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Fusion of transgene and interspecies hybridization enhances seed yield and root rot disease resistance in *Jatropha curcas*

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Abstract

Perennial woody plants play an indispensable role in sustainable production. To shorten their juvenile phase and improve health, *Jatropha curcas*, a classic bioenergy crop, serves as a model for a breeding strategy integrating transgenes with interspecific hybridization. Specifically, overexpressing *JcFT* (*JcFT-OE*) *J. curcas* exhibiting early flowering were crossed with *Jatropha integerrima* exhibiting bright flowers and robust stems. The resulting hybrids were then backcrossed (BC) with *JcFT-OE* plants to refine the desired traits. The F1 generation displayed early flowering and an intermediary type of parents, with hard stems, more xylem, lower seed yields, and higher C18:2 content in the seed oil compared to those of wildtype and *JcFT-OE* transgenic plants. The BC1 generation showed early flowering, diverse shapes of fruit and seeds, and higher seed yield than the F1 generation. Among these lines, BC1-3 produced the highest yield, while the seed yields of *J. integerrima*, F1 and BC1-1 had notably low yields attributed to fruit dropping. Notably, F1 and BC1 plants demonstrated enhanced resistance to root rot caused by *Lasiodiplodia theobromae* and lignin contents of stems compared to *JcFT-OE*, a trait inherited from *J. integerrima*. Overall, the hybrid plants inherited desirable traits such as precocity and root rot resistance from their parents, resulting in higher seed yields in BC1 individuals. Blending transgenes with hybridization in *Jatropha curcas* enriches traits, boosting yields and disease resistance in woody plants. Furthermore, *FT* overexpression has substantial superiority in accelerating the breeding process in woody trees.

1 | INTRODUCTION

Jatropha curcas (physic nut), a perennial woody plant in the Euphorbiaceae family, has high seed oil content (30–40%), making it ideal for biodiesel production. Its biodiesel is efficient, low-emission, and reduces greenhouse gases (Achten et al. 2008). This species is drought-tolerant, grows well in marginal lands, and does not compete with food crops, making it suitable for tropical arid regions. It prevents soil erosion, improves soil structure, and provides farmers with additional income. By-products like seed shells and cake can be used as fertilizer, feed, or biomass energy (Openshaw 2000, Alherbawi et al. 2021).

Due to the late start of breeding research on *Jatropha curcas*, several major challenges persist, including limited genetic diversity, unstable seed yield and oil content, insufficient stress resistance, and an unsystematic breeding plan. To address these issues, the following strategies can be systematically implemented (Montes and Melchinger 2016). The initial focus should be on the systematic collection and assessment of germplasm resources, using phenotypic and genotypic analyses to identify accessions that demonstrate high yield, increased oil content, and strong tolerance to stress. Second, genetic variation can be generated through cross-breeding, followed by the selection of hybrid combinations demonstrating heterosis (Sujatha and Prabakaran 2003). Besides, the breeding process should take into account

various traits, including yield, oil content, stress resistance, and disease tolerance, to develop high-quality varieties that are well-suited for diverse ecological environments.

A 'fast-track breeding' strategy has been proposed to shorten the breeding cycle of woody plants (Putterill and Varkonyi-Gasic 2016, Petri et al. 2018). Despite the challenges associated with transformation in woody species, some trees have proven amenable to successful transgene integration through the efforts of plant breeders. Notably, the overexpression of *FT* (*FLOWERING LOCUS T*) genes in certain angiosperm trees has been used to overcome their long juvenile phase (Böhlenius et al. 2006), including citrus (Endo et al. 2005), apple (Kotoda et al. 2010), poplar (Hsu et al. 2011), *Jatropha curcas* (Li et al. 2014, Ye et al. 2014), eucalypt (Klocko et al. 2016), and blueberry (Gao et al. 2016). Although transgenesis and hybridization have been widely applied in woody plants, a combined strategy for tree breeding has not been clearly proposed in recent years. To shorten the breeding cycle and accelerate directed breeding in woody plants, we propose combining transgenes with hybridization to create a complementary approach. This strategy will be applied to *J. curcas*. In the past decade, researchers have gradually improved the transformation efficiency in *J. curcas* (Pan et al. 2010, Fu et al. 2015, Liu et al. 2020). Through this optimized transformation process, several transgenic lines exhibiting desirable breeding features have been developed, including early-flowering plants by overexpressing *JcFT* (Li et al. 2014, Ye et al. 2014).

In actual cultivation, root rot caused by *Lasiodiplodia theobromae* in *J. curcas* has been reported in several planting regions, leading to leaf abscission, stem rotting and yield reduction (Latha et al. 2009, Adandonon et al. 2014). Sujatha (2001) found that *J. integerrima* exhibited greater resistance to insects compared to other *Jatropha* species. Laosatit et al. (2019) found several desirable traits in the progenies from *J. curcas* and *J. integerrima* interspecific hybrids concerning seed/oil yield and quality. Based on these findings, *J. integerrima* may have considerable genetic potential for resistance to diseases. This study aimed to tackle the pathogens linked to planting by using transgenic plants of *JcFT* and crossing them with *J. integerrima*. The goal was to boost resistance to root rot disease and increase seed yield in *J. curcas*. This study's approach integrates interspecific hybridization and backcrossing, assisting the incorporation of desirable traits with transgenic early maturation characteristics. This approach is conducive to the development of superior varieties and has a positive impact on biodiesel production and environmental sustainability.

2 | MATERIALS AND METHODS

2.1 | Plant materials and conditions

Wildtype seeds of *J. curcas* (*Jc*-WT) and *J. integerrima* (*Ji*) were collected from the Xishuangbanna Tropical Botanical Garden (XTBG; 21°54′ N, 101°46′ E, 580 m above sea level) of the Chinese Academy of Sciences, located in Mengla County, Yunnan Province, Southwest China. Seed germination and seedling growth were conducted in the same greenhouse (28 ± 2°C, 14/10 h of light/dark). The homozygous plants and seeds of *AtSUC2:JcFT* (*JcFT-OE*) were obtained from a previous study (Li et al. 2014).

2.2 | The processes of cross and backcross

A interspecific cross was performed between *JcFT-OE* transgenic *J. curcas* (female parent) and *J. integerrima* (male parent). The backcross was performed between *JcFT-OE J. curcas* (female parent) and male flowers of F1-1 plant with pink flowers (male parent, Figure 1I). Eighteen-month-old adult plants with an adequate number of flowers were used as hybrid parents in April 2018. Prior to hybridization, unopened male flowers of the female parents were completely emasculated, while unopened female flowers were retained. Subsequently, the entire emasculated inflorescences were enclosed in mesh bags. When the female flowers of the female parents opened, and the pollen from the male parents became visible, mature pollen collected from the male flowers of *J. integerrima* plants was applied to the stigmas and petals of the *JcFT-OE* plants. After hand pollination, the inflorescences were immediately re-bagged with mesh bags. After two months, the mature seeds were collected and germinated in a greenhouse for further analysis. Initially, we aimed to obtain self-pollinating seeds from the F1 plants; however, only a few seeds were gathered through manually assisted self-crossing in the first year due to frequent fruit drops in the F1 plants (Figure S2). Consequently, we opted to backcross with *JcFT-OE* as the female parent to improve fruit-setting ability.

2.3 | Identification of hybrid progenies

To identify hybrid seedlings, DNA samples isolated from the young leaves of each seedling were analyzed by PCR using *JcActin1* primers (sense primer: CTCCTCTCAACCCCAAAGCCAA; antisense primer: CACCAGAATCCAGCACGATACCA). Based on the difference in the size of the amplified products by *JcActin1* primers between *J. curcas* and *J. integerrima*, samples exhibiting two bands (approximately 500 and 750 bp) in agarose gel electrophoresis were classified as the hybrid generation.

