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The *Jatropha curcas KASIII* gene alters fatty acid composition of seeds in *Arabidopsis thaliana*

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Abstract

Jatropha curcas L. is a perennial, drought-resistant, and non-food oilseed crop. The fatty acid composition of seed oil, especially the ratio of 16- to 18-carbon fatty acids, has a direct impact on the biodiesel quality. In plants, fatty acid chain lengths are mainly determined by the plastidial fatty acid synthase complex which includes three β-ketoacyl-acyl carrier protein synthases (KASs), KASI, KASII, and KASIII. The KASIII is thought to play a rate-limiting role in fatty acid synthesis. Here, we report the functional characterization of a putative *JcKASIII* gene from *Jatropha curcas* using *Arabidopsis thaliana* L. as model system. The transcripts of *JcKASIII* were detected in all tissues examined and increased in seeds. Overexpression of *JcKASIII* in *Arabidopsis* led to an increased content of palmitic acid and a higher ratio of 16- to 18-carbon fatty acids. Moreover, functional analysis of *JcAKSIII* in *kasI* or *kasII* knock down *Arabidopsis* mutants revealed that the composition of seed oil changed. Taken together, these results suggest that heterologous *JcKASIII* could function as one of the major regulators of fatty acid composition.

Additional key words: fatty acid synthase, mutants, transgenic plants.

Introduction

Jatropha curcas L. (a common name physic nut), which belongs to the *Euphorbiaceae* family, is a perennial, drought-resistant small tree. *Jatropha* is widely grown in many tropical and sub-tropical areas and has emerged as attractive candidate crop for biodiesel production (Becker *et al*. 2008, Berchmans *et al*. 2008). It is a non-food crop with a high oil content and can grow on degraded soils and waste lands unsuitable for food crops. *Jatropha* seed oil has rather a high 18-carbon (C18) fatty acid content (stearic, oleic, linoleic, and linolenic acid content is 3.7 - 9.8, 34.3 - 45.8, 29.0 - 44.2, and 0 - 0.3 %, respectively) and a low C16 fatty acid content (palmitic and palmitoleic acid content is 14.1 - 15.3 and 0 - 1.3 %, respectively) (Gubitz *et al*. 1999). Fatty acid composition in seed oils directly affects fuel properties. A high content of long-chain saturated fatty acids reduce the cold-temperature flow properties whereas a high content of polyunsaturated fatty acids can increase the rate of oxidation and NO_x emission (Durrett *et al*. 2008). Hence, a key strategy for *Jatropha's* genetic breeding is to produce plants with better quality of seed oil.

In plants, fatty acid chain lengths and saturation are determined by the type II fatty acid synthase (FAS) complex in plastids. The FAS complex, which comprises a set of discrete enzymes, mainly generates saturated palmitic acid (C16:0) and stearic acid (C18:0), monounsaturated palmitoleic acid (C16:1) and oleic acid (C18:1), and polyunsaturated linoleic acid (C18:2)

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Abbreviations: ACP - acyl carrier protein; CN - cetane number; DAF - days after flowering; DAS - days after sowing; FA - fatty acid; FAD - fatty acid desaturase; FAS - fatty acid synthesis; KAS - β-ketoacyl-acyl carrier protein synthase; RT-PCR - reverse transcription PCR; SAD - stearoyl-ACP desaturase; VIGS - virus-induced gene silencing; WT - wild type.
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and linolenic acid (C18:3). Three major classes of enzymes, termed as β-ketoacyl-acyl carrier protein synthase I, II, and III (KASI, KASII, and KASIII), which require an acyl-carrier protein (ACP) for catalytic activities, are involved in chain-length determination (Nishida *et al*. 2004). The KASIII, an initial enzyme of fatty acid chain elongation, catalyzes the condensation reaction of malonyl-ACP with acetyl-CoA to yield ketobutyryl-ACP. Subsequent condensation reactions are catalyzed by KASI and KASII. The KASI uses butyryl- to myristyl-ACP as substrate to produce hexanoyl- to palmitoyl-ACP, and KASII uses C16:0-ACP as substrate to produce stearoyl-ACP (Shimakata and Stumpf 1982, Clough *et al*. 1992, Biswas *et al*. 2013). Molecules elongated to C18:0-ACP are efficiently desaturated by stromal stearoyl-ACP desaturase to form C18:1-ACP. These fatty acids are ultimately exported to the endoplasmic reticulum, or they enter the prokaryotic glycerolipid pathway (Li-Beisson *et al*. 2010).

