Silencing of the Ortholog of \textit{DEFECTIVE IN ANther DEHISCENCE 1} Gene in the Woody Perennial \textit{Jatropha curcas} Alters Flower and Fruit Development

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Abstract: \textit{DEFECTIVE IN ANther DEHISCENCE 1} (DAD1), a phospholipase A1, utilizes galactolipids (18:3) to generate $\alpha$-linolenic acid (ALA) in the initial step of jasmonic acid (JA) biosynthesis in \textit{Arabidopsis thaliana}. In this study, we isolated the \textit{JcDAD1} gene, an ortholog of \textit{Arabidopsis DAD1} in \textit{Jatropha curcas}, and found that it is mainly expressed in the stems, roots, and male flowers of \textit{Jatropha}. \textit{JcDAD1}-RNAi transgenic plants with low endogenous jasmonate levels in inflorescences exhibited more and larger flowers, as well as a few abortive female flowers, although anther and pollen development were normal. In addition, fruit number was increased and the seed size, weight, and oil contents were reduced in the transgenic \textit{Jatropha} plants. These results indicate that \textit{JcDAD1} regulates the development of flowers and fruits through the JA biosynthesis pathway, but does not alter androecium development in \textit{Jatropha}. These findings strengthen our understanding of the roles of JA and DAD1 in the regulation of floral development in woody perennial plants.

Keywords: DAD1; flower and fruit development; physic nut; jasmonic acid; RNA interference

1. Introduction

Jasmonic acid (JA) is one of several major phytohormones that plays a pivotal role in modulating plant growth and development, as well as in responding to various abiotic and biotic stresses [1–4]. JA is synthesized from $\alpha$-linolenic acid (ALA) via the octadecanoid pathway [5,6]. The initial step of JA biosynthesis is the release of ALA from fatty acids in \textit{Arabidopsis} chloroplasts, which is performed by two types of enzymes, fatty acid desaturases (FADs) and phospholipase A1 (PLA1) [7]. FADs convert diunsaturated fatty acids (18:2) to triunsaturated fatty acids (18:3), whereas PLA$_1$ proteins, including DONGLE (DGL), \textit{DEFECTIVE IN ANther DEHISCENCE 1} (DAD1), and other lipases, hydrolyze the triunsaturated fatty acids in glycerides and phospholipids to produce free ALA [7]. Three sequential steps are catalyzed by 13-lipoxygenases (13-LOXs), allene oxide synthase (AOS), and allene oxide cyclase (AOC): oxygenation of ALA to 13-hydroperoxylinoleic acid (13-HPOT), dehydrogenation of 13-HPOT to the unstable compound 12,13-epoxyoctadecatrienoic acid (12,13-EOT), and cyclization of 12,13-EOT to 9(S,13S)-12-oxo-phytodienoic acid (OPDA), respectively [8–13]. OPDA is synthesized in chloroplasts, transported to the peroxisomes by the transport protein peroxisomal ABC transporter 1 (PXA1) [14] and then converted to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic
acid (OPC-8:0) by 12-oxo-phytodienoic acid reductase (OPR) [12]. Subsequently, OPC-8:0 in peroxisomes is converted to JA by three cycles of β-oxidation [15–17]. Eventually, JA is catalyzed via jasmonic acid carboxyl methyltransferase (JMT) to methyl jasmonate (MeJA) or by jasmonic acid-amino acid synthetase to jasmonic acid-amino acids, such as jasmonic acid-isoleucine (JA-Ile) [18].

The DAD1 gene encodes a chloroplastic glycerolipid lipase that belongs to the PLA₁ family and acts in the initial step of JA biosynthesis [19–21]. In Arabidopsis, the defective in anther dehiscence 1 (dad1) mutant displays a male sterility phenotype resulting from decreased endogenous JA levels, deficient filament elongation, nonviable pollen, and abnormal anther dehiscence, all of which can be rescued by exogenous treatment with linolenic acid or methyl jasmonate [22]. Arabidopsis plants with mutations in genes encoding key enzymes in JA biosynthesis (e.g., fad3−2 fad7−2 fad8, lox3 lox4, aos) exhibit similar phenotypes [23–25]. Transgenic Arabidopsis overexpressing DAD1 shows various pale color phenotypes because of the destruction of chloroplasts caused by the excessive accumulation of DAD1 protein in these organelles [22,26]. Antisense suppression of DADI in Brassica rapa induces male sterility and delays or inhibits flower opening [27]. In rice (Oryza sativa), mutation of EXTRA GLUME 1 (EG1), which is a homolog of Arabidopsis DAD1, causes a decrease in the endogenous JA level, resulting in a changed spikelet morphology that includes altered floral organ number, increased glume-like structures and defective floral meristem determinacy [28]. In maize (Zea mays), Tasselseed1 (TS1) encodes a plastid-targeted 13-LOX that acts in JA biosynthesis [29,30], and two oxophytodienoate reductase genes, OPR7 and OPR8, also participate in JA production [31]. Mutations of either TS1 or OPR7/OPR8 lead to the conversion of tassel inflorescence from staminate to pistillate [28,30–32]. These results indicate that JA plays diverse roles in flower development and sex determination. The functions of DAD1 have been successively revealed in herbaceous plants [19,33–37] but are still unknown in woody perennial plants.