2.4 | Phenotype analysis

An electronic vernier caliper was employed to accurately measure the sizes of leaves, fruits, and seeds, achieving a precision of 0.1 mm. The ImageJ software was used to calculate the leaf area based on images of leaves with scales. The flowering time was defined as the number of days from seed germination until the inflorescence bud could be observed. The numbers of inflorescences and infructescences of *Jc*-WT, *JcFT-OE*, *J. integerrima*, and F1 plants were recorded and analyzed in five individuals. For the BC1 generation, the branches

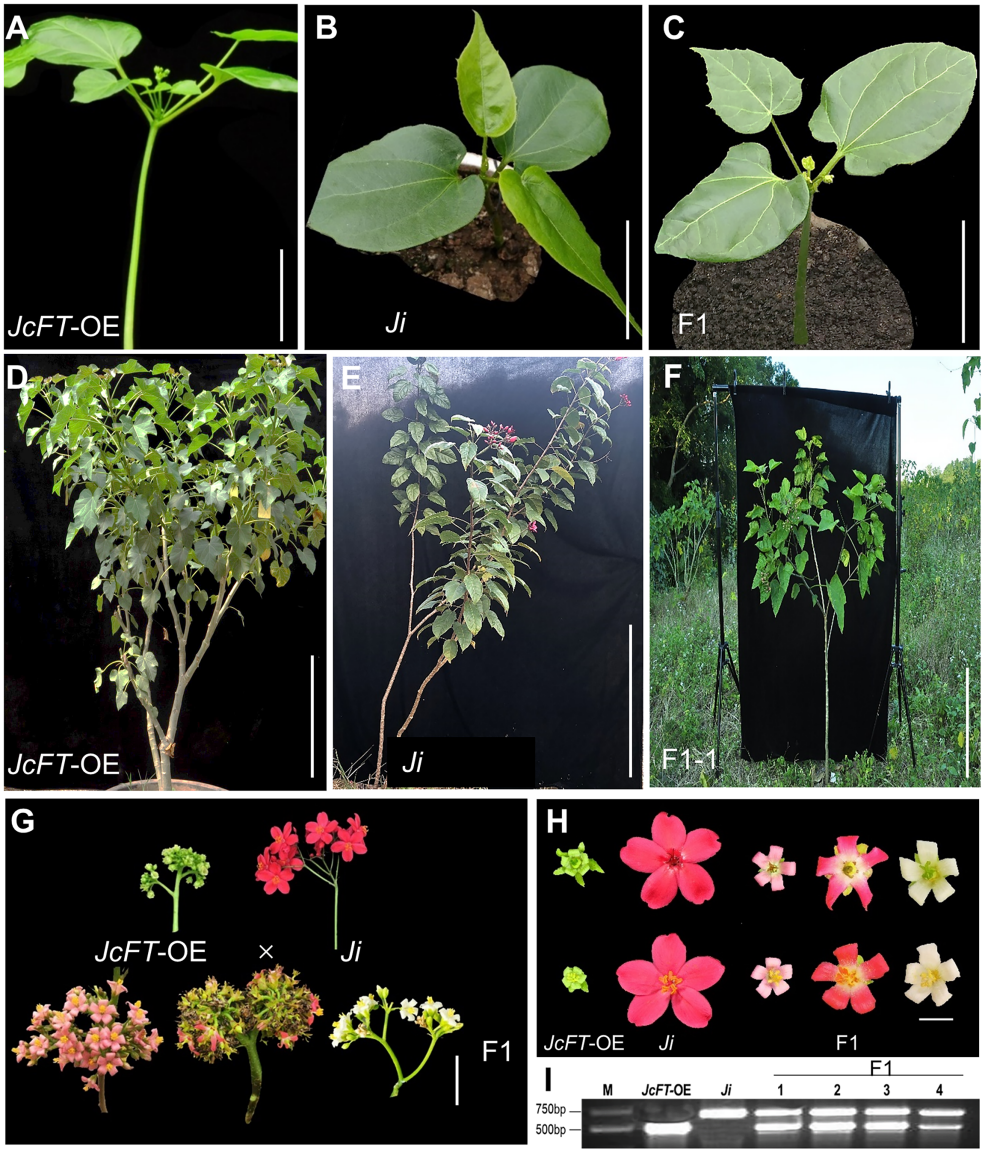

FIGURE 1 **Seedlings and flowers of *JcFT-OE*, *J. integerrima* and F1 plants.** (A-C) One-month-old seedlings and (D-F) 18-month-old adult plants of *JcFT*-OE (mother), *Ji* (father) and F1; A-C, bar = 5 cm; D-F, bar = 50 cm. (G) Inflorescences of *JcFT*-OE, *Ji* (above), and three F1 plants (below); bar = 1 cm. (H) Female (top) and male flowers (bottom) of *JcFT*-OE, *Ji* and F1 from left to right; bar = 1 cm. (I) Identification of parents and F1 generation by PCR using *JcActin1* primers.

of BC1-1/2/3 were grafted onto *J. curcas*-WT plants to increase the population, and five grafted plants of each line were used to record flowering time. The yellow ripe fruits from each line were collected, and their sizes were measured. The percentage of four-carpel fruits was calculated by dividing the number of four-carpel fruits by the total fruit count. Prior to seed analysis, the seeds were dried at 37°C until constant weight. The seed yield of each line was recorded for three consecutive years. For stem analysis, six-month-old grafted plants were compared based on their stem components. The thicknesses of the stem, phloem, xylem, and pith were measured using an electronic vernier caliper in cross-sections, and the percentages of each layer's thickness were calculated as the ratio of each layer to the total stem thickness. Lignin analyses of the stems were performed using a lignin content detection kit (Boxbio) following the manufacturer's instructions (Kong et al. 2023). The solution was measured at 280 nm using a NanoDrop UV–Vis Spectrophotometer, and the lignin content was calculated based on the absorbance.

2.5 | Seed oil determination by time-domain nuclear magnetic resonance (TD-NMR)

The mature seeds were dried at 65°C until constant weight. According to the operation manual, the oil content of mature seeds and kernels (approximately 6 g/oil measurement) was determined using a minispec MQ-one Seed Analyzer (Bruker Optik GmbH) and compared with a standard curve generated from pure *J. curcas* oil as a reference. The seeds of each line were analyzed in three biological replications.

2.6 | Seed oil determination by Soxhlet extraction method

The mature seeds were dried at 65°C until constant weight, after which the dried kernels were separated from the seed coats. After crushing by a high-speed grinder and sieving through a 16-mesh screen, the dried seed kernel powder was obtained to extract seed oil.

An accurately weighed 2 g of dried kernel powder was placed in pre-degreased filter paper bags. These bags containing the samples were subsequently placed in an automatic Soxhlet apparatus (Soxtec 2050, FOSS, Denmark), and 70 mL of petroleum ether was added to the condensate tube as the extraction solvent, heating at 55°C for 12 h. After the extraction process, a mixture of petroleum ether and seed oil was obtained and transferred to a rotary evaporator at 80°C (the maximum boiling point of petroleum ether) for 1.5 h. The remaining lipid was then dried at 65°C to eliminate any residual water and petroleum ether. The seed oil was weighed, and the seed oil content was calculated using the following equation: % Oil = $[(W_b - W_a) \times 100]$/kernel weight.

Where W_a is the weight of the empty flask, and W_b is the weight of the flask containing the extracted oil. Each experiment was repeated three times.

2.7 | Determination of fatty acid composition by gas chromatography–mass spectrometry (GC–MS)

The transesterification reaction and analysis methods for fatty acid composition were performed according to Kumar and Das (2018). Each seed oil sample (approximately 100 μL) was treated with methyl esters using 1 mL of methanol-sulfuric acid (2.5 M) at 70°C in a thermostatic water bath for 30 minutes. Subsequently, the crude methyl esters were extracted with 1 mL of hexane before being injected into the gas chromatograph (GC). The individual fatty acid methyl esters were analyzed using an Agilent Technologies 7890A gas chromatograph equipped with a 5975C mass spectrometer (Agilent Technologies). A polar DB-WAX capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Agilent Technologies) was employed for separation. The flow rate of the helium carrier gas was set at 1 mL min^{-1}. One microliter of the sample was injected in split mode at a ratio of 1:30. The oven temperature was initially held at 40°C for 2 min, increased to 220°C at a rate of 5°C min^{-1}, and then maintained at 220°C for 10 min (total run time 48 min). The temperatures of the front inlet, transfer line, and ion source were set at 250, 250, and 230°C, respectively. The mass spectrometer (MS) operated at 70 eV with a mass range of m/z 35–500. The identification of each fatty acid methyl ester was confirmed by comparing the mass spectra and retention times with those of authentic standards analyzed under the same conditions.