A plant *KASIII* gene was first isolated from *Spinacia oleracea* (Tai and Jaworski 1993) and then from other plant species (Tai *et al*. 1994, Slabaugh *et al*. 1995, Chen and Post-Beittenmiller 1996, Jones *et al*. 2003, Stoll *et al*. 2006, Li *et al*. 2008a). Jaworski *et al.* (1989) found that KASIII activity correlates with the rate of fatty acid synthesis in spinach homogenates suggesting that KASIII may play a rate-limiting role in fatty acid synthesis. In *Brassica napus* plants overexpressing *KASIII,* decreased amounts of C18:1 fatty acids and increased amounts of C18:2 and C18:3 fatty acids were detected, and it was proposed that fatty acid biosynthesis is not controlled by one rate-limiting enzyme but is controlled by a number of component enzymes of the fatty acid biosynthetic machinery (Verwoert *et al*. 1995). Overexpression of *ChKASIII* from *Cuphea hookeriana* in tobacco, rapeseed, and *Arabidopsis thaliana* under the control of a constitutive or seed-specific promoter globally increases C16:0 fatty acid content (Dehesh *et al*. 2001). Moreover, HaKASIII from *Helianthus annuus* can use medium-chain acyl-ACPs as primer substrates to produce (at least) 10-carbon-ACPs (González-Mellado *et al*. 2010). Knockout of the regulatory site of KASIII enhances short- and medium-chain acyl-ACP synthesis (Abbadi *et al*. 2000). A bacterial strain lacking KASIII fails to grow in the absence of supplementation with exogenous long-chain unsaturated fatty acids (Lai and Cronan 2003). The *kas3* mutant of *Arabidopsis*, which was isolated based on the hypersensitivity of photosystem II to a low temperature, carries a single point mutation that causes an amino acid modification in the KASIII enzyme, reducing total fatty acid content to 75 % of the wild-type at 23 °C (Takami *et al*. 2010). Therefore KASIII, which acts as the key enzyme of the fatty acid synthesis pathway, is not only determinant of the initiation of fatty acid synthesis, but also of the extension of the C16 fatty acid chain, which finally determines the ratio of C16 to C18 fatty acids.

In *Jatropha*, some genes involved in the fatty acid biosynthesis pathway have been identified, including *steraroyl-acyl carrier protein desaturase* (*JcSAD)* (Luo *et al*. 2006), *JcKASIII* (Li *et al.* 2008a), *acyl-acyl carrier protein thioesterase* (*JcFATB1)* (Wu *et al*. 2009), *JcKASII* (Wei *et al.* 2012), and *fatty acid desaturase (JcFAD2)* (Qu *et al*. 2012). Although *in vitro* regenerated *Jatropha* plants have been obtained from various explants, such as cotyledons (Li *et al*. 2008b), leaves (Thepsamran *et al*. 2008), hypocotyls (Lu *et al*. 2003), and nodes (Datta *et al*. 2007, Shrivastava and Banerjee 2008), these regeneration systems have not translated into efficient genetic transformation. Therefore, *Arabidopsis* is still considered as suitable model system for functional complementation studies (Wei *et al*. 2012, Kim *et al*. 2014).

The aim of this study was to investigate the function of *Jatropha* KASIII in regulation of C16 and C18 fatty acid biosynthesis by combining expression analysis in *Jatropha* with functional characterization in *Arabidopsis*.

