974. 10.1186/1471-2164-15-974

425 Pan B-Z, and Xu Z-F. 2011. Benzyladenine treatment significantly increases the seed yield of the biofuel plant

426    Jatropha curcas. *Plant Growth Regul* 30:166-174.

427 Pospisilova H, Jiskrova E, Vojta P, Mrizova K, Kokas F, Cudejkova MM, Bergougnoux V, Plihal O, Klimesova J,

428    Novak O, Dzurova L, Frebort I, and Galuszka P. 2016. Transgenic barley overexpressing a cytokinin

429    dehydrogenase gene shows greater tolerance to drought stress. *New Biotechnol* 33:692-705.

430    10.1016/j.nbt.2015.12.005

431 Rao GR, Korwar GR, Shanker AK, and Ramakrishna YS. 2008. Genetic associations, variability and diversity in

432    seed characters, growth, reproductive phenology and yield in *Jatropha curcas* (L.) accessions. *Trees*

433    22:697-709. 10.1007/s00468-008-0229-4

434 Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. *Annu Rev Plant Biol* 57:431-449.

435 Sato S, Hirakawa H, Isobe S, Fukai E, Watanabe A, Kato M, Kawashima K, Minami C, Muraki A, and Nakazaki N.

436    2010. Sequence analysis of the genome of an oil-bearing tree, *Jatropha curcas* L. *DNA Res* 18:65-76.

437 Schmulling T, Werner T, Riefler M, Krupkova E, and Bartrina y Manns I. 2003. Structure and function of cytokinin

438    oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. *J Plant Res* 116:241-252.

439    10.1007/s10265-003-0096-4

440 Shimizu-Sato S, Tanaka M, and Mori H. 2009. Auxin–cytokinin interactions in the control of shoot branching. *Plant*

441    *Mol Biol* 69:429.

442 Sun J, Niu Q-W, Tarkowski P, Zheng B, Tarkowska D, Sandberg G, Chua N-H, and Zuo J. 2003. The Arabidopsis

443    *AtIPT8/PGA22* gene encodes an isopentenyl transferase that is involved in de novo cytokinin biosynthesis.

444    *Plant Physiol* 131:167-176.

445 Suttle JC, and Banowetz G. 2000. Changes in cis‐zeatin and cis‐zeatin riboside levels and biological activity

446    during potato tuber dormancy. *Physiologia Plantarum* 109:68-74.

447 Takei K, Sakakibara H, and Sugiyama T. 2001. Identification of genes encoding adenylate isopentenyltransferase, a

448    cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J Biol Chem* 276:26405-26410.

449    10.1074/jbc.M102130200

450 Takei K, Takahashi T, Sugiyama T, Yamaya T, and Sakakibara H. 2002. Multiple routes communicating nitrogen

451    availability from roots to shoots: a signal transduction pathway mediated by cytokinin. *J Exper Bot* 53:971-

452    977.

453 Takei K, Yamaya T, and Sakakibara H. 2004. *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin

454    hydroxylases that catalyze the biosynthesis of *trans*-Zeatin. *J Biol Chem* 279:41866-41872.

455    10.1074/jbc.M406337200

456 Tanaka M, Takei K, Kojima M, Sakakibara H, and Mori H. 2006. Auxin controls local cytokinin biosynthesis in the

457    nodal stem in apical dominance. *Plant J* 45:1028-1036.

458 Taya Y, Tanaka Y, and Nishimura S. 1978. 5′-AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*.

459    *Nature* 271:545-547.

460 Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, and Sakakibara H. 2012. Arabidopsis lonely guy

461    (LOG) multiple mutants reveal a central role of the LOG‐dependent pathway in cytokinin activation.

462    *Plant J* 69:355-365.

463 Tsai Y-C, Weir NR, Hill K, Zhang W, Kim HJ, Shiu S-H, Schaller GE, and Kieber JJ. 2012. Characterization of
464   genes involved in cytokinin signaling and metabolism from rice. *Plant Physiol* 158:1666-1684.

465 Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, and Schmulling T. 2003. Cytokinin-deficient
466   transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of
467   cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15:2532-2550.
468   10.1105/tpc.014928

469 Werner T, Motyka V, Strnad M, and Schmulling T. 2001. Regulation of plant growth by cytokinin. *Proc Natl Acad*
470   *Sci U S A* 98:10487-10492. 10.1073/pnas.171304098

471 Wu P, Zhou C, Cheng S, Wu Z, Lu W, Han J, Chen Y, Chen Y, Ni P, Wang Y, Xu X, Huang Y, Song C, Wang Z,
472   Shi N, Zhang X, Fang X, Yang Q, Jiang H, Chen Y, Li M, Wang Y, Chen F, Wang J, and Wu G. 2015.
473   Integrated genome sequence and linkage map of physic nut (*Jatropha curcas* L.), a biodiesel plant. *Plant*
474   *Journal* 81:810-821. 10.1111/tpj.12761

475 Yamasaki S, Fujii N, and Takahashi H. 2005. Hormonal regulation of sex expression in plants. *Vitam Horm* 72:79-
476   110.

477 Yang S, Yu H, Xu Y, and Goh CJ. 2003. Investigation of cytokinin‐deficient phenotypes in *Arabidopsis* by ectopic
478   expression of orchid *DSCKX1*. *FEBS Lett* 555:291-296. 10.1016/s0014-5793(03)01259-6

479 Zhang L, He L-L, Fu Q-T, and Xu Z-F. 2013. Selection of reliable reference genes for gene expression studies in the
480   biofuel plant *Jatropha curcas* using real-time quantitative PCR. *International Journal of Molecular*
481   *Sciences* 14:24338-24354. 10.3390/ijms141224338

482

483 **Figure legends**

484 **Figure 1 Basic scheme for the cytokinin biosynthesis and degradation pathways.** Solid arrows indicate

485 pathways with genes that are known, and dotted arrows indicate pathways with genes that remain to be identified.