Jatropha curcas L., a woody perennial plant, is considered an important biofuel plant with economic value because it contains a high content of seed oil (30–40%) that can be processed into biodiesel and aviation oil [38–41]. Typically, Jatropha is monoecious, with separate female and male flowers on the same inflorescence [42,43]. In a previous study, we speculated that JcDAD1, an ortholog of Arabidopsis thaliana DAD1, might participate in the abortion of male flowers during the transition from monoecy to gynoecy in Jatropha [44]. In this study, we repressed expression of JcDAD1 by gene silencing to investigate its function in the regulation of flower and fruit development in Jatropha.

2. Results

2.1. Characterization of the JcDAD1 Gene

We obtained a JcDAD1 cDNA library (GenBank accession no. 105643375) from the Jatropha transcriptome [44]. The lengths of the JcDAD1 genomic sequence and coding sequence (CDS) are 1537 bp and 1323 bp, respectively (http://jcdb.xtbg.ac.cn/). The JcDAD1 gene, which encodes 440 amino acids, is located on the fifth chromosome of Jatropha [45]. Similar to Arabidopsis and rice, JcDAD1 possesses only one exon accompanied by no introns (Figure 1A), indicating that DAD1 is evolutionarily conserved among these plants. Phylogenetic analysis showed that JcDAD1 has a close relationship with HbDAD1 and RcDAD1 (Figure 1B). In addition, the JcDAD1 protein containing a phospholipase A1 domain belongs to a member of the alpha/beta-hydrolase superfamily of proteins, which is similar to that of Arabidopsis [46].

The tissue-specific expression analysis showed that JcDAD1 is primarily expressed in the stems, roots, and male flowers but has low expression levels in other tissues of Jatropha (Figure 2). In Arabidopsis, expression of DAD1 is highly restricted to the filaments of stamens, which is consistent with the function of JA in promoting water transport by synchronizing anther dehiscence, pollen maturation, and flower opening [22]. In rice, expression of the EG1 gene (an ortholog of DAD1) is high in inflorescence primordia, but weak in developing floral primordia, which is in accordance with its role in early flower development [28,47]. However, the transcript of tomato LeLID1 (a homolog of Arabidopsis
DAD1) is hardly detected in reproductive organs (buds, flowers, or fruits) but strongly expressed in germinating seedlings, where the encoded protein functions as a TAG lipase [48]. These results show that DAD1 genes have various expression patterns in different species, indicating that they may have different functions.

Figure 1. Structural and phylogenetic analysis of DAD1 genes among *Jatropha* and other species. (A) Genomic organization of DAD1. (B) Phylogenetic analysis of deduced DAD1 proteins. EG1, a rice ortholog of *Arabidopsis* DAD1. Al, *Arabidopsis lyrata*; At, *Arabidopsis thaliana*; Cp, *Carica papaya*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Hb, *Hevea brasiliensis*; Jc, *Jatropha curcas*; Os, *Oryza sativa*; Re, *Ricinus communis*. The GenBank accession numbers in the phylogenetic tree are listed in Supplementary Table S1.

Figure 2. Tissue-specific expression analysis of JcDAD1 in different tissues of adult *Jatropha* plants. Three biological replicates and three technical replicates were prepared for qRT-PCR. JcActin was used as the internal reference. Error bars represent standard errors (n = 3). FF, female flowers; IF, inflorescences; MF, male flowers; ML, mature leaves; P, pericarps; R, roots; Se, seeds; St, stems; YL, young leaves.

2.2. JcDAD1 Gene Silencing Increased Inflorescence Branching, Flower Number, and Flower Size

To investigate the functionality of the JcDAD1 gene, we transformed the JcDAD1-RNAi construct into *Jatropha* plants and identified 16 independent JcDAD1-RNAi transgenic lines (T1 generation), in which the transcript of JcDAD1 is repressed in inflorescence buds (Figure 3). The order of inflorescence
branches was increased to the fifth-order branches in the transgenic inflorescence, whereas the wild type (WT) inflorescence had only fourth-order branches (Figure 4). Compared with the WT plants, notable increases in the female flower number (approximately three-fold) and male flower number (three- to five-fold) per inflorescence were observed in the transgenic Jatropha plants (Figure 5A–F), which is similar to the phenotype of the Arabidopsis dad1 mutant [22]. The flower size of the transgenic plants was conspicuously larger than that of the WT plants (Figure 6A–L), and the average diameters of the transgenic female and male flowers increased by 2–4 mm (Figure 6M). These results show that JcDAD1 participates in flower development and promotes flower production and floral organ growth in Jatropha.

![Figure 3](image-url)  
**Figure 3.** Relative expression levels of JcDAD1 in inflorescence buds of the wild type (WT) plants and JcDAD1-RNAi transgenic Jatropha lines (L2, L3, L9, L10, and L12). Two biological replicates and three technical replicates were prepared for qRT-PCR assays. JcActin was used as the internal reference. Error bars represent standard errors (n = 2). Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (p < 0.01). * Significant difference (p < 0.05).