2.8 | Inoculation of root rot pathogen and evaluation of plant resistance

For root rot of *J. curcas*, a serious crop-reducing root disease, it has been reported that its primary pathogen is *L. theobromae* in Benin and India (Latha et al. 2009, Adandonon et al. 2014). According to these studies, we also isolated the same pathogen from black-rotted tissues of two-year-old diseased plants affected by root rot (planted in the field of XTBG), identified by DNA sequencing. The purified mycelia of *L. theobromae* with 1.5 mL of 10% sterilized glycerol were stored at −80°C. Healthy branches from each plant line, approximately 20 cm in length, were cut at the same position and artificially wounded to create equal-sized injuries (approximately 50 mm^2) using a dissecting needle or scalpel. Subsequently, the activated mycelia were inoculated on the spots of each branch, which were then wrapped in sterilized absorbent cotton and stored in zigzag bags disinfected with 75% alcohol, and sterile water was used as a control. The treated branches were placed in an artificial climate chamber (28 ± 2°C, 14 h of light and 10 h of darkness) for one week. After that, the health status and lesion sizes of the inoculated and control wound spots were measured and compared among *Jc*-WT (wildtype), *JcFT-OE*, *Ji*, F1, BC1-1, BC1-2, and BC1-3 stems. The disease score was calculated as the average ratio of lesion area to wound area (as control) of three plants of each line from three replications.

2.9 | qRT-PCR analysis

Total RNA was extracted from frozen *Jatropha* tissues using the method described by (Ding et al. 2008). The first-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara, Dalian). The cDNA templates were diluted fivefold with sterile double-distilled water, and qRT-PCR was performed using SYBR® Premix Ex Taq™ II (Roche Diagnostics) on a Roche 480 Real-Time PCR Detection System (Roche Diagnostics). The primers used for qRT-PCR are listed in Table S1. The qRT-PCR was conducted on three independent biological replicates, with three technical replicates per sample. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method as described by (Livak and Schmittgen 2001). The transcript levels of specific genes were normalized to those of the *JcActin1* gene.

3 | RESULTS

3.1 | F1 generation of *JcFT-OE* × *J. integerrima* was obtained through interspecific crossing

Previously, an early-flowering *Jatropha curcas* transgenic plant with overexpressed *JcFT* was obtained by our group (Li et al. 2014), and its flowering time was advanced to approximately 14 days (Figure 1A, Table 1). To further integrate the desirable traits in *J. curcas*, interspecific crosses were attempted between *J. curcas* and *J. integerrima*, aiming to combine beneficial characteristics from both species and develop a superior cultivar. The female parent was a one-year-old *JcFT-OE J. curcas*, characterized by early flowering. The male parent was one-year-old *J. integerrima*, renowned for its prolonged flowering period and bright petal colour (Figure 1D-E). During the early flowering stage of *JcFT-OE*, the mature pollen of *J. integerrima* was hand-pollinated to the stigma of emasculated inflorescences of *JcFT-OE J. curcas*. After bagging for ten days, ovary enlargement of the

TABLE 1 Cross and backcross generations exhibited early flowering and different ratios of female/male flowers.

Genotype	Flowering time (day)	Num. of female flower	Num. of male flower	Female: male flowers
J. curcas-WT	201.58 ± 11.85^{a}	8.75 ± 1.42^{bc}	143.63 ± 23.45^{c}	1: 16.75
JcFT-OE	14.35 ± 4.25^{c}	10.43 ± 4.26^{b}	175.70 ± 18.53^{b}	1: 16.85
J. integerrima	94.25 ± 14.53^{b}	5.61 ± 3.23^{c}	55.83 ± 8.15^{e}	1: 9.95
F1	15.55 ± 4.63^{c}	7.52 ± 3.64^{b}	82.64 ± 10.83^{d}	1: 10.98
BC1-1	16.14 ± 4.16^{c}	0.61 ± 0.33^{d}	95.83 ± 14.03^{d}	1: 157.10
BC1-2	15.78 ± 3.62^{c}	17.65 ± 4.51^{a}	215.36 ± 18.64^{a}	1: 12.20
BC1-3	15.43 ± 4.50^{c}	19.40 ± 3.06^{a}	226.80 ± 38.81^{a}	1: 11.69

Note: The F1 generation was obtained by crossing *JcFT-OE* (female parent) and *J. integerrima* (male parent). The BC1 generation was obtained by backcrossing *JcFT-OE*. Each line contains five individuals of each line that were counted. The number of inflorescences was 15 in each group, and values are the mean ± standard deviation. Values with different letters (a-c) are significantly different from each other ($p < 0.05$, t test).

pollinated female flowers was observed with the naked eye, and subsequently, these fruits turned yellow and ripened more than a month later. After harvesting and planting the bagged fruits and seeds, several hybrid plants were identified by PCR with *JcActin1* primers. Three types of banding patterns were observed in the agarose gel electrophoresis image: approximately 500 bp band of *J. curcas*, approximately 750 bp band of *J. integerrima*, and a combination of both bands representing the F1 plants. In this study, four F1 plants were confirmed (Figure 1I). Additionally, one-month-old seedlings of *JcFT-OE*, *J. integerrima*, and the F1 were cultivated in cups with a diameter of 20 cm. Both *JcFT-OE J. curcas* and the F1 generation plants exhibited extremely early blooming after one month, whereas *J. integerrima* plants did not display any signs of early blooming (Figure 1A-C).

3.2 | Comparison of leaf, flower, fruit and seed phenotypes among F1 and their parents

Many visible distinctions emerged among one-year-old trees of *JcFT-OE*, *J. integerrima*, and the F1 generation. The most pronounced phenotypic characteristics were evident in the leaves, specifically regarding their size, shape, number of lobes, and degree of flatness. The leaves of F1 plants exhibited a combination of traits from both parent species: the leaf shape and size resembled those of the female parent (*JcFT-OE*), while the leaves of *J. integerrima* were well-known for their fiddle shape. The leaves of F1 plants had a palm-like vein structure with five main veins, similar to those of *J. curcas*. Both the F1 and *JcFT-OE* plants have five leaf lobes, whereas *J. integerrima* has three (Figure S1A). Comparing the inflorescence and flowers of these individuals, the *JcFT-OE* plants developed smaller and more compact inflorescences with smaller light green flowers. In contrast, the *J. integerrima* plants produced larger incompact inflorescence with the largest red flowers. The F1 generations developed middle-type inflorescence with pink flowers, white, and dark red (Figure 1G-H) flowers. The inflorescences and flowers of the F1 plants displayed intermediate traits, with sizes resembling those of the female parent and colours more similar to those of the male parent. The desirable trait of early flowering was inherited by the F1 generation, which flowered approximately half a month close to the *JcFT-OE* plants (Table 1). The number of female and male flowers, along with their ratios in the F1 plants, fell between those of their parents. The fruit shape of the F1 plants closely resembled that of the *JcFT-OE*, while the mature fruits of *J. integerrima* were smaller, featured distinct and deep dorsal sutures, and exhibited a shattering habit (Figure 2F). The seed size of the F1 plants was intermediate between that of *JcFT-OE* and *J. integerrima*, while the colour and shape of the F1 seeds were similar to those of *J. integerrima* (Figure 2O).

3.3 | Adult plants of *J. integerrima* and the F1 generation exhibited obvious fruit dropping

The fruit-setting rates of *J. integerrima* and the F1 generation were lower than those of *JcFT-OE*, as frequent fruit drop was observed (Figure S2). During ovary swelling, the fruit stems of *J. integerrima* often broke spontaneously, making mature seed harvesting extremely difficult (Figure S2B). Fortunately, fruit drop was alleviated to some extent in the F1 generation (Figure S2H). To enhance the fruit-setting rate and seed yield of the F1 generation, backcrossing with the *JcFT-OE* line should be the next necessary step.