486 The enzymes are marked by red frames. The iP, Z and their ribosides inside the dotted boxes could be degraded by

487 CKX. cZ, *cis*-zeatin; DMAPP, dimethylallyldiphosphate; CKX, cytokinin oxidase/dehydrogenase; cZ, *cis*-zeatin;

488 DMAPP, dimethylallyl diphosphate; iP, N6-(Δ2- isopentenyl)adenine; IPT, adenosine phosphate-

489 isopentenyltransferase; LOG, LONELY GUY; tRNA-IPT, tRNA-isopentenyltransferase; tZ, *trans*-zeatin; Z, zeatin.

490 This figure was modified and redrawn from reference (Kudo et al. 2010).

491 **Figure 2 Genomic organization of *IPT* and *CYP735A* family members in *Jatropha* and *Arabidopsis.*** (a) *IPT*

492 family members; (b) *CYP735A* family members. At, *Arabidopsis thaliana*; Jc, *Jatropha curcas*.

493

494 **Figure 3 Genomic organization of *CKX* family members in *Jatropha* and *Arabidopsis.*** At, *Arabidopsis*

495 *thaliana*; Jc, *Jatropha curcas*.

496

497 **Figure 4 Neighbour-joining phylogenetic tree for IPT and CYP735A family members in various species.**

498 (a) IPT family members; (b) CYP735A family members. At, *Arabidopsis thaliana*; Jc, *Jatropha curcas*; Os,

499 *Oryza sativa*; Rc, *Ricinus communis.*

500

501 **Figure 5 Neighbour-joining phylogenetic tree for CKX family members in various species.** At,

502 *Arabidopsis thaliana*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Rc, *Ricinus communis.*

503

504 **Figure 6 Expression of *JcIPTs* in various *Jatropha* tissues.** (a) to (f) are expression patterns

505 of *JcIPT1*, *JcIPT2*, *JcIPT3*, *JcIPT5*, *JcIPT6*, and *JcIPT9*, respectively. The qRT-PCR results were obtained

506 from two independent biological replicates and three technical replicates for each sample. R, roots; SAM, shoot

507 apical meristems; ST, stems; YL, young leaves; ML, mature leaves; FB, flower buds; FF, female flowers; MF,

508 male flowers; FRU, fruits; SD, seeds; 0 DAP, unfertilized ovules; 10 DAP, seeds at 10 days after pollination;

509 20 DAP, seeds at 20 days after pollination; 30 DAP, seeds at 30 days after pollination; DAP, days after

510 pollination.

511

512 **Figure 7 Expression of *JcCYP735A* and *JcCKXs* in various *Jatropha* tissues.** (a) to (h) are expression

513 patterns of *JcCYP735A*, *JcCKX1*, *JcCKX2*, *JcCKX3*, *JcCKX4, JcCKX5, JcCKX6, and JcCKX7*, respectively.

514 Values in the Y- axis of (e) are displayed in scientific notation. e, exponent. The qRT-PCR results were

515 obtained from two independent biological replicates and three technical replicates for each sample. R, roots;

516 SAM, shoot apical meristems; ST, stems; YL, young leaves; ML, mature leaves; FB, flower buds; FF, female

517 flowers; MF, male flowers; FRU, fruits; SD, seeds.

518

519 **Figure 8 Generation and phenotypic variation of *Jccyp735a* mutants of *Jatropha* obtained by the**

520 **CRISPR-Cas9 system.** (a) Different types of *JcCYP735A* mutation generated by the CRISPR-Cas9-

521 mediated gene silencing in the progenies (T1 generation) of the three transgenic lines L1, L2, and L3. The

522 blue characters show the selected target site sequences. The characters in purple box show the protospacer

523 adjacent motif (PAM) sequences. The red character indicates the nucleotide insertion. WT, wild-type. (b-d)

524 Cytokinin concentrations (b), the heights (c), and appearance (d) of the four-month-old seedlings (T1) of

525 *Jccyp735a* mutant lines and WT. Error bars represent the standard deviation (SD) of three (b) or five (c)

526 biological replicates. Asterisks indicate statistically significant differences compared with WT (p < 0.05). In

527 (d), bar = 5 cm.

528

529 **Additional files**

530 **Supplemental Figures**

531 **Figure S1 Flower buds, ovules and seeds of *Jatropha* used in this study**. A, Flower buds from the FB1 stage.

532 Bar = 1 cm. B, Flower buds from the FB2 stage. Bar = 1 cm. C, Unfertilized *Jatropha* pistil, from which the

533 ovule was obtained. Bar = 1 mm. D, Morphology of unfertilized ovules. Bar = 1 mm. E, Morphology of fruits

534 and seeds. The first row contains fruits at different developmental periods. The second row shows the seeds of

535 the corresponding fruits. Bar = 1 cm. 0 DAP, unfertilized ovules; 10 DAP, seeds at 10 days after pollination;

536 20 DAP, seeds at 20 days after pollination; 30 DAP, seeds at 30 days after pollination; DAP, days after

537 pollination.

Figure 1

Basic scheme for the cytokinin biosynthesis and degradation pathways.

Solid arrows indicate pathways with genes that are known, and dotted arrows indicate pathways with genes that remain to be identified. The enzymes are marked by red frames. The iP, Z and their ribosides inside the dotted boxes could be degraded by CKX. cZ, *cis*-zeatin; DMAPP, dimethylallyldiphosphate; CKX, cytokinin oxidase/dehydrogenase; cZ, *cis*-zeatin; DMAPP, dimethylallyl diphosphate; iP, N6-(Δ2- isopentenyl)adenine; IPT, adenosine phosphate-isopentenyltransferase; LOG, LONELY GUY; tRNA-IPT, tRNA-isopentenyltransferase; tZ, *trans*-zeatin; Z, zeatin. This figure was modified and redrawn from reference (Kudo et al. 2010).