![Figure 4](image-url)  
**Figure 4.** Comparison of inflorescence branching in the JcDAD1-RNAi transgenic plants with that in the wild type (WT) plants. The graph depicts inflorescence branching in WT plants (A) and the transgenic lines L2 (B), L3 (C), and L10 (D). The numbers (1–5) represent different orders of branching. Bars = 2.0 cm.
Figure 5. Number of flowers per inflorescence was increased in the JcDAD1-RNAi transgenic plants. (A–D) Inflorescences of wild-type (WT) plants (A), and inflorescences of transgenic lines L2 (B), L3 (C), and L10 (D); bars = 1.0 cm. (E,F) Comparison of female (E) and male (F) flower number per inflorescence in the transgenic lines (L2, L3 and L10) with those in the WT plants, \( n \geq 8 \). Error bars represent the standard deviations. Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (\( p < 0.01 \)).

Figure 6. Comparison of the flower morphology between the wild-type (WT) and JcDAD1-RNAi transgenic plants. (A) Female flowers in the WT (left) and transgenic line L2 (right). (B,C) Female flowers of the transgenic lines L3 and L10, respectively. (D) Male flowers of the WT (left) and transgenic line L2 (right) \textit{Jatropha} plants. (E,F) Male flowers of the transgenic lines L3 and L10, respectively. (G) Anatomy of female flowers of the WT (left) and transgenic line L2 (right). (H) and (I) Anatomy of the female flowers of the transgenic lines L3 and L10, respectively. (J) Anatomy of the male flowers of the WT (left) and transgenic line L2 (right). (K,L) Anatomy of the male flowers of the transgenic lines L3 and L10, respectively. Bars = 0.5 cm shown in (A–F); bars = 1.0 cm shown in (G–L). (M) Statistics of female and male flower size in the WT and transgenic JcDAD1-RNAi plants, \( n \geq 31 \). Pe, petal; Pi, pistil; Se, sepal; St, stamen. Error bars represent the standard deviations. Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (\( p < 0.01 \)).
2.3. JcDAD1 Gene Silencing Caused the Abortion of Some Female Flowers but Did Not Affect Anther Dehiscence

At the late stage of flower development (in the inflorescences of 21–30 days after emergence), a portion of flowers were abortive and most were female flowers in the transgenic inflorescences, while flower development was normal in the WT inflorescences (Figure 7A–L). Abortion might be caused by a deficiency of the nutrient supply. In Arabidopsis, the dad1 mutant displays a defect in anther dehiscence [22], although obvious phenotypic changes in anthers and pollens were not observed in the transgenic Jatropha (Figure 8). These results suggest that DAD1 may play different roles in the regulation of anther dehiscence and pollen development in Arabidopsis and Jatropha.

Figure 7. Abortion of florets in transgenic Jatropha plants. (A,B) Inflorescences of the wild type (WT) and transgenic line L10, respectively. The red arrow refers to the normal floret in (A) or the abnormal florets in (B). (C,D) Normal female flowers of WT and transgenic L10 Jatropha, respectively. (E) Abortive female flowers of transgenic L10 Jatropha. (F–H) Anatomy of female flowers from (C–E). (I) Normal male flowers in the WT (left) and transgenic Jatropha L10 (right). (J) Abortive male flowers of the transgenic Jatropha L10. (K,L) Anatomy of the male flowers from (I,J), respectively. Bars = 1.0 cm shown in (A,B); bars = 1.0 mm shown in (C–L).
2.4. JcDAD1 Gene Silencing Affected Jatropha Yield Traits

Compared with that of WT Jatropha, the fruit number per infructescence in the transgenic plants was increased by 2–3-fold (Figure 9A–E). The transgenic Jatropha plants produced smaller fruit and seeds in length and width (Figure 10A–G), and had a lighter ten-seed weight (Figure 10H), and lower seed oil content (Figure 10I). The results indicate that JcDAD1 regulates fruit development and therefore affects the traits of seeds in Jatropha.
Figure 9. Fruit number per infructescence was increased in the JcDAD1-RNAi transgenic plants. (A–D) Infructescences of the wild type (WT) and transgenic lines L2, L3, and L10, respectively; scale bars = 5.0 cm. (E) Comparison of the fruit number per infructescence between the WT and transgenic lines, n ≥ 5. Error bars represent the standard deviations. Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (p < 0.01).

Figure 10. Decrease in the fruit size, seed size, seed weight, and seed oil content in the JcDAD1-RNAi transgenic plants. (A) Fruits from the wild type (WT) and transgenic Jatropha plants. The red line with the double-headed arrow represents the fruit length (31.69 mm), and the cyan line represents the fruit width (27.62 mm). (B,C) Seed length and width in the WT and transgenic Jatropha plants. (D,E) Fruit length and width in the WT and transgenic Jatropha plants, n ≥ 34. (F,G) Seed length and width in the WT and transgenic Jatropha plants, n ≥ 120. (H) Ten-seed weight in the WT and transgenic Jatropha plants, n ≥ 14. (I) Seed oil content in the WT and transgenic Jatropha plants, n ≥ 14. Scale bars = 2.0 cm. L2, L3 and L10 represent different transgenic lines. Error bars represent standard deviations. Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (p < 0.01).
2.5. JcDAD1 Gene Silencing Reduced Endogenous JA and JA-Ile Contents in Jatropha Inflorescences

To determine whether the endogenous jasmonate contents in the transgenic Jatropha plants were affected by JcDAD1, two types of jasmonate (JA and the bioactive form JA-Ile) were measured in developing inflorescences (15–20 days after emergence) from the WT and transgenic Jatropha plants. Based on the results, the concentrations of both JA and JA-Ile were significantly decreased in the transgenic Jatropha inflorescences (Figure 11), indicating that JcDAD1 is a key positive regulator of JA biosynthesis.