3.4 | The BC1 generation exhibited early flowering and different female/male flower ratios

After the phenomenon of young fruit drop in F1 plants was observed, backcrossing (BC) was implemented with their female parent, *JcFT-OE*, to increase seed yield. Candidate seeds from the first BC generation were harvested and planted in pots. After two weeks, the mature leaves of these plants were screened using PCR with *JcActin1* primers. Samples exhibiting two length bands (approximately 750 and 500 bp) were identified as identified as the BC1 generation. Consequently, 19 out of 24 plants were confirmed as BC1 using agarose gel electrophoresis (Figure S3). Meanwhile, the first inflorescence bloomed on the *JcFT-OE*, F1, and BC1 plants. The BC1 plants exhibited a similar

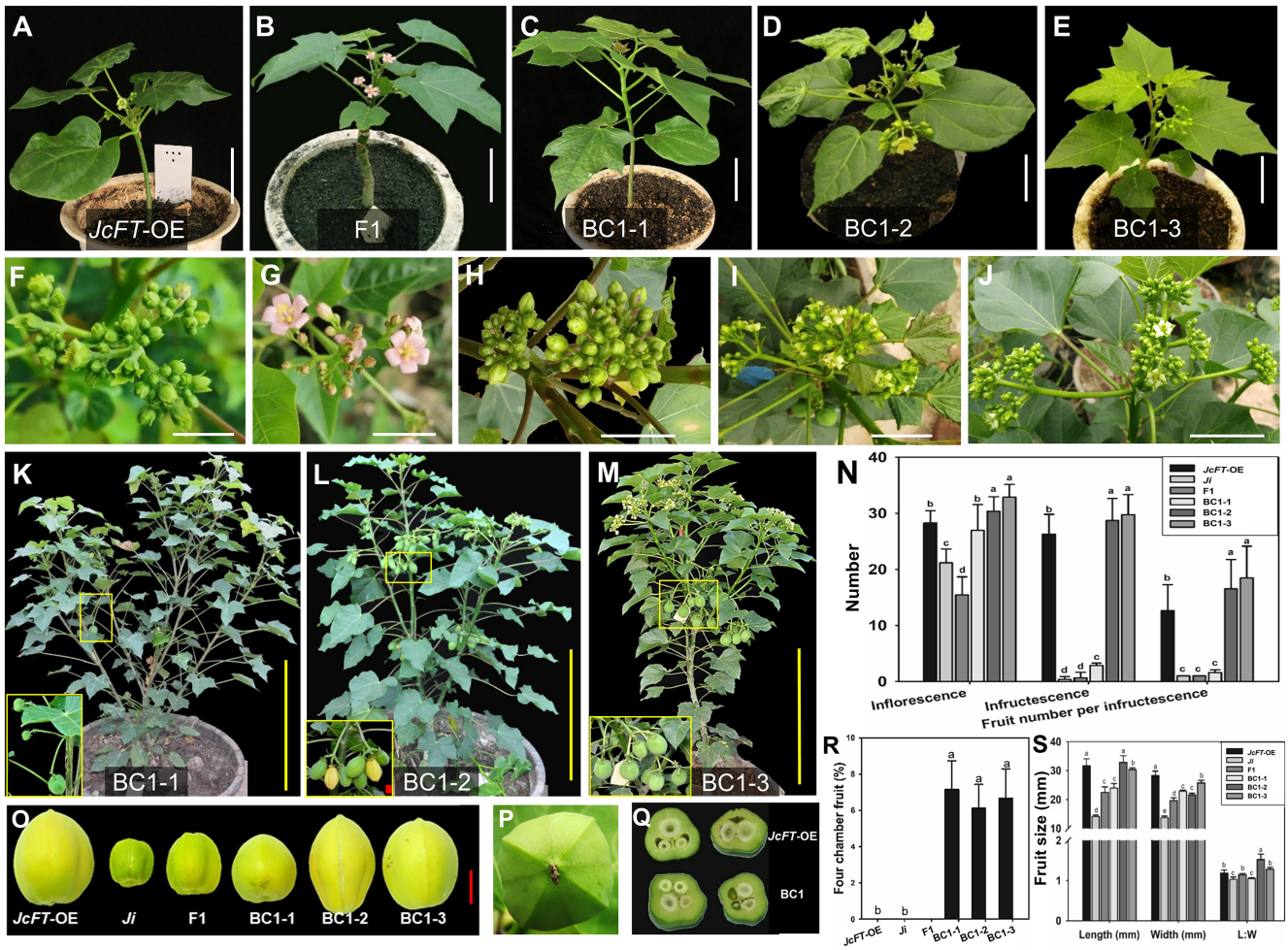

FIGURE 2 **Early flowering BC1 generation by crossing between *JcFT-OE* and F1**. (A-E) One-month-plants of *JcFT-OE*, F1, and BC1-1/2/3 plants. (F-J) Inflorescences of *JcFT*-OE, F1, and BC1-1/2/3 plants. (K-M) Three-month-old BC1-1/2/3 adult plants, bars = 50 cm. (N) The numbers of inflorescences, infructescences and fruits of per infructescence. (O) Variant fruit shapes of six genotypes. (P) A four-carpel fruit of BC1 plants. (Q) Cross sectional view of fruits, and top two belong to *JcFT-OE*, bottom two belong to BC1. (R) The percentage of four-chamber fruits. (S) The fruit length, width and L: W of six strains. White bar = 5 cm, yellow bar = 50 cm, red bar = 1 cm. Values with different letters are significantly different from each other ($p < 0.05$, t test).

flowering time of approximately 15 days, with a noticeably shorter juvenile phase compared to wildtypes of *J. curcas* and *J. integerrima* plants (Table 1). Three BC1 lines were randomly selected for further observation, representing high, medium, and low yield, respectively. After one month, the *JcFT-OE*, F1, and BC1-1/2/3 plants transitioned to reproductive growth, displaying different inflorescences (Figure 2F-J). On the one hand, the flower colours of BC1-1/2/3 were consistent with those of the female parent (*JcFT-OE*), while the flowers of the F1 plants remained pink as usual (Figure 2B and G). On the other hand, the inflorescence structures of the BC1-2/3 plants exhibited a longer axis than that of the BC1-1 plants (Figure 3H-I).

The numbers of female and male flowers and their ratios exhibited significant differences among *JcFT-OE*, *J. integerrima*, and three lines of BC1. BC1-2/3 displayed a higher number of female flowers, similar to *JcFT-OE* plants, while BC1-1 showed the opposite. The number of male flowers in BC1-1 was less than half that of BC1-2/3 flowers, approaching that in *J. integerrima* and F1 flowers. These variations resulted in higher ratios of female/male flowers in the BC1-2/3 plants (Table 1).

In terms of plant architecture, BC1 plants resembled *JcFT-OE* plants. Specifically, focusing on their infructescences, more infructescences and more fruits were observed in BC1-2/3 plants than in BC1-1 plants (Figure 2 N).

3.5 | Variant fruit shapes and four-carpel fruits appeared on BC1 plants

First, a large difference in fruit shape was observed between *JcFT-OE* and *J. integerrima*, with *J. integerrima* plants producing smaller fruits (Figure 2O). Similarly, variations in fruit shapes were noted among the