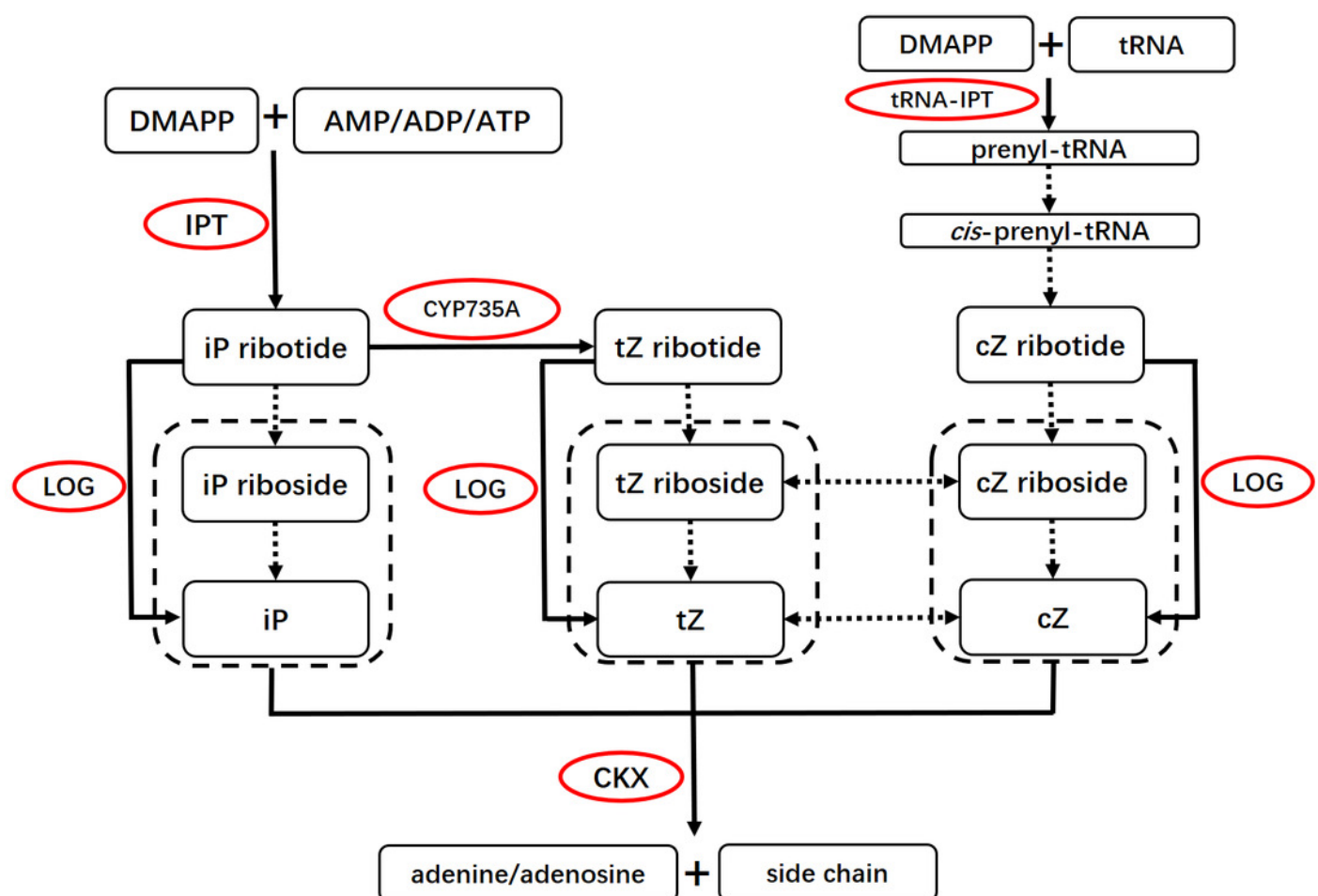



Figure 2

Genomic organization of *IPT* and *CYP735A* family members in *Jatropha* and *Arabidopsis.*

(a) *IPT* family members; (b) *CYP735A* family members. At, *Arabidopsis thaliana*; Jc, *Jatropha curcas.*