Materials and methods

Jatropha curcas L. plants were propagated from seeds collected from clones of elite individuals with a high oil content selected in our laboratory. The plants were grown in a trial land at the Forestry Science Institute of Haikou City, Hainan Province, China. *Arabidopsis thalliana* L. cv. Columbia-0 (Col-0) was used as wild type (WT) genetic background in all experiments. The seeds were surface sterilized and then sown on a Murashige and Skoog (MS) medium and stratified at 4 °C for 48 h in the dark before germination. The MS medium was supplemented with 20 μg cm^{-3} hygromycin or 25 μg cm^{-3} kanamycin to screen resistant transgenic plants. In addition, primary transformants carrying the bar selection marker were directly screened by spraying the plants with a herbicide *Barsta* (250 mg dm^{-3}) (Zhang *et al.* 2006). The plants were grown in a growth chamber at a temperature of 22 °C, a 16-h photoperiod, and an irradiance of 120 μmol m^{-2} s^{-1}.

To generate an antiserum that recognizes JcKASIII, an amino acid sequence consisting of seven specific peptide sequences from *Jatropha* KASIII was designed and synthesized by the *Abmart Company* (Shanghai, China), and the synthesized peptide was used for antibody production in a rabbit using standard immunization protocols. The polyclonal antiserum was affinity purified using a *HiTrap Protein-G HP* column (*Amersham Pharmacia Biotech*, Piscataway, USA) and further purified using an immunosorbent column coupled

with a GST-JcKASIII fusion protein. The antiserum was evaluated by immunoblotting and shown to be highly specific. Total protein extracts were prepared as described previously by Fan *et al*. (2009).

Western blot analyses were performed as described Fan *et al.* (2009) with minor modifications. Total protein was separated by SDS-PAGE on 10 % (m/v) polyacrylamide gels, and the polypeptides were transferred to nitrocellulose membranes (0.45 mm; *Amersham Life Science*) in a medium consisting of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20 % (v/v) methanol. After rinsing the blotted membranes in Tris-buffered saline (TBS) containing 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl, the membranes were pre-incubated for 1.5 h in a blocking buffer containing 3 % (m/v) bovine serum albumin dissolved in TBS supplemented with 0.05 % (v/v) *Tween-20* (TBST1) and incubated with gentle shaking for 2 h at room temperature with appropriate antibodies (generally diluted 1:2 000 in the blocking buffer). Following extensive washes with TBST1 (three washes for 10 min per wash), the membranes were incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:1 000 diluted in TBST1) at room temperature for 1.5 h and washed three times (10 min per wash) with TBST2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1 % *Tween-20*) and TBS, respectively. The locations of the antigenic proteins on the membranes were visualized by incubating the membranes in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

The *kasI* mutant was obtained from the *WISCDLOX* collection (CS853792). This mutant carries a T-DNA insertion in the 3'-UTR (downstream of the TGA stop codon) of *KASI* (AT5G46290.3), which was confirmed by PCR amplification using primers KASI-LP/RP. A T-DNA left boundary primer WISCDLOXP745 was used in combination with a gene-specific primer to confirm the T-DNA insertion. The *kasII* mutant was obtained from the *GABI-KAT* collection (CS350573). This mutant carries a T-DNA insertion in the 5'-UTR (upstream of the ATG start codon) of *KASII* (AT1G74960), which was confirmed by PCR amplification using primers KASII-LP/RP. A T-DNA left boundary primer GABILBo8409 was used in combination with a gene-specific primer to confirm the T-DNA insertion.

Total RNA was extracted from harvested samples with a *TRIzol* reagent according to the manufacturer's instructions (*Invitrogen*, Carlsbad, USA) and digested with DNaseI (*TaKaRa*, Shiga, Japan) to remove the genomic DNA. First-strand cDNA was synthesized using *Superscript*III reverse transcriptase (*Invitrogen*). Semi-quantitative RT-PCR analysis was performed according to Qiu *et al*. (2014). The expression of *JcKASIII* in *Jatropha* was examined with a primer combination JcKASIII-F1/R1. The *JcACTIN* was used as internal control and expression was quantified using the *Image J* software. The relative expression of *JcKASIII* was normalized with *JcACTIN* (Barrero *et al*. 2007). Quantitative RT-PCR analysis was performed with a *Roche* (Indianapolis, USA) *LightCycler 480* using the *SYBR Green* detection protocol (*TaKaRa*). The expressions of *KASI*, *KASII*, and *KASIII* were examined using primers KASI-F/R, KASII-F/R, and KASIII-F/R, respectively. The relative expressions of *KASI*, *KASII*, and *KASIII* in the transgenic plants were individually normalized to those of the *ACTIN* gene (AT5G09810) and then compared to those of the WT, respectively. Data presented are averages from at least three biological replicates with SD. Statistical analyses were performed with Student's *t*-test. The primers used in this study are described in Table 1 Suppl.