![Figure 11](image-url) Contents of JA (A) and JA-Ile (B) in early developing inflorescences from the wild type (WT) and the transgenic Jatropha plants. FW, fresh weight; JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine. Error bars represent the standard deviations (n ≥ 3). Student’s t-test was performed to analyze significant differences. ** Extremely significant difference (p < 0.01).

3. Discussion

JA, a pivotal phytohormone, plays diversified functions in inflorescence and flower development [49]. The initial step of JA biosynthesis is catalyzed by DAD1 [19–21,50]. The Arabidopsis dad1 mutant generally displayed male sterility and abnormal anther dehiscence that can be rescued by exogenous methyl jasmonate (MeJA) treatment [22]. Similarly, antisense inhibition of BrDAD1 also resulted in male sterility in Brassica rapa [27]. Compared with the male sterile phenotypes in Arabidopsis and Brassica, EG1, an ortholog of Arabidopsis DAD1, controlled both empty glume fate and spikelet development in rice [22,27,28,47,49]. In this study, JcDAD1 silencing caused a decrease of endogenous JA levels and an increase in flower number and flower size in the JcDAD1-RNAi transgenic inflorescences (Figures 3, 5, 6 and 11), suggesting that JcDAD1 regulates flower development by controlling JA levels in Jatropha inflorescences. However, the JcDAD1-RNAi transgenic plants did not exhibit obviously abnormal androecium (Figure 8), which is not consistent with that in Arabidopsis, thus implying that JA could play different roles in regulating androecium development in Jatropha and Arabidopsis. Furthermore, the phenotype of male flowers showed increased size but normal fertility in the transgenic plants, which is inconsistent with the speculation that JcDAD1 might contribute to male abortion in gynoecious Jatropha plants [44]. It is possible that JcDAD1 may function in adjusting the balance between reproductive development and stress response via the JA synthetic pathway because DAD1 can simultaneously act in flower development and wound defense and inhibit pathogen infection [7,22]. In general, cell size or cell number, which are mainly controlled by the genes involved in the biosynthesis or signal transduction of auxin, ethylene, cytokinin, and brassinosteroid, determine flower size in the plant kingdom [51,52]. An increase in flower size in JcDAD1-RNAi transgenic Jatropha plants (Figure 6) suggests that JA might participate in the regulation of flower size in woody perennial plants. In the transgenic Jatropha plants, some female flowers and a few male flowers were abortive at the early stage of inflorescence development (Figure 7), which is likely caused by insufficient nutrient supply due to the generation of more flowers in a single inflorescence.
In summary, this study suggests that DADI genes are involved in the JA biosynthesis pathway and play diverse roles in regulating flower development among different species.

To investigate whether abnormal flower phenotypes of the transgenic plants can be rescued by exogenous jasmonate treatment, JA (2.5 or 5 mM) and MeJA (200 or 400 µM) solutions were sprayed onto the emerging transgenic inflorescence buds in the field. Unfortunately, whether JA/MeJA treatment could recover flowers in the transgenic inflorescences to normal size or normal flower number could not be clarified. Given the lack of control of environmental cues in the field trials, an optimized scheme will be designed for conducting experiments in future studies. Treatment with exogenous gibberellic acid (GA) was found to increase the number of female flowers in *Jatropha* [53–55], similar to the phenotypes of the *JcDAD1*-RNAi transgenic plants (Figure 5E). Moreover, both exogenous benzyladenine (BA) treatment and flower-specific overproduction of endogenous cytokinins (CKs) promoted the total flower number and female flower number in *Jatropha* [38,56], which again resembled the phenotypes of the *JcDAD1*-RNAi transgenic plants (Figure 5E,F). These results indicate that JA may act together with GA and/or CK to regulate flower development in *Jatropha*. However, the underlying molecular mechanism remains unclear and needs further investigation in the future.

4. Materials and Methods

4.1. Plant Materials

Two-year-old WT and *JcDAD1*-RNAi transgenic *Jatropha* plants were cultivated on the hillside land of an experimental field located in the Xishuangbanna Tropical Botanical Garden (XTBG; 21°54’ N, 101°46’ E; 580 m in altitude) of the Chinese Academy of Sciences, Menglun County, Yunnan Province, Southwest China [38]. Inflorescences from the WT and transgenic T1 generation plants were collected for morphologic observation. Roots, stems, young leaves, mature leaves, developing inflorescences (15–20 days after emergence), inflorescence buds, female flowers, male flowers, pericarps, and seeds were harvested from WT or transgenic plants, immediately frozen in liquid nitrogen and then stored at −80 °C for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis.

4.2. Sequence Alignment and Phylogenetic Analysis

The gene structures of DADI in different species were analyzed by the Gene Structure Display Server (GSDS2.0, Center for Bioinformatics, Peking University, China) with default settings [57]. The deduced DADI amino acid sequences for phylogenetic analysis from NCBI databases were used. A phylogenetic tree was constructed with the neighbor-joining statistical method, the Poisson model, and the bootstrap method applied in 1000 replications via MEGA (version 7.0) software (http://www.megasoftware.net/).