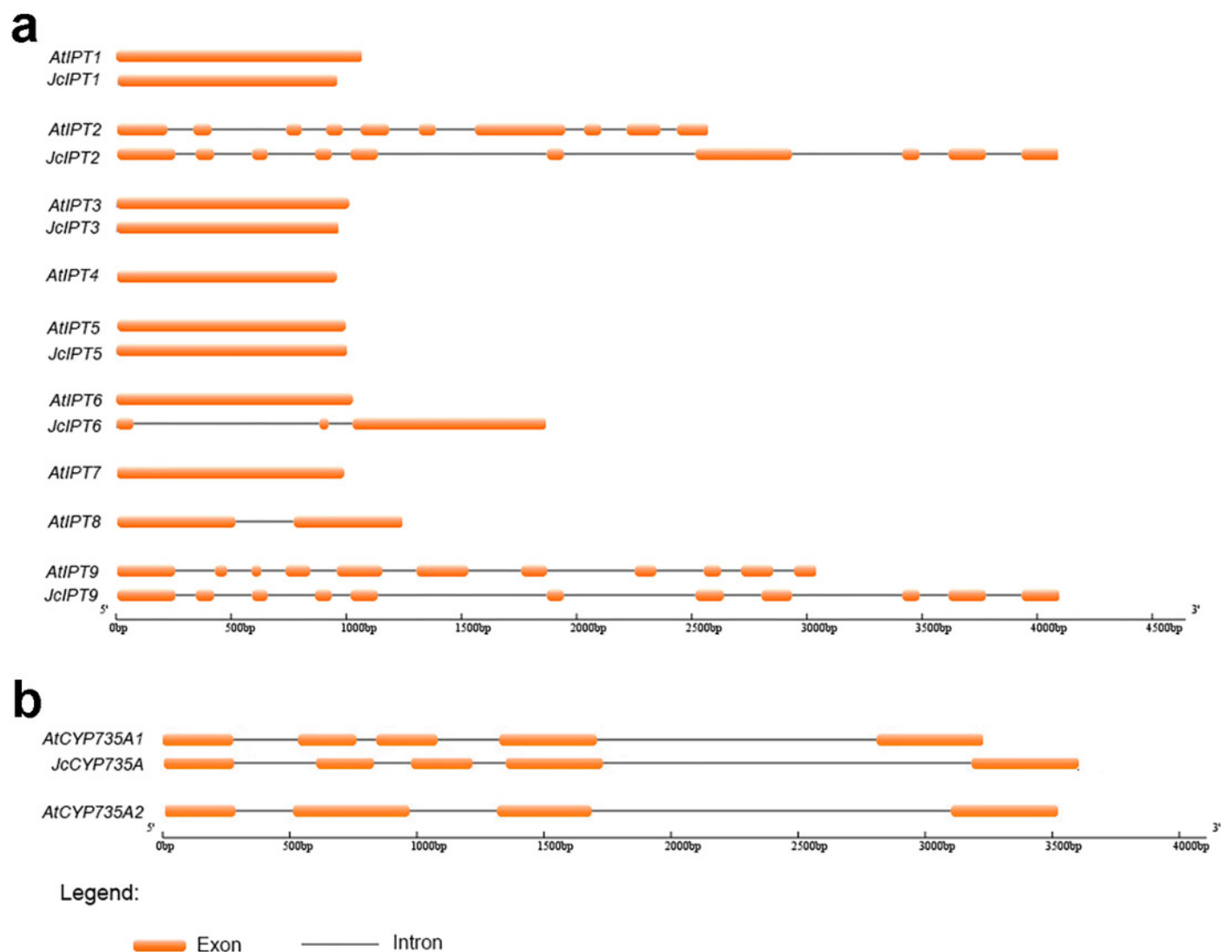



Figure 3

Genomic organization of *CKX* family members in *Jatropha* and *Arabidopsis.*

At, *Arabidopsis thaliana*; Jc, *Jatropha curcas.*

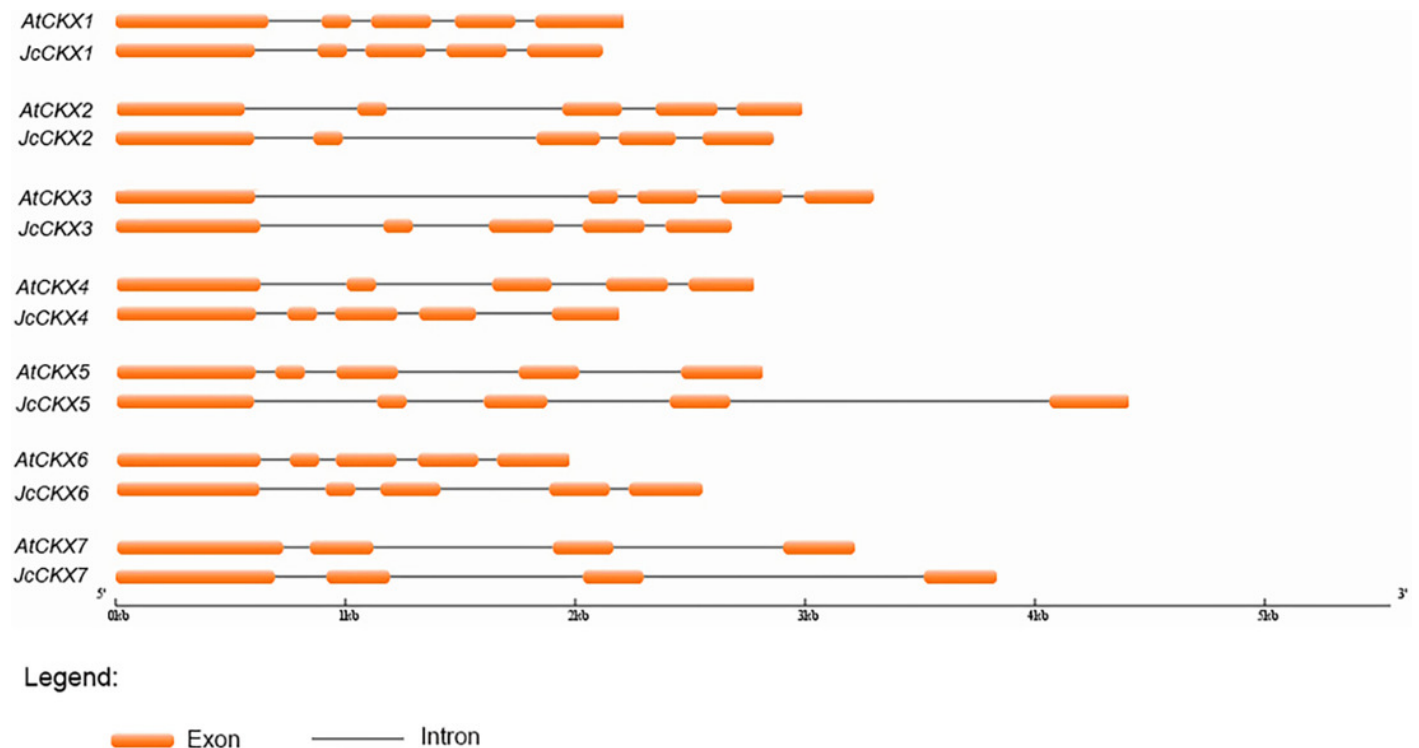



Figure 4

Neighbour-joining phylogenetic tree for IPT and CYP735A family members in various species.