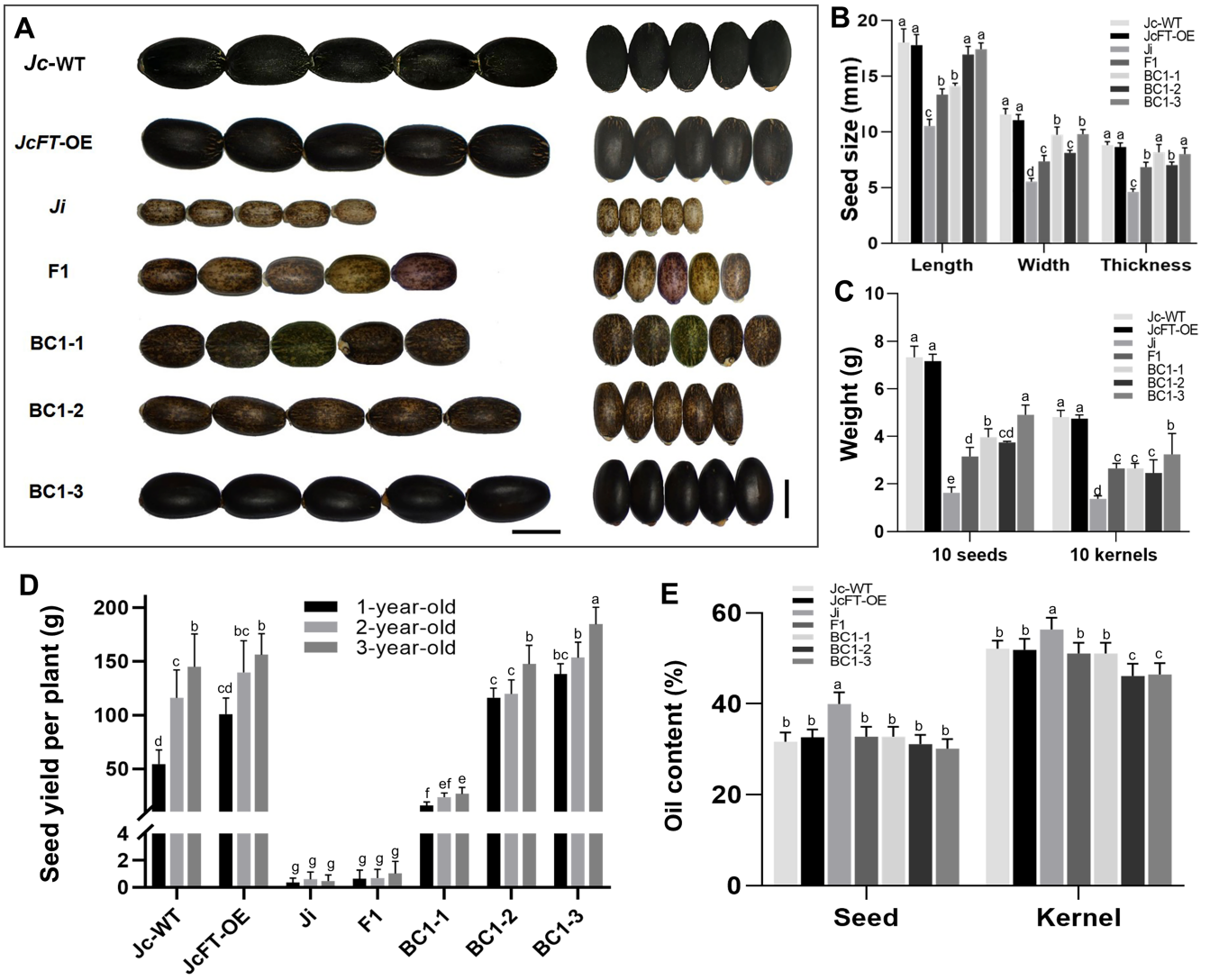

FIGURE 3 **Comparison of seed traits.** (A) five-seed length (left) and width (right), bars = 1 cm; (B) Characterization of seed length, width, and thickness among six lines, $n = 15$; (C) Weight of 10 seeds and 10 kernels of six lines, $n = 5$; (D) Seed yields per plant of six lines. (B-D) Data are represented as the mean ± SD. (E) Seed and kernel oil contents of six lines. Bars with different letters are significantly different from each other ($p < 0.05$, t test).

F1 and BC1 generations. Specifically, the F1 and BC1-1 lines exhibited fruits with a middle shape, while the fruit shape of BC1-3 plants closely resembled that of *JcFT-OE*, and that of BC1-2 displayed a spindly olive shape (Figure 2O). Statistical analysis of fruit sizes indicated the following order from largest to smallest: *JcFT-OE*, BC1-3, BC1-2, BC1-1, F1, and *J. integerrima*. The longest fruits were found in BC1-2 plants, while the widest fruits were observed in *JcFT-OE* plants. Besides, BC1-2 fruits exhibited the greatest length-to-width ratio (Figure 2S).

Second, the size and number of inflorescences and infructescences were counted and compared among the six lines. The inflorescence size in F1 plants was mostly reduced, followed by *J. integerrima*. Notably, obvious variations in the number of infructescences and fruits were observed among the lines. Moreover, BC1-1 plants exhibited the lowest fruit count, while a greater number of fruits were observed in *JcFT-OE* and BC1-2/3 plants (Figure 2 N). Infructescence and fruit development were particularly challenging in the F1 and BC1-1 plants. During the fruit developmental process, a considerable proportion of fruit drop was observed in *J. integerrima*, F1, and BC1-1 plants (Figure S2).

Interestingly, a few four-carpel fruits were observed in three lines of the BC1 generation (Figure 2P-Q). However, during the cultivation of *J. curcas* and *J. integerrima*, three-carpel fruits predominated in almost all fruits. In the cross-sectional views of the fruits, three or two ovules developed in three-carpel fruits, while four ovules were present in the four-carpel fruits of the BC1 plants (Figure 2P-Q). The data revealed that the percentage of four-carpel fruits was approximately 6% to 7% across all three BC1 lines, while the other plants (including *JcFT-OE*, *J. integerrima*, and F1) produced only three-carpel fruits (Figure 2R).

3.6 | Comparison of seed traits among *JcFT-OE J. curcas*, *J. integerrima*, F1 and BC1

A variety of shapes and colours of seeds were identified in wildtype *J. curcas*, *JcFT-OE*, *J. integerrima*, F1, and BC1 plants. The size order corresponded with their fruits, with *Jc*-WT, *JcFT-OE*, BC1-3, and BC1-2 exhibiting the longest seed lengths. As a hybrid of *JcFT-OE* and *J. integerrima*, the seeds of the F1 generation were similar to their male parent, *J. integerrima*, exhibiting a light seed coat with darker spots. In comparison to the BC1 generation, the seed coat of BC1-1/2 retained the characteristics of their male parent (F1), while that of BC1-3 was more similar to the female parent (*JcFT-OE*, Figure 3A). The seeds of *J. integerrima*, F1, and BC1-2 were smaller in both width and thickness (Figure 3B). Besides, the *JcFT-OE* plants exhibited the largest and heaviest seeds, whereas the BC1-3 plants produced the second largest seeds, with seed coats comparable in size to those of the *JcFT-OE* plants. In terms of kernel weight, the kernels of BC1-3 were heavier than those of *J. integerrima* and other offspring (Figure 3C). For three consecutive years, seed yields per plant were measured and compared among seven lines, each line containing five clonal plants. In the first year, lines BC1-3 and BC1-2 yielded over 100 g of seeds per plant, considerably surpassing the production of the other lines, which included *JcFT-OE*, *Jc*-WT, and BC1-1 (Figure 3D). However, the seed yields per plant of *J. integerrima* and F1 remained below 2 g. During the second and third years, the seed yields of each line were increased by different degrees. In a comparison of seed yields among seven lines of three-year-old plants, the BC1-3 plants demonstrated the highest yields, while the BC1-1 plants exhibited considerably lower yields than the other BC1 variants (Figure 3D). However, the seeds of *J. integerrima* and F1 plants were difficult to harvest, resulting in the lowest yield due to fruit drop (Figure 3D). Thus, the yield of hybrid offspring was successfully increased by the backcross experiment.

3.7 | Comparison of oil content and oil composition among *JcFT-OE*, *J. integerrima*, F1, and BC1 plants

After harvesting the seeds from six lines (*JcFT-OE*, *J. integerrima*, F1, and BC1-1/2/3), the oil content and seed composition were analyzed. It was evident that the highest oil content was found in the seeds and kernels of *J. integerrima*, followed by those of *JcFT-OE* and F1 plants, with no significant difference in kernel percentage among the six lines (Figure 3E). Furthermore, the distinct fatty acid compositions of the extracted seed oils from the six lines were clearly revealed by GC–MS. The gas chromatograms of the six lines showed similar peaks in *JcFT-OE*, F1, and BC1-1/2/3 plants, showing higher levels of linoleic acid (C18:2), oleic (C18:1), and palmitic (C16:0), while the seed oil of *J. integerrima* contained more C18:2 and less C18:1 (Figure S4). The principal components of these seed oils included C18:2, C18:1, and C16:0, followed by stearic acid (C18:0), with minimal amounts of palmitoleic acid (C16:1) and linolenic acid (C18:3), listed in descending order of abundance (Figure S4). The oil composition of F1 plants was intermediate between their parents, *JcFT-OE* and *J. integerrima*. The BC1-1 plants have distinct component profiles, with higher levels of C16:0 and lower levels of C18:2 compared to BC1-2/3.