4.3. Isolation of *JcDAD1* cDNA

Total RNA was extracted from *Jatropha* leaves using the pBIOZOL Plant Total RNA Extraction Reagent (BioFlux, Hangzhou, China) following the manufacturer’s instructions. Then, first-strand cDNA was synthesized with 1.0 µg of the extracted RNA using the TAKARA PrimeScript™ RT Reagent Kit (TAKARA, Dalian, China). A 153 bp fragment (TCCGTCAATCAGATGGAGATACGTGCTTAGCTCGTGACATGTGGCCACGTCTCTACTCGCTTCTGTAACCAACT) was PCR-amplified from the synthesized cDNA using primers carrying appropriate restriction enzyme cutting sites (all primers used in this study are listed in Supplementary Table S2). The PCR products were subsequently cloned into a pEASY-Blunt Zero cloning vector (TransGen Biotech, Beijing, China) with the appropriate restriction enzymes and sequenced.
4.4. RNAi Silencing Vector Construction and Transformation

To construct the JcDAD1-RNAi expression vector, the sense and antisense fragments of JcDAD1 were cloned into the pJL10 binary vector [58] in opposing orientations on either side of a pdk intron to produce an invert repeat driven by the 35S promoter. The expression vector was transformed into Jatropha with Agrobacterium strain EHA105 as described previously [59]. The positive transgenic plants were confirmed by PCR and qRT-PCR.

4.5. Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed using SYBR® Premix Ex Taq™ II (TAKARA, Dalian, China) on a Roche 480 Real-Time PCR Detection System (Roche Diagnostics, Mannheim, Germany). At least two biological replicates and three technical replicates for all samples were applied in the qRT-PCR analysis. We used the 2^−∆∆CT method described by Livak and Schmittgen [60] to analyze the data. The JcActin gene was used to normalize the transcript levels of specific genes of Jatropha.

4.6. Phenotypic Analysis of Flowers

The phenotypes of heterozygous (T1 generation) transgenic Jatropha plants were analyzed using a 3D Super Depth digital microscope (Smart Zoom 5, Carl Zeiss, Germany).

4.7. Quantitation of the JA and JA-Ile Contents

The 15- to 20-day-old inflorescences of the JcDAD1-RNAi transgenic and WT plants were harvested, frozen rapidly in liquid nitrogen and stored at −80 °C for measuring the JA and JA-Ile contents. The measurement method was described previously [61].

5. Conclusions

Silencing of JcDAD1 reduced endogenous jasmonate contents in inflorescences, increased the size and number of flowers, and caused the abortion of a few female flowers in Jatropha. Furthermore, the JcDAD1-RNAi transgenic Jatropha plants displayed increases in fruit number and decreases in seed size, weight and oil contents. However, compared with Arabidopsis, anther and pollen development in the JcDAD1-RNAi transgenic Jatropha plants was normal. These results indicate that JcDAD1 participates in JA biosynthesis and acts in regulating flower and fruit development in Jatropha. JcDAD1 is also highly expressed in the stems and roots, implying that JcDAD1 not only plays important roles in reproductive growth, but may also have undiscovered roles in vegetative growth. Since synergy may occur among JA, GA, and/or CK in regulating flower development of Jatropha, interactions of JA and other phytohormones to control flower development in Jatropha will be investigated in further work.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/23/8923/s1.

Author Contributions: C.-J.X. and M.-L.Z. contributed equally to this study. M.-S.C. and Z.-F.X. conceived the study; C.-J.X. and M.-L.Z. designed and executed experiments; C.-J.X. analyzed data and prepared figures and tables. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.
Abbreviations

aos allene oxide synthase

DAD1 DEFECTIVE IN ANther DEHISCENCE 1

EG1 EXTRA GLume 1

fad fatty acid desaturase

JA jasmonic acid

JA-Ile jasmonic acid-isoleucine

JcDAD1-RAI Jatropha curcas DEFECTIVE IN ANther DEHISCENCE 1-RNA interference

lox lipoxygenase

qRT-PCR transcriptase-polymerase chain reaction

References

1. Šimura, J.; Antoniadi, I.; Širôká, J.; Tarkowská, D.; Strnad, M.; Ljung, K.; Novák, O. Plant Hormonomics: Multiple Phytohormone Profiling by Targeted Metabolomics. Plant Physiol. 2018, 177, 476–489. [CrossRef]

2. Balfagóñ, D.; Sengupta, S.; Gómez-Cadenas, A.; Fritschi, F.B.; Azad, R.K.; Mittler, R.; Zandalinas, S.I. Jasmonic Acid Is Required for Plant Acclimation to a Combination of High Light and Heat Stress. Plant Physiol. 2019, 181, 1668–1682. [CrossRef] [PubMed]

3. Smith, S.M.; Li, C.; Li, J. Hormone function in plants. In Hormone Metabolism and Signaling in Plants, 1st ed.; Li, J., Li, C., Smith, S.M., Eds.; Academic Press: London, UK, 2017; pp. 1–38.