(a) IPT family members; (b) CYP735A family members. At, *Arabidopsis thaliana*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Rc, *Ricinus communis.*

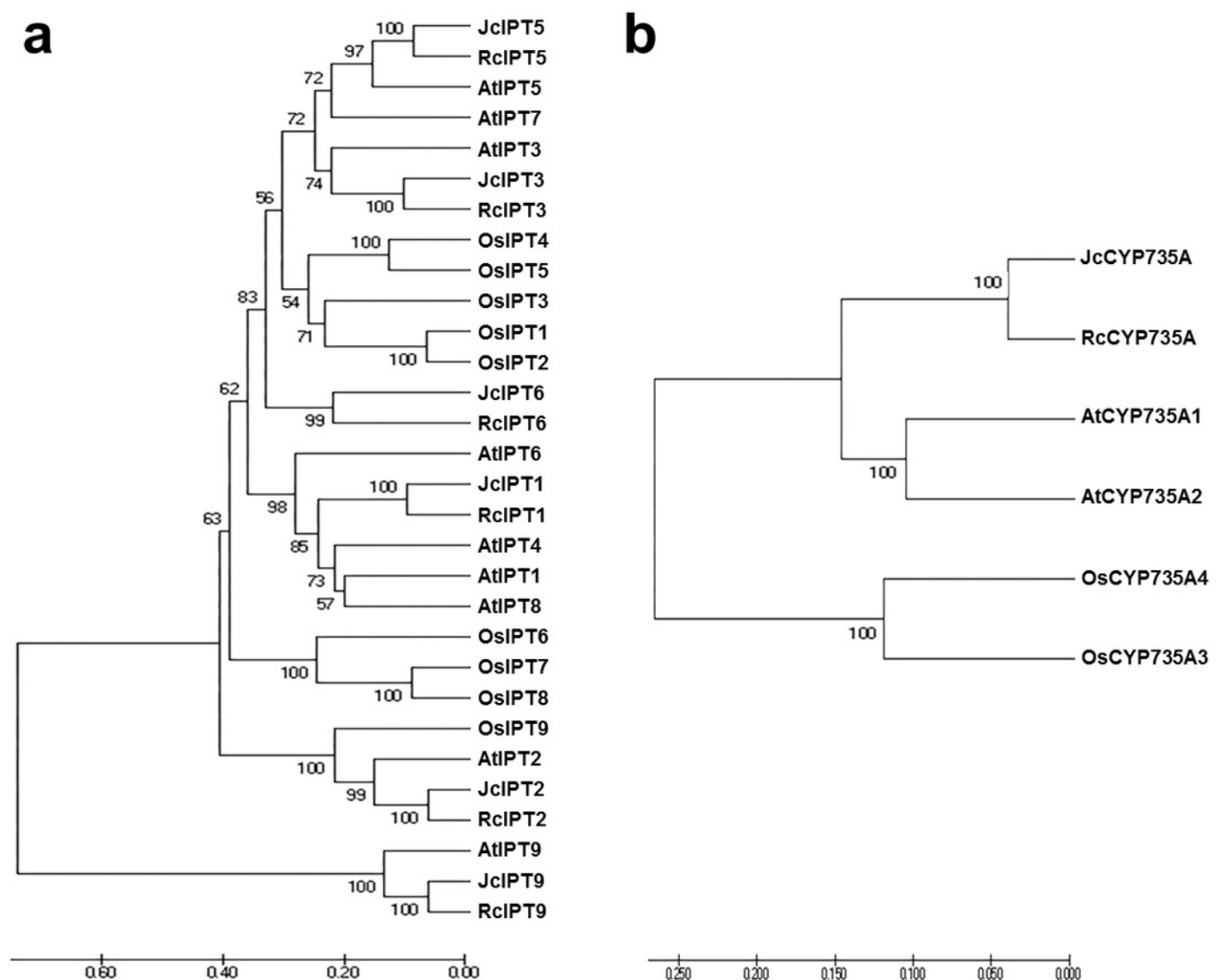



Figure 5

Neighbour-joining phylogenetic tree for CKX family members in various species.

At, *Arabidopsis thaliana*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Rc, *Ricinus communis.*