A 1.2 kb sequence containing the *JcKASIII* gene was amplified by PCR using primers JcKASIII-F/R, digested with restriction endonucleases XbaI and SacI, and ligated to a similarly digested XbaI/SacI site of the pBI121 vector to generate the *P35S:JcKASIII* construct (Fan *et al.* 2009). A 209 bp fragment, which is not homologous to any other *Arabidopsis* sequences encoding KASI or KASII enzymes, downstream of the ATG start codon of *KASIII* (AT1G62640) was amplified from an *Arabidopsis* cDNA template using primers AtKASIII-RNAi-attB1/attB2 and subsequently cloned into the pDONR207 vector, sequenced, and recombined into the pFRH5941 binary vector to generate the *KASIII-RNAi* construct following the supplier's instructions (*Invitrogen*). The above constructs were then introduced into the *Agrobacterium tumefaciens* strain LBA4404 and used to transform *Arabidopsis* plants using the floral dip method (Clough and Bent 1998).

Seeds of *Arabidopsis* were germinated on an MS medium (*Sigma-Aldrich*, St. Louis, USA) solidified with 0.7 % (m/v) agar containing 1.0 % (m/v) sucrose or glucose, or in the absence of sucrose and grown in a vertical position. The results presented are from at least 30 seedlings from individual transgenic lines. The root growth experiments were repeated twice with similar results.

Arabidopsis seeds were used to determine their fatty acid content. Fatty acid methyl esters were prepared according to Li-Beisson *et al*. (2010) with a few modifications. A total of 20 µg C17:0 TAG (triheptadecanoin) was added as internal standard. Gas chromatography-mass spectrometry (GC-MS) was performed using a *Finnigan* (Markham, Canada) *TRACE* GC-MS system with a *DB-1* column (*Finnigan*). For each genotype, at least two independent transgenic lines were analyzed. For each line, three biological replicates were performed and presented as means ± SD. Statistical analyses were performed with Student's *t*-test.

Results

First, we cloned the *JcKASIII* cDNA fragment with degenerated primers using RACE-PCR. The *JcKASIII* is a single copy gene encoding a protein that shares 80 % sequence identity with *KASIII* from other plants and is thought to be involved in carbon-chain elongation (Li *et al.* 2008a). To analyze JcKASIII abundance, the anti-