3.8 | *J. integerrima*, F1, and BC1 plants have stronger resistance to root rot compared to *JcFT-OE J. curcas* plants

In recent years, *J. curcas* plants have been affected by root rot disease in various regions, including Xishuangbanna (Southeast China), Tamil Nadu (Southeast India), and Benin (West Africa). This disease has led to symptoms such as rotting, blackening, and even plant death. Previous research has indicated that the pathogenic fungus causing *Jatropha* root rot is *Lasiodiplodia theobromae* (Latha et al. 2009, Adandonon et al. 2014). We hypothesized that plants with mature stems with a higher xylem ratio, such as *J. integerrima*, *JcFT-OE*, and BC1 plants, might have greater resistance to root rot. Consequently, we compared the resistance to root rot in hybrid and BC1 plants with their parents. Initially, we identified diseased plants with black spots at the basal part of the stem in the XTBG field. Next, *L. theobromae* fungi were isolated from the identified rot samples three times. Subsequently, the inoculation of *L. theobromae* hyphae was conducted on six-month-old healthy stems of *J. integerrima*, *JcFT-OE*, F1, and BC1 plants using spots of the same size and water as a control treatment. After one week, some of the healthy stems inoculated with *L. theobromae* exhibited blackening and rot (Figure 4A-G).

The results showed that JcFT-OE plants were the most severely affected, with the largest lesions. In contrast, J. integerrima plants were the healthiest, with minimal lesions (Figure 4H). The lesion sizes of F1, BC1-2, and BC1-3 plants were also significantly smaller than those of *JcFT-OE* and BC1-1 plants (Figure 4H). Overall, *J. integerrima* plants exhibited higher resistance to *L. theobromae*, and the resistance of F1 and partial BC1 plants was enhanced through crossbreeding and backcrossing. Consequently, the strategy of interspecies hybridization between the two *Jatropha* species was successful in improving resistance to root rot disease in *J. curcas* breeding.Additionally, the stem component was compared among these plants. According to the transverse and longitudinal photos of the stems, the percentage of phloem, xylem, and pith were calculated. The stems of *J. integerrima* had the largest proportion of xylem, followed by those of F1, BC1-3, BC1-2, and BC1-1 plants (Figure 5A-B). Furthermore, the results of stem lignin contents showed that the highest value in *J. integerrima* stems and the lignin contents of F1 and BC1 generations were in the middle, significantly higher than that of *Jc*-WT and *JcFT*-OE (Figure 5C). To determine the reasons for the stronger resistance and higher xylem and lignin ratios in F1 and BC1 generations, we detected and compared the expression levels of 16 genes associated with pathogenesis, jasmonic acid biosynthesis, and lignin biosynthesis in young stems of *JcFT-OE*, *Ji*, F1, and BC1-1/2/3 plants (Figure 4I, 5D). First, the pathogenesis-related gene *PR1-1* showed significantly higher expression in the BC1-2/3 stems, with expression levels 32-fold and

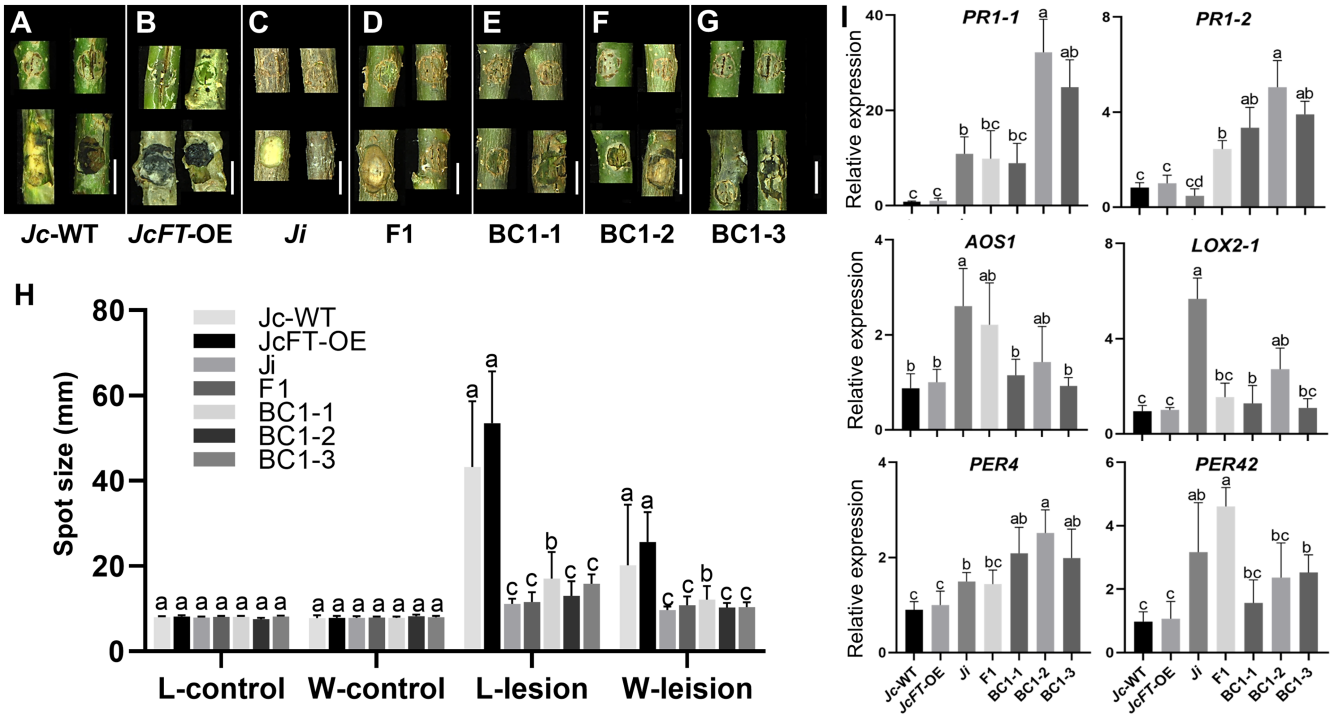

FIGURE 4 **Comparison of resistance to root rot by *Lasiodiplodia theobromae*.** (A-G) Six-month-old branches of six lines one week after inoculated with hyphae of *L. theobromae* (lower), water as control (upper). Bar = 5 cm. (H) The length (L) and width (W) of controls and lesion spots, $N = 15$. (I) Relative expression levels of genes related with pathogenesis and biosynthesis of jasmonic acid. PR: pathogenesis-related protein; AOS: allene oxide synthase; LOX: linoleate 13S-lipoxygenase; PER: peroxidase. Data are represented as the mean ± SD, each data contains three biological repetitions. Bars with different letters are significantly different from each other ($p < 0.05$, t test).

25-fold greater than those in *JcFT-OE*. Additionally, *PR1-2* was more highly expressed in three BC1 lines than in their parent plants (Figure 4I). Second, jasmonates (JA), a kind of defence signalling molecule, are also involved in plant resistance to pathogens (Ghorbel et al. 2021). *Allene oxide synthase 1* (*AOS1*), a key gene involved in JA biosynthesis, exhibited higher expression in *Ji* and F1 plants. A similar expression profile was observed in another JA biosynthesis-related gene, *LOX2-1* (linoleate 13S-lipoxygenase 2–1). Peroxidases (PER) play an important role in plant defence against pathogens by activating metabolic processes such as phenolic oxidation and lignification (Shigeto and Tsutsumi 2016). The higher expression of *PER4* was detected in BC1 stems than in their parents (Figure 4I). Another member of *PER* genes, *PER42*, was also significantly high-expressed in the stems of *Ji* and F1 plants (Figure 4I). In the lignin biosynthesis pathway, the expression levels of the phenylalanine ammonia-lyase *PAL1* and *PAL2* were lowest in *JcFT*-OE, while other lines with *Ji* lineage exhibited markedly higher expressions. Moreover, downstream genes involved in lignin biosynthesis, including *4CL1/2*, *CCR1*, *CAD9/14*, and *COMT1-1/1–2*, displayed similar expression patterns with the highest expression found in the stems of *Ji* or BC1-2 and the lowest was in *JcFT*-OE stems. Therefore, the genes associated with pathogenesis and the biosynthesis of JA and lignin were upregulated in the stems of *Ji*, F1, and BC1. Additionally, the expression levels of genes related to JA and lignin biosynthesis were highest in the stems of *Ji*.