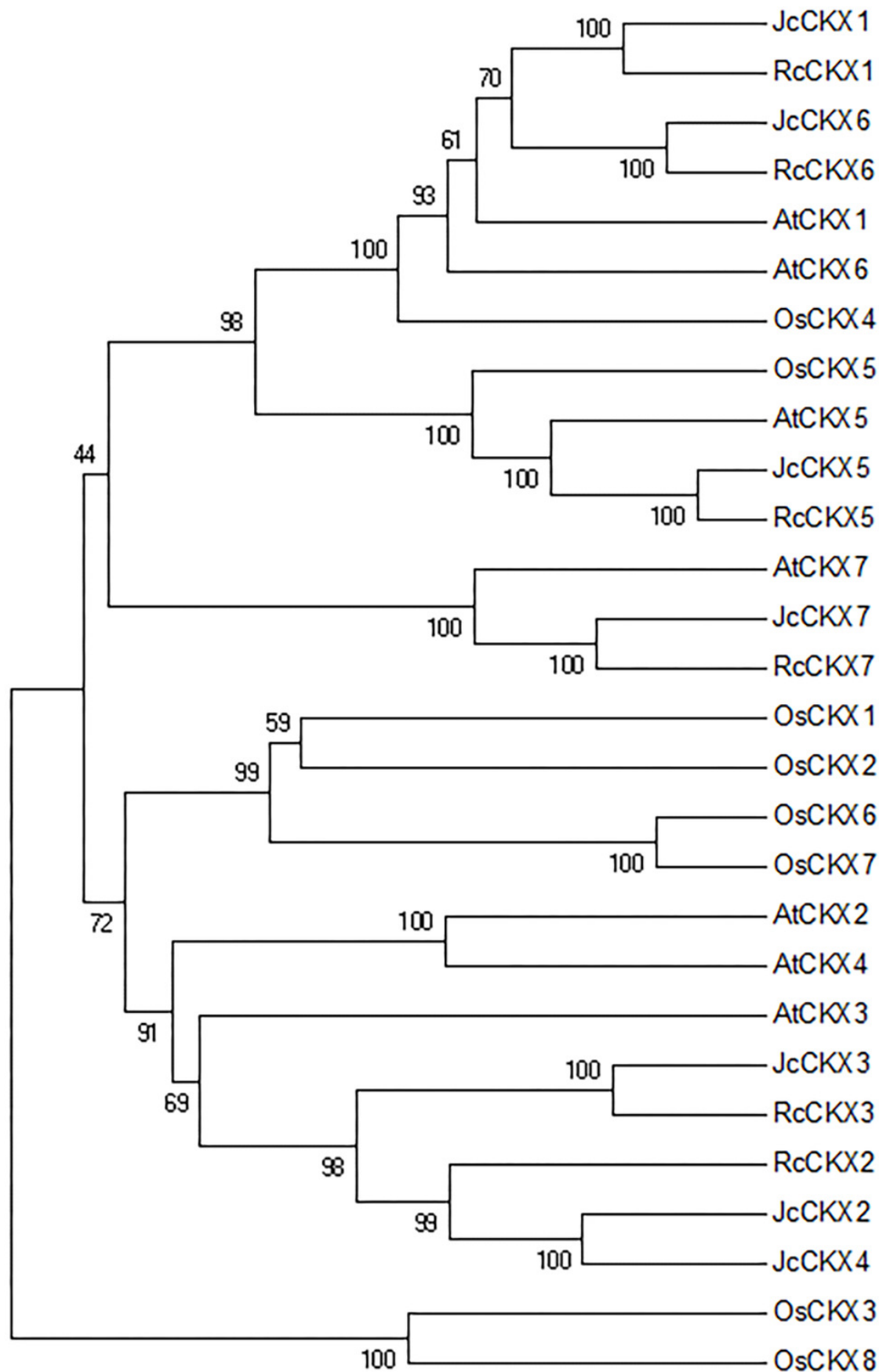



Figure 6

Expression of *JcIPTs* in various *Jatropha* tissues.

(a) to (f) are expression patterns of JcIPT1 , JcIPT2 , JcIPT3 , JcIPT5 , JcIPT6 , and JcIPT9 , respectively. The qRT-PCR results were obtained from two independent biological replicates and three technical replicates for each sample. R, roots; SAM, shoot apical meristems; ST, stems; YL, young leaves; ML, mature leaves; FB, flower buds; FF, female flowers; MF, male flowers; FRU, fruits; SD, seeds; 0 DAP, unfertilized ovules; 10 DAP, seeds at 10 days after pollination; 20 DAP, seeds at 20 days after pollination; 30 DAP, seeds at 30 days after pollination; DAP, days after pollination.

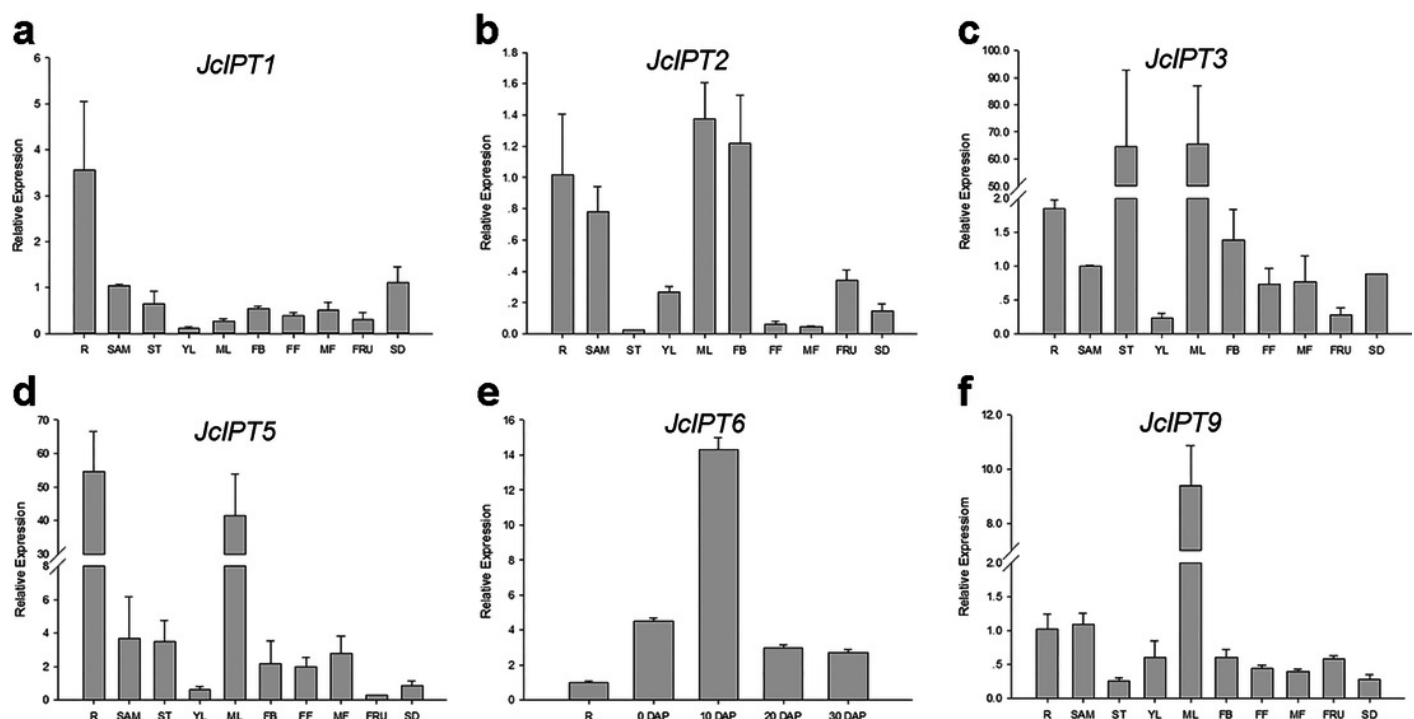



Figure 7

Expression of *JcCYP735A* and *JcCKXs* in various *Jatropha* tissues.

(a) to (h) are expression patterns of JcCYP735A , JcCKX1 , JcCKX2 , JcCKX3 , JcCKX4, JcCKX5, JcCKX6, and JcCKX7 , respectively. Values in the Y- axis of (e) are displayed in scientific notation. e, exponent. The qRT-PCR results were obtained from two independent biological replicates and three technical replicates for each sample. R, roots; SAM, shoot apical meristems; ST, stems; YL, young leaves; ML, mature leaves; FB, flower buds; FF, female flowers; MF, male flowers; FRU, fruits; SD, seeds.