4. Creelman, R.A.; Mullet, J.E. Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. USA 1995, 92, 4114–4119. [CrossRef] [PubMed]

5. Vick, B.A.; Zimmerman, D.C. Biosynthesis of Jasmonic Acid by Several Plant Species. Plant Physiol. 1984, 75, 458–461. [CrossRef] [PubMed]

6. Agrawal, G.K.; Tamogami, S.; Han, O.; Iwahashi, H.; Rakwal, R. Rice octadecanoid pathway. Biochem. Biophys. Res. Commun. 2004, 317, 1–15. [CrossRef] [PubMed]

7. Ellinger, D.; Stingl, N.; Kubigsteltig, I.I.; Bals, T.; Juenger, M.; Pollmann, S.; Berger, S.; Schuennemann, D.; Mueller, M.J. DONGLE and DEFECTIVE IN ANther DEHISCENCE1 lipases are not essential for wound-and pathogen-induced jasmonate biosynthesis: Redundant lipases contribute to jasmonate formation. Plant Physiol. 2010, 153, 114–127. [CrossRef] [PubMed]

8. Bannenberg, G.; Martinez, M.; Hamberg, M.; Castresana, C. Diversity of the Enzymatic Activity in the Lipoxygenase Gene Family of Arabidopsis thaliana. Lipids 2008, 44, 85. [CrossRef] [PubMed]

9. Howe, G.A.; Schilmiller, A.L. Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. 2002, 5, 230–236. [CrossRef]

10. Laudert, D.; Pfannschmidt, U.; Lottspeich, F.; Holländ-Czytko, H.; Weiler, E.W. Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol. Biol. 1996, 31, 323–335. [CrossRef] [PubMed]

11. Brash, A.R.; Baertschi, S.W.; Ingram, C.D.; Harris, T.M. Isolation and characterization of natural allene oxides: Unstable intermediates in the metabolism of lipid hydroperoxides. Proc. Natl. Acad. Sci. USA 1988, 85, 3382–3386. [CrossRef] [PubMed]

12. Schaller, A.; Stintzi, A. Enzymes in jasmonate biosynthesis-Structure, function, regulation. Phytochemistry 2009, 70, 1532–1538. [CrossRef] [PubMed]

13. Stenzel, I.; Hause, B.; Miersch, O.; Kurz, T.; Maucher, H.; Weichert, H.; Ziegler, J.; Feussner, I.; Wasternack, C. Jasmonate biosynthesis and the allene oxide cyclase family of Arabidopsis thaliana. Plant Mol. Biol. 2003, 51, 895–911. [CrossRef] [PubMed]

14. Baker, A.; Carrier, D.J.; Schaelder, T.; Waterham, H.R.; van Roermund, C.W.; Theodoulou, F.L. Peroxisomal ABC transporters: Functions and mechanism. Biochem. Soc. Trans. 2015, 43, 959–965. [CrossRef] [PubMed]

15. Browse, J. Jasmonate passes muster: A receptor and targets for the defense hormone. Annu. Rev. Plant Biol. 2009, 60, 183–205. [CrossRef] [PubMed]

16. Koo, A.J.K.; Howe, G.A. Catabolism and deactivation of the lipid-derived hormone jasmonoyl-isoleucine. Front. Plant Sci. 2012, 3, 19. [CrossRef] [PubMed]
17. Ghasemi Pirbalouti, A.; Sajjadi, S.E.; Parang, K. A review (research and patents) on jasmonic acid and its derivatives. *Arch. der Pharm.* 2014, 347, 229–239. [CrossRef] [PubMed]

18. Zhai, Q.; Yan, C.; Li, L.; Xie, D.; Li, C. Jasmonates. In *Hormone Metabolism and Signaling in Plants*, 1st ed.; Li, J., Li, C., Smith, S.M., Eds.; Academic Press: London, UK, 2017; pp. 243–272.

19. Ghelli, R.; Brunetti, P.; Napoli, N.; De Paolis, A.; Cecchetti, V.; Tsuge, T.; Serino, G.; Matsui, M.; Mele, G.; Rinaldi, G. A newly identified flower-specific splice variant of *AUXIN RESPONSE FACTOR8* regulates stamen elongation and endothecium lignification in Arabidopsis. *Plant Cell* 2018, 30, 620–637. [CrossRef]

20. Zhang, C.; Lei, Y.; Lu, C.; Wang, L.; Wu, J. MYC2, MYC3, and MYC4 function additively in wounding-induced jasmonic acid biosynthesis and catabolism. *J. Integr. Plant Biol.* 2020, 62, 1159–1175. [CrossRef]

21. Wasternack, C.; Hause, B. Jasmonates: Biosynthesis, Perception, Signal Transduction and Action in Plant Stress Response, Growth and Development. An Update to the 2007 Review in Annals of Botany. *Ann. Bot.* 2013, 111, 1021–1058. [CrossRef]

22. Ishiguro, S.; Kawai-Oda, A.; Ueda, J.; Nishida, I.; Okada, K. The *DETECTIVE IN ANther DEHISCENCE* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. *Plant Cell* 2001, 13, 2191–2209. [CrossRef]

23. McConn, M.; Browse, J. The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant. *Plant Cell* 1996, 8, 403–416. [CrossRef] [PubMed]

24. Park, J.H.; Halitschke, R.; Kim, H.B.; Baldwin, I.T.; Feldmann, K.A.; Feyereisen, R. A knock-out mutation in *BrDAD1*, in *Brassica* causes male sterility that is restorable with jasmonic acid treatment. *Mol. Breed.* 2003, 11, 325–336. [CrossRef]