4 | DISCUSSION

Perennial woody plants, such as *Jatropha curcas*, are essential for sustainable agriculture due to their biofuel potential and ecological benefits (Kumar and Kamari 2020, Abobatta 2021). However, the long juvenile phase, plant disease and relatively low seed yield of *J. curcas* have limited its economic viability (Arockiasamy et al. 2021). The interspecific hybridization between *J. curcas* (*JcFT-OE* transgenic line) and *J. integerrima*, along with subsequent backcross, has provided considerable insights into the genetic enhancement of *Jatropha* species. This study successfully integrated desirable traits such as early flowering, enhanced seed yield, and improved resistance to root rot disease. Below, we provide an in-depth discussion regarding the significance of these findings in the context of plant breeding, genetic inheritance, and disease resistance mechanisms.

The early flowering phenotype of *JcFT-OE*, achieved through the overexpression of the *JcFT* gene (Li et al. 2014), was stably inherited by the F1 generation. This outcome emphasizes the effectiveness of *JcFT* in controlling flowering time across various hybrid backgrounds, aligning with observations in other plant species where *FT* homologs assist reproductive development (Andrés and Coupland 2012, Klocko et al. 2016, Song et al. 2019). interspecific hybridization between *JcFT-OE J. curcas* and *J. integerrima* produced F1 progeny with intermediate phenotypic traits, combining leaf morphology, floral architecture, and fruit characteristics from both parents. The F1

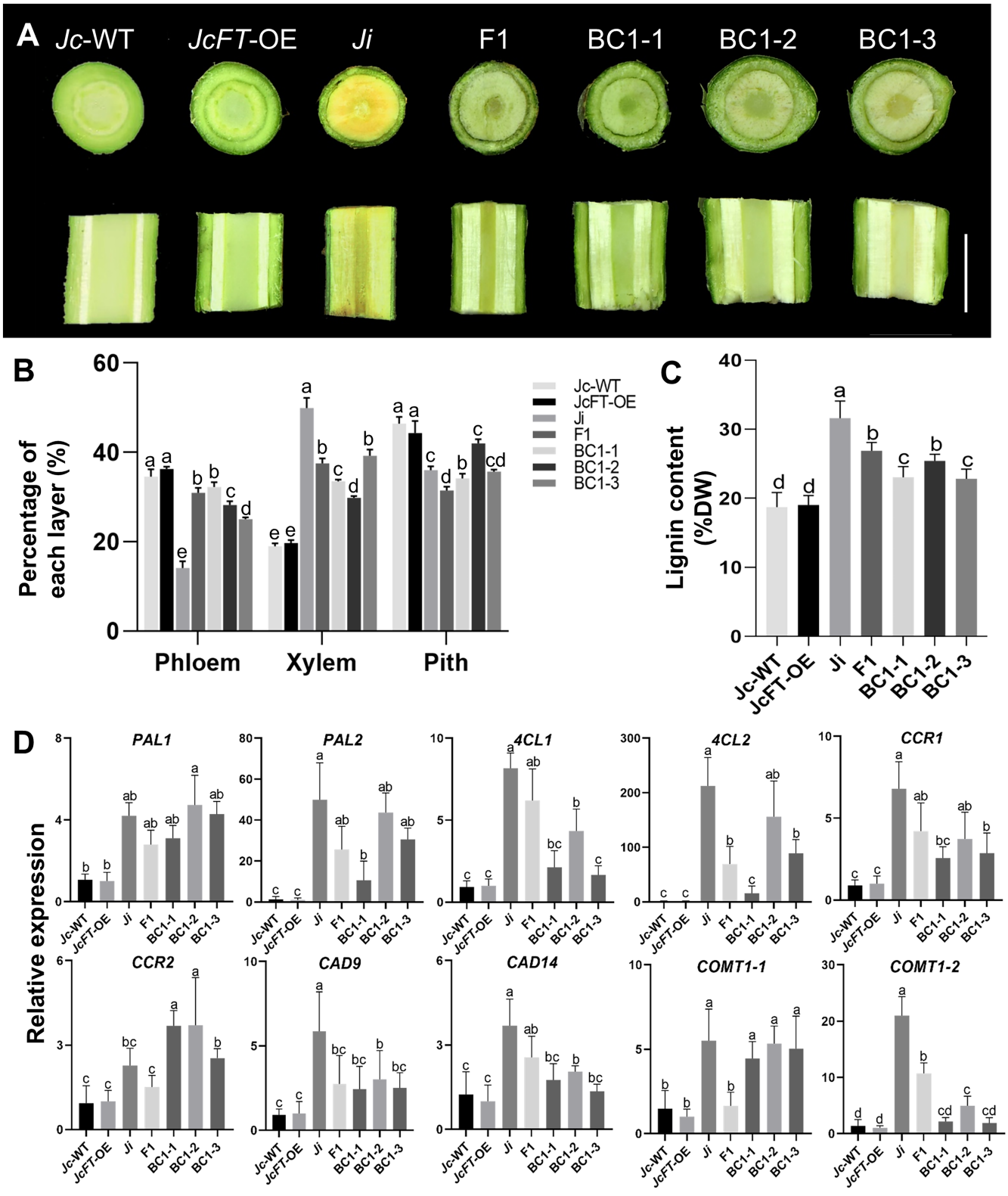

FIGURE 5 **Comparison of stem component and expression levels of genes related with lignin biosynthesis**. (A) Cross section (upper) and longitudinal section (lower) of six-month-old plant stems of six lines. Bar = 1 cm. (B) The percentages of phloem, xylem and pith in stem. The areas of phloem, xylem and pith were calculated according to each diameter. (C) Lignin content of stems ($n = 5$). (D) Relative expression levels of genes related with lignin biosynthesis. PAL: phenylalanine ammonia-lyase; 4CL: 4-coumarate--CoA ligase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; COMT: caffeic acid 3-O-methyltransferase. RNA samples were extracted from young stems of these plants. Three biological replicates were prepared for qRT-PCR assays. *JcActin1* was used as the internal reference. Data are represented as the mean ± SD. Bars with different letters are significantly different from each other based on three biological replications ($p < 0.05$, t test; $n = 3$).

generation exhibited leaves with five lobes and palmate venation resembling *J. curcas*, while flower colour (pink, white, and dark red) and inflorescence size reflected contributions from *J. integerrima* (Figure 1G-H, S1A). This phenotypic intermediacy aligns with classical hybrid vigour patterns observed in interspecific crosses (Bar-Zvi et al. 2017, Laosatit et al. 2019). The early flowering characteristic of *JcFT-OE* was consistently observed in the F1 generation, which bloomed approximately 15 days earlier than the wild-type *J. curcas* (Table 1). This finding emphasizes the stability of *JcFT* overexpression in promoting accelerated reproductive development (Li et al. 2014).

Moreover, there are notable differences in fruit morphology, seed characteristics, and seed oil composition among *JcFT-OE*, *J. integerrima*, F1, and BC1 plant variants. However, seeds of *J. integerrima* and F1 were smaller and lighter, consistent with hybrid results in Laosatit et al. (2019). The intermediate seed size and *J. integerrima*-like seed coat patterns in F1 plants further indicate paternal influence on seed traits, consistent with studies on cytoplasmic inheritance in hybrids (Baskin and Baskin 2019). However, BC1 progeny exhibited phenotypic diversity, including variant inflorescence structures and fruit shapes (Figure 2O), suggesting segregation of alleles controlling developmental pathways. Integrating genomic tools, such as marker-assisted selection, could accelerate the development of high-yielding *Jatropha* cultivars with enhanced agronomic resilience (Hasan et al. 2021). Notably, the emergence of four-carpel fruits in BC1 plants (6–7% frequency) represents a novel phenotypic variation, as both parental species predominantly produce three-carpel fruits. This finding suggests that hybridization may disrupt genetic pathways regulating carpel development, potentially offering opportunities for increased seed yield due to additional ovules (Figure 2P-Q). Similar carpel variations have been reported in other species, where genetic recombination or epigenetic changes during hybridization alter floral organ development (Mahmood et al. 2024). BC1-3 seeds, which closely resembled *JcFT-OE* in size and coat pattern, produced the highest kernel weight and seed yield (>100 g/plant), outperforming other lines (Figure 3A-D). This aligns with studies showing that backcrossing can restore desirable agronomic traits while maintaining hybrid vigour (Warschefsky et al. 2016).