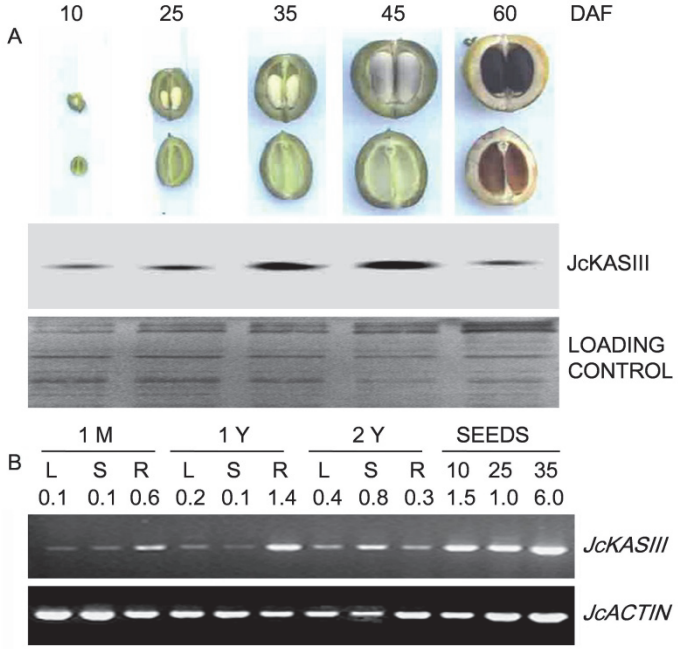

Fig. 1. The expression pattern of *JcKASIII* in *Jatropha*. *A* – The protein expression pattern of *JcKASIII* in seeds. The upper panel indicates the time course of seed development from 10 to 60 d after flowering (DAF). The middle panel shows the abundance of JcKASIII protein in seeds by immunoblotting with the specific rabbit anti-JcKASIII polyclonal antibody. Total protein SDS-PAGE staining with Coomassie blue indicates a loading control (the lower panel). *B* - Semi-quantitative RT-PCR analysis of *JcKASIII* expression. L - leaf, S - stalk, R - root, 1 M - 1-month-old, 1 Y - 1-year-old, 2 Y - 2-years-old. The *JcACTIN* was performed as internal control. Numbers above gel lanes indicate the ratio of the *JcKASIII* products after normalization with *JcACTIN*. The experiments were repeated twice with similar results.

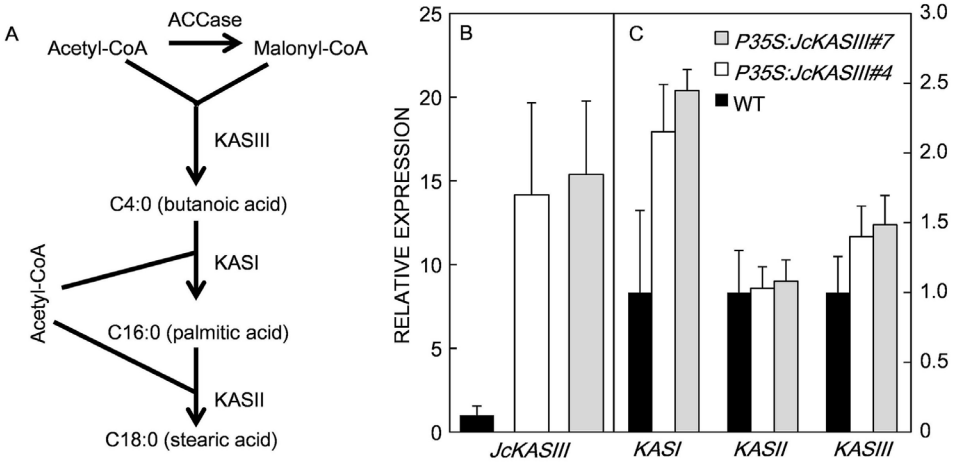

Fig. 2. The relative expressions of *JcKASIII*, *KASI*, *KASII*, and *KASIII* in *P35S:JcKASIII* seedlings. *A* - schematic diagram of the fatty acid biosynthesis pathway. KASI, KAS II, and KAS III - β-ketoacyl-acyl carrier protein synthase I, II, and III. This scheme was modified from Biswas *et al*. 2013. *B* - qRT-PCR analysis of *JcKASIII* expression in *P35S:JcKASIII* seedlings. *C* - qRT-PCR analysis of *KASI*, *KASII*, and *KASIII* expression in *P35S:JcKASIII* seedlings. The relative expression is calculated by comparison with that of WT (set as 1.0). Means ± SE, $n = 3$.