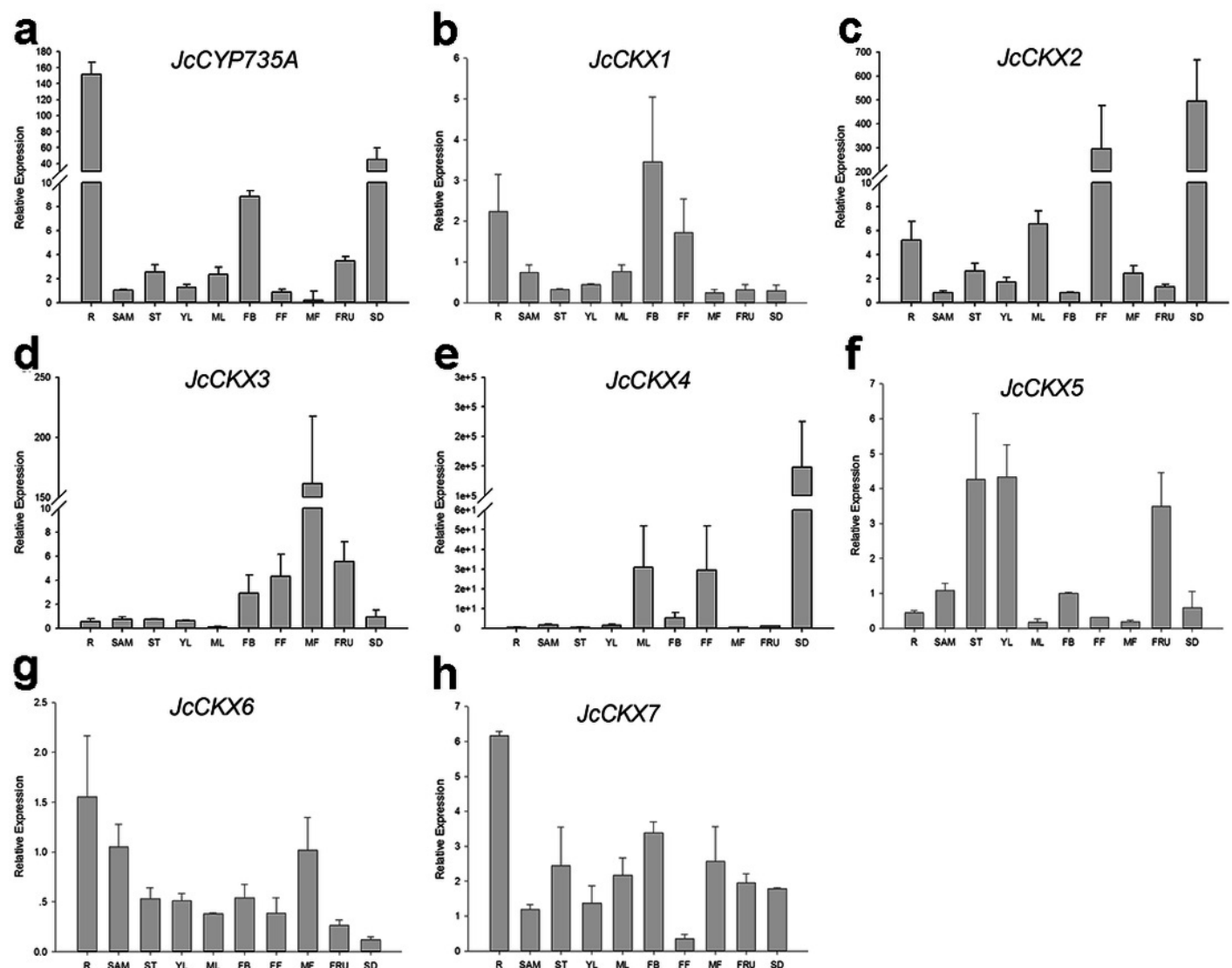
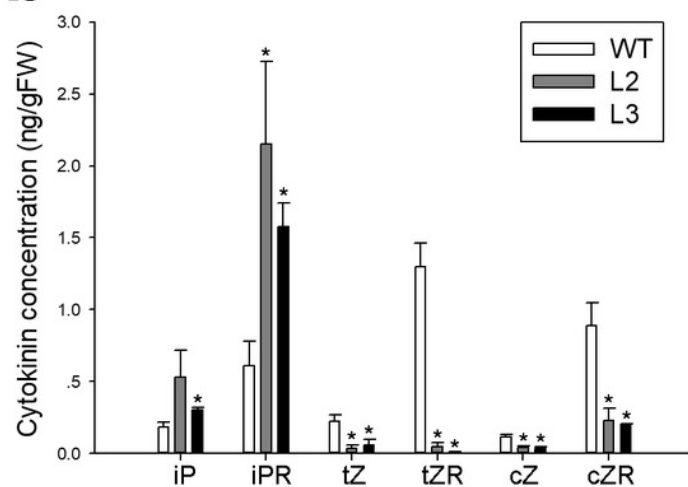
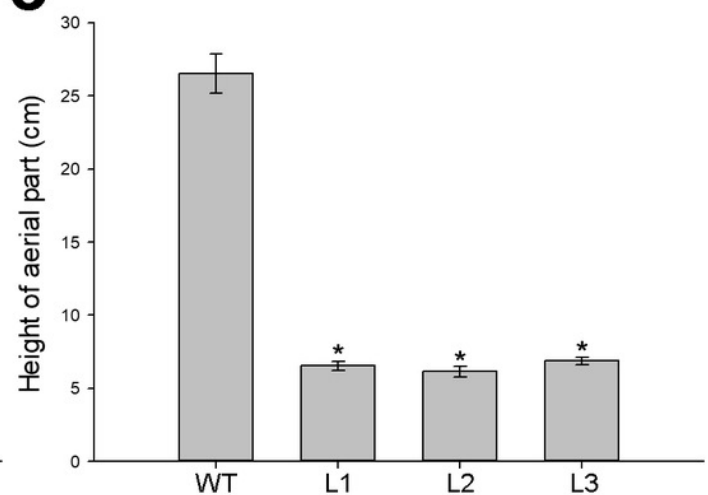
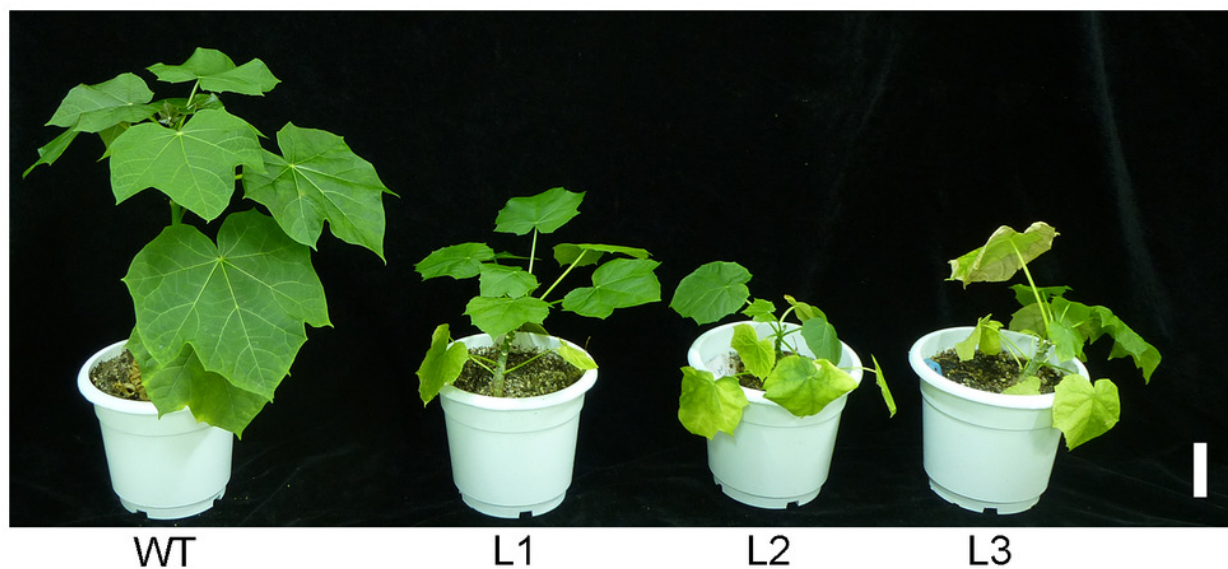