However, fruit drop remained a challenge in F1 and BC1-1 plants (Figure 3D, S2), likely due to incomplete introgression of traits stabilizing fruit retention (Dariva et al. 2021). Addressing this issue through further backcrossing or genetic modification could enhance seed yield stability. Backcrossing to *JcFT-OE* generated BC1 progeny with improved fruit-setting rates, particularly in BC1-2 and BC1-3 lines, which displayed higher female flower ratios and infructescence numbers (Figure 2 N, Table 1). The restoration of *JcFT-OE*-like floral ratios in BC1-2/3 suggests that backcrossing effectively reintroduced genetic factors favouring female flower development, a critical determinant of seed yield (Ajayi et al. 2024). These findings emphasize the effectiveness of backcrossing in addressing hybrid vulnerabilities while retaining sought-after characteristics, as evidenced by other crop breeding initiatives (Abdul and Masmoudi 2025). Additionally, oil content and fatty acid composition analysis revealed that *J. integerrima* had the highest oil content, while F1 and BC1 plants exhibited intermediate profiles (Figure 3E, S4). The oil composition of F1 and BC1-2/3 plants was dominated by linoleic (C18:2) and oleic (C18:1) acids, similar to *JcFT-OE*, whereas BC1-1 showed distinct profiles with higher palmitic acid (C16:0) levels (Figure S4). These variations indicate that backcrossing can adjust oil quality, making Jatropha suitable for specific industrial applications (Alherbawi et al. 2021, Rathnakumar and Sujatha 2022).

Particularly, our research indicates that *J. integerrima* and its hybrid offspring (F1 and BC1) display markedly enhanced resistance to *L. theobromae*-induced root rot compared to *JcFT-OE* plants. *J. integerrima* displayed minimal lesion formation, while *JcFT-OE* was highly susceptible (Figure 4A-G), aligning with prior reports of *L. theobromae* as a major pathogen in *Jatropha* cultivation regions (Adandonon et al. 2014). The improved resistance observed in F1 and BC1 plants, especially BC1-2 and BC1-3, emphasizes the effectiveness of interspecific hybridization and backcrossing in transfer resistance traits from *J. integerrima* to *J. curcas*. This result emphasizes the important potential of interspecific hybrids to enhance disease resistance in crops (Warschefsky et al. 2016). The superior resistance in *J. integerrima* and its progeny correlated with higher xylem ratios and lignin content in stems, suggesting mechanical and biochemical reinforcement against pathogen invasion. Gene expression analysis showed a notable increase in pathogenesis-related genes (*PR1-1*, *PR1-2*), JA biosynthesis genes (*AOS1*, *LOX2-1*), and lignin biosynthesis genes (*PAL1/2*, *4CL1/2*) in resistant lines (Figure 4I, 5D). The upregulation of the *PR* gene indicates that the horse carcass activates PR protein to enhance the defence mechanism in the face of root rot bacteria attacks. PR proteins play an important role in systemic acquired resistance and allergic reactions in plants (Jain and Khurana 2018). Genes associated with JA biosynthesis, such as *AOS1* and *LOX2-1*, are upregulated in resistant plants, which is consistent with the role of JA in plant defence mechanisms, suggesting that JA plays an important role in regulating plant resistance to pathogens (Wasternack and Song 2017). JA signalling is known to mediate defence against necrotrophic pathogens (Ghorbel et al. 2021). Lignin, an essential element of cell walls, acts as a physical barrier and is associated with disease resistance in plants (Bagniewska-Zadworna et al. 2014, Yadav and Chattopadhyay 2023). Lignin biosynthesis directly influences key traits in crop breeding, such as stem strength, disease resistance, and seed yield stability, while also impacting biofuel production potential through lignin's role in cell wall composition and structural integrity (Yoon et al. 2015). Moreover, peroxidases (*PER4*, *PER42*), which play an essential role in lignification (Shigeto and Tsutsumi 2016), likely synergistically enhance resistance in *J. integerrima* and its progeny. The highest expression of these genes in *J. integerrima* stems further emphasizes its genetic contribution to hybrid vigour. These results validate interspecific hybridization as a viable strategy for improving root rot resistance in *Jatropha* breeding programs. Future studies should focus on functional validation of candidate genes (e.g., *PR1-1*, *PAL1*) and field trials to assess trait stability across environments. Integrating these resistant lines into breeding pipelines could mitigate

yield losses and enhance the sustainability of *Jatropha* cultivation in disease-prone regions.

The present study is subject to certain limitations. Specifically, the population of crossbred and backcrossed offspring is relatively small. If the backcross progeny population were measured for yield and evaluated for resistance to root rot, the range of variation in target traits might broaden, potentially leading to the identification of more outstanding individual plants. Moreover, current investigations into the pathogenesis of plant root rot resistance, including aspects such as pathogenesis, jasmonic acid synthesis, and lignin synthesis, have primarily focused on comparing the expression levels of genes associated with these processes. In future research, the mechanisms underlying the resistance to root rot in *J. integerrima* and its hybrids have yet to be fully elucidated. The RNA-Seq analysis allows for the investigation of the coordinated regulatory network of the jasmonic acid (JA) and lignin pathways in disease-resistant lines. Further analysis of secondary metabolites and transcriptome data is required to gain a comprehensive understanding of the resistance mechanisms. Future research could focus on constructing diverse populations, which would enable the effective application of Genome-Wide Association Studies (GWAS) to identify genetic loci associated with seed traits and resistance to root rot in *Jatropha*. (Kaur et al. 2023).

Interspecific hybridization within the *Jatropha* genus has proven to be a valuable strategy for genetic improvement, showing major promise for the improvement of essential agronomic characteristics such as seed yield, oil quality parameters, and genetic diversity (Maghuly and Laimer 2013). Several researchers emphasized the critical importance of integrating advanced genetic analyses, including high-throughput molecular marker systems and comprehensive phenotypic evaluation protocols for the precise identification and selection of superior hybrid genotypes (Sudheer et al. 2009, Tanya et al. 2011). Future research should focus on the genetic structures and regulatory networks of key traits and explore male sterility systems to improve hybrid breeding (Du et al. 2020, Bohra et al. 2025). The accumulated evidence from this study supports the creation of improved *Jatropha* varieties with better agricultural performance and increased biofuel output, thereby contributing to sustainable energy solutions (Divakara et al. 2010, Kumar and Kamari 2020).

5 | CONCLUSIONS

The breeding strategy integrating transgenic technology and interspecific hybridization has been successfully applied to combine multiple advantageous traits in the *Jatropha genus*, including early flowering, novel flower colouration, increased female-to-male flower ratio, enhanced seed yield, and improved resistance to root rot. This approach demonstrates considerable potential for the breeding and genetic improvement of woody tree species. Moreover, the overexpression of *FT* in transgenic plants greatly speeds up the breeding cycle of perennial woody species, thereby optimizing crop improvement initiatives.

AUTHOR CONTRIBUTIONS

Xue Bai performed the experiments, analyzed the data, and wrote this manuscript. Yiqing Su revised the paper. Zhonghong Huang completed the isolation and identification of the pathogenic fungi. Ping Huang and Tong Cheng assisted in inoculating the branches with pathogenic fungi. Mingyong Tang conceived the experiments and revised the manuscript.

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DATA AVAILABILITY STATEMENT

The data and materials used in this study are available from the corresponding authors upon reasonable request. All data generated or analyzed during this study are included in this published article and supplementary information. Sequence data included in our manuscript can be obtained from the publicly available genome of Jatropha curcas (https://www.ncbi.nlm.nih.gov/bioproject/38697) under the following accession numbers: JcFT (NP_001295681) and JcACTIN1 (NM_112764).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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