25. Cai, Q.; Yuan, Z.; Chen, M.; Yin, C.; Luo, Z.; Zhao, X.; Liang, W.; Hu, J.; Zhang, D. Jasmonic acid regulates spikelet development in rice. *Nat. Commun.* 2014, 5, 1–13. [CrossRef] [PubMed]

26. Lunde, C.; Kimberlin, A.; Leiboff, S.; Koo, A.J.; Hake, S. *Tasselseed5* overexpresses a wound-inducible enzyme, *ZmCYP94B1*, that affects jasmonate catabolism, sex determination, and plant architecture in maize. *Commun. Biol.* 2019, 2, 1–11. [CrossRef] [PubMed]

27. Acosta, I.F.; Laparra, H.; Romero, S.P.; Schmelz, E.; Hamberg, M.; Mottinger, J.P.; Moreno, M.A.; Dellaporta, S.L. *tasselseed1* is a lipoxygenase affecting jasmonic acid signaling in sex determination of maize. *Science* 2009, 323, 262–265. [CrossRef]

28. Yan, Y.; Christensen, S.; Isakete, T.; Engelberth, J.; Meeley, R.; Hayward, A.; Emery, R.N.; Kolomiets, M.V. Disruption of *OPR7* and *OPR8* reveals the versatile functions of jasmonic acid in maize development and defense. *Plant Cell* 2012, 24, 1420–1436. [CrossRef]

29. Browse, J. Jasmonate: Preventing the maize tassel from getting in touch with his feminine side. *Sci. Signal.* 2009, 2, pe9. [CrossRef]

30. Ito, T.; Ng, K.-H.; Lim, T.-S.; Yu, H.; Meyerowitz, E.M. The homeotic protein AGAMOUS controls late stamen development by regulating a jasmonate biosynthetic gene in Arabidopsis. *Plant Cell* 2007, 19, 3516–3529. [CrossRef] [PubMed]

31. Kim, S.-G.; Lee, S.; Kim, Y.-S.; Yun, D.-J.; Woo, J.-C.; Park, C.-M. Activation tagging of an Arabidopsis SRI-RELATED SEQUENCE gene produces abnormal anther dehiscence and floral development. *Plant Mol. Biol.* 2010, 74, 337–351. [CrossRef] [PubMed]

32. Cecchetti, V.; Altamura, M.M.; Brunetti, P.; Petrocelli, V.; Falasca, G.; Ljung, K.; Costantino, P.; Cardarelli, M. Auxin controls Arabidopsis anther dehiscence by regulating endothecium lignification and jasmonic acid biosynthesis. *Plant J.* 2013, 74, 411–422. [CrossRef]