Figure 8

Generation and phenotypic variation of *Jccyp735a* mutants of *Jatropha* obtained by the CRISPR-Cas9 system.

(a) Different types of *JcCYP735A* mutation generated by the CRISPR-Cas9-mediated gene silencing in the progenies (T1 generation) of the three transgenic lines L1, L2, and L3. The blue characters show the selected target site sequences. The characters in purple box show the protospacer adjacent motif (PAM) sequences. The red character indicates the nucleotide insertion. WT, wild-type. (b-d) Cytokinin concentrations (b), the heights (c), and appearance (d) of the four-month-old seedlings (T1) of *Jccyp735a* mutant lines and WT. Error bars represent the standard deviation (SD) of three (b) or five (c) biological replicates. Asterisks indicate statistically significant differences compared with WT ($p < 0.05$). In (d), bar = 5 cm.

a

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JcCYP735A
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                    241 bp                                        280 bp
                    5'- ACCTCGCCCTCTTACCGGAAACATCTTAGACGTGACCTCT -3'
                    3'- TGGAGCGGGAGAATGGCCTTTGTAGAATCTGCACTGGAGA -5'
                        PAM
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```
WT    GCGTGGCCCTGAACCTCGCCC-TCTTACCGGAAACATCTTAGACGTGACCTCTTTTGTCTCCCA
L1    GCGTGGCCCTGAACCTCGCC-----TACCGGAAACATCTTAGACGTGACCTCTTTTGTCTCCCA
L2    GCGTGGCCCTGAACCTCGCCCCTCTTACCGGAAACATCTTAGACGTGACCTCTTTTGTCTCCCA
L3    GCGTGGCC------------------------------------------TCTTTTGTCTCCCA
```

Legend:

━━━ Exon   ──── Intron

b

c

d

Content of endogenous CKs in flower buds and seeds of *Jatropha* in different developmental stages (ng/gFW).

FB1, flower buds of less than 5 mm in length; FB2, flower buds of 2-3 cm in length; 0 DAP, unfertilized ovule; 10 DAP, seeds of 10 days after pollination; 20 DAP, seeds of 20 days after pollination; DAP, days after pollination; N.D., not detected; N.Q., not quantified.

1   **Table 1.** Content of endogenous CKs in flower buds and seeds of *Jatropha* in different developmental stages
2   (ng/gFW).

Samples	iP	iPR	iP9G	tZ	tZR	tZ9G	DZ	DZR
FB1	0.14±0.01	1.50±0.03	N.D.	1.21±0.03	12.55±0.78	N.Q.	1.27±0.09	9.24±0.15
FB2	24.86±0.83	15.24±0.24	N.D.	2.49±0.17	0.89±0.08	N.D.	3.31±0.19	0.97±0.03
0 DAP	3.97±0.40	1.20±0.07	N.D.	0.79±0.08	0.08±0.007	N.D.	0.5±0.06	N.D.
10 DAP	3.96±0.19	0.71±0.02	N.D.	22.61±2.42	1.56±0.09	0.06±0.001	6.42±0.24	0.18±0.02
20 DAP	0.31±0.01	0.78±0.05	N.D.	149.28±11.60	39.76±1.80	0.75±0.03	28.90±2.68	16.56±0.86

3   FB1, flower buds of less than 5 mm in length; FB2, flower buds of 2-3 cm in length; 0 DAP, unfertilized ovule;
4   10 DAP, seeds of 10 days after pollination; 20 DAP, seeds of 20 days after pollination; DAP, days after
5   pollination; N.D., not detected; N.Q., not quantified.