33. Ruduš, I.; Terai, H.; Shimizu, T.; Kojima, H.; Hattori, K.; Nishimori, Y.; Tsukagoshi, H.; Kamiya, Y.; Seo, M.; Nakamura, K.; et al. Wound-induced expression of *DETECTIVE IN ANther DEHISCENCE1* and DAD1-like...
lipase genes is mediated by both CORONATINE INSENSITIVE1-dependent and independent pathways in Arabidopsis thaliana. Plant Cell Rep. 2014, 33, 849–860. [CrossRef] [PubMed]
37. Peng, Y.J.; Shih, C.F.; Yang, J.Y.; Tan, C.M.; Hsu, W.H.; Huang, Y.P.; Liao, P.C.; Yang, C.H. A RING-type E3 ligase controls anther dehiscence by activating the jasmonate biosynthetic pathway gene DEFECTIVE IN ANther DEHISCENCE1 in Arabidopsis. Plant J. 2013, 74, 310–327. [CrossRef] [PubMed]
38. Pan, B.Z.; Xu, Z.F. Benzyladenine Treatment Significantly Increases the Seed Yield of the Biofuel Plant Jatropha curcas. J. Plant Growth Regul. 2011, 30, 166–174. [CrossRef]
39. Dasumniata, D.; Miftahudin, M.; Triadiati, T.; Hartana, A. Short Communication: Sex types in flowering of Jatropha curcas, an emerging model for woody energy plants. BMC Genom. 2019, 20, 958. [CrossRef] [PubMed]
40. Vakinin, Y.; Yermiyahu, U.; Bar-Tal, A.; Samocha, Y. Global maximization of Jatropha oil production under semi-arid conditions by balancing vegetative growth with reproductive capacity. GCB Bioenergy 2018, 10, 382–392. [CrossRef]
41. Mazumdar, P.; Singh, P.; Babu, S.; Siva, R.; Harikrishna, J.A. An update on biological advancement of Jatropha curcas L.: New insight and challenges. Renew. Sustain. Energy Rev. 2018, 91, 903–917. [CrossRef]
42. Triadiati, T.; Kurniati, K.; Widyaustuti, U.; Dasumiati, D. Androgynomonoecious Jatropha curcas: Chromosomes, Isozymes, and Flowers Gender. HAYATI J. Biosci. 2019, 26, 139–146.
43. Dasumiata, D.; Miftahudin, M.; Triadiati, T.; Hartana, A. Short Communication: Sex types in flowering of Jatropha curcas. Biodiversitas 2017, 18, 275–279.
44. Chen, M.-S.; Pan, B.-Z.; Fu, Q.; Tao, Y.-B.; Martinez-Herrera, J.; Niu, L.; Ni, J.; Dong, Y.; Zhao, M.-L.; Xu, Z.-F. Comparative transcriptome analysis between gynoecious and monoecious plants identifies regulatory networks controlling sex determination in Jatropha curcas. Front. Plant Sci. 2017, 7, 1953. [CrossRef] [PubMed]
45. Chen, M.-S.; Niu, L.; Zhao, M.-L.; Xu, C.; Pan, B.-Z.; Fu, Q.; Tao, Y.-B.; He, H.; Hou, C.; Xu, Z.-F. De novo genome assembly and Hi-C analysis reveal an association between chromatin architecture alterations and sex differentiation in the woody plant Jatropha curcas. GigaScience 2020, 9, 1–12. [CrossRef] [PubMed]
46. Zapata, L.; Ding, J.; Willing, E.-M.; Hartwig, B.; Bezdan, D.; Jiao, W.-B.; Patel, V.; Velikkakam James, G.; Koornneef, M.; Ossowski, S.; et al. Chromosome-level assembly of Arabidopsis thaliana Ler reveals the extent of translocation and inversion polymorphisms. Proc. Natl. Acad. Sci. USA 2016, 113, E4052–E4060. [CrossRef]
47. Li, H.; Xue, D.; Gao, Z.; Yan, M.; Xu, W.; Xing, Z.; Huang, D.; Qian, Q.; Xue, Y. A putative lipase gene EXTRA GLUME1 regulates both empty-glume and spikelet development in rice. Plant J. 2009, 57, 593–605. [CrossRef]
48. Matsu, K.; Fukutomii, S.; Ishii, M.; Kajiwara, T. A tomato lipase homologous to DAD1 (LeLID1) is induced in post-germinative growing stage and encodes a triacylglycerol lipase. FEBS Lett. 2004, 569, 195–200. [CrossRef]
49. Yuan, Z.; Zhang, D. Roles of jasmonate signalling in plant inflorescence and flower development. Curr. Opin. Plant Biol. 2015, 27, 44–51. [CrossRef]
50. Yan, Y.; Borrego, E.; Kolomiets, M. Jasmonate biosynthesis, perception and function in plant development and stress responses. In Lipid Metabolism; InTech: London, UK, 2013; pp. 393–442.
51. Krizek, B.A.; Anderson, J.T. Control of flower size. J. Exp. Bot. 2013, 64, 1427–1437. [CrossRef]
52. Nishijima, T. Large Flower Size: Molecular Basis and Role of Cytokinins. Int. J. Mol. Sci. 2012, 13, 999–1006. [CrossRef] [PubMed]
53. Makwana, V.; Shukla, P.; Robin, P. GA application induces alteration in sex ratio and cell death in Jatropha curcas. Plant Growth Regul. 2010, 61, 121–125. [CrossRef]
54. Hui, W.K.; Wang, Y.; Chen, X.Y.; Zayed, M.Z.; Wu, G.J. Analysis of Transcriptional Responses of the Inflorescence Meristems in Jatropha curcas Following Gibberellin Treatment. Int. J. Mol. Sci. 2018, 19, 432. [CrossRef] [PubMed]
55. Li, J.L.; Pan, B.Z.; Niu, L.J.; Chen, M.S.; Tang, M.Y.; Xu, Z.F. Gibberellin Inhibits Floral Initiation in the Perennial Woody Plant Jatropha curcas. J. Plant Growth Regul. 2018, 37, 999–1006. [CrossRef]
56. Ming, X.; Tao, Y.-B.; Fu, Q.; Tang, M.; He, H.; Chen, M.-S.; Pan, B.Z.; Xu, Z.F. Flower-Specific Overproduction of Cytokinins Altered Flower Development and Sex Expression in the Perennial Woody Plant Jatropha curcas L. Int. J. Mol. Sci. 2020, 21, 640. [CrossRef] [PubMed]
57. Hu, B.; Jin, J.; Guo, A.Y.; Zhang, H.; Luo, J.; Gao, G. GSDS 2.0: An upgraded gene feature visualization server. Bioinformatics (Oxf. Engl.) 2015, 31, 1296–1297. [CrossRef] [PubMed]
58. Li, C.; Fu, Q.; Niu, L.; Luo, L.; Chen, J.; Xu, Z.-F. Three TFL1 homologues regulate floral initiation in the biofuel plant *Jatropha curcas*. *Sci. Rep.* 2017, 7, 43090. [CrossRef] [PubMed]

59. Fu, Q.; Li, C.; Tang, M.; Tao, Y.-B.; Pan, B.-Z.; Zhang, L.; Niu, L.; He, H.; Wang, X.; Xu, Z.-F. An efficient protocol for *Agrobacterium*-mediated transformation of the biofuel plant *Jatropha curcas* by optimizing kanamycin concentration and duration of delayed selection. *Plant Biotechnol. Rep.* 2015, 9, 405–416. [CrossRef]

60. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCT method. *Methods* 2001, 25, 402–408. [CrossRef]

61. Wu, J.; Hettenhausen, C.; Meldau, S.; Baldwin, I.T. Herbivory Rapidly Activates MAPK Signaling in Attacked and Unattacked Leaf Regions but Not between Leaves of *Nicotiana attenuata*. *Plant Cell* 2007, 19, 1096–1122. [CrossRef]

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