JcKASIII antibody was produced and subjected to ELISA and protein dot blot analysis which confirmed its efficiency and specificity. We investigated in detail the JcKASIII protein content during *Jatropha* seed development from 10 d after flowering (DAF) to 60 DAF by the Western blot and found that the JcKASIII protein was present in all examined developmental stages, reaching its highest content at 45 DAF and decreasing during late fruit development from 45 to 60 DAF (Fig. 1*A*). We also performed the semi-quantitative RT-PCR analysis to examine the temporal and spatial expression patterns of *JcKASIII*. The results revealed that *JcKASIII* was expressed in all tissues examined including roots, stems, and leaves, but the content was much higher in seeds (Fig. 1*B*). The expression of *JcKASIII* during seed development was significantly higher at 35 DAF than at 10 and 25 DAF (Fig. 1*B*), which is consistent with previously published qRT-PCR results (Li *et al.* 2008). In *Arabidopsis*, *AtKASIII* is also expressed more in seeds than in roots, leaves, and stems. Furthermore, since plant *de novo* fatty acid synthesis occurs in plastids (Li-Beisson *et al*. 2010), we decided to check the subcellular localization of the GFP-fused JcKASIII protein in transiently transformed *Arabidopsis* protoplasts and found that this protein was located in some areas of chloroplasts (Fig. 1 Suppl.). Taken together, these results suggest that *JcKSIII* was mainly expressed in seeds and there was a rapid accumulation of fatty acids during later stages of seed development.

To study a potential role of JcKASIII in the overall regulation of the fatty acid biosynthesis *in planta* (Fig. 2*A*), *JcKASIII* was transformed into *Arabidopsis* WT plants under the control of the CaMV-35S promoter. We examined a total of 20 independent kanamycin-resistant transgenic lines and analyzed *JcKASIII* expression by the RT-PCR from cDNA of the transgenic lines and WT to confirm that the *JcKASIII* gene was correctly transcribed in *Arabidopsis* (data not shown). Then we selected two lines for further analysis in which *JcKASIII* was elevated nearly 15-fold at 28 d after sowing (DAS) (Fig. 2*B*). Dehesh *et al*. (2001) suggested that FAS enzymes may be coordinately regulated to counteract changes brought about by genetic overexpression. To determine whether the introduced heterologous KASIII enzyme brought any change into the FAS enzymes, we examined changes in the expression of the three *AtKAS* genes in transgenic plants. The results reveal that the expression of *KASI* in the *P35S:JcKASIII* seedlings was up-regulated nearly 2-fold compared to that of the WT whereas the expressions of *KASII* and *KASIII* were unchanged (Fig. 2*C*). Then we analyzed fatty acid composition in seeds of these two *P35S:JcKASIII* homozygous lines by the GC-MS. The *P35S:JcKASIII* seeds exihibited a significant increase in the content of C16:0 (up to 18 % of total fatty acids), C16:1 (up to 0.9 %), and C18:2 (up to 32.7 %) fatty acids when compared to that of the WT (8.6 %, 0.3 %, and 28.8 %, respectively) (Fig. 4*A*). By contrast, the content of long chain C20:0 and C20:1 fatty acids in the *P35S:JcKASIII* seeds decreased. Moreover, the ratio of the total C16 fatty acid content to the total C18 fatty acid content (C16/C18) in the *P35S:JcKASIII* seeds increased more than two-fold (0.295) when compared to that of the WT (0.140) (Table 1).

Table 1. The ratio of C16 to C18 fatty acids (C16 - the sum of C16:0 and C16:1; C18 - the sum of C18:0, C18:1, C18:2, and C18:3). Means ± SD from three biological replicates; * - significant differences between transgenic lines and WT at $P < 0.001$ according to Student's *t*-test. One representative line from each genotype is presented.

Genotype	C16/C18
WT	0.140 ± 0.031
P35S:JcKASIII#7	0.295 ± 0.012*
KASIII-RNAi#2	0.163 ± 0.005
P35S:JcKASIII/KASIII-RNAi#25	0.306 ± 0.051*
kasI	0.113 ± 0.037
P35S:JcKASIII/kasI#1	0.180 ± 0.006
kasII	0.145 ± 0.034
P35S:JcKASIII/kasII#4	0.167 ± 0.003

We further tested whether the function of JcKASIII can rescue the effect of the *Arabidopsis kasIII* mutant on fatty acid content. Since potential *KASIII* null mutants could not be identified from the *Arabidopsis* T-DNA insertion databases, we used an RNAi approach to study the function of JcKASIII. Firstly, we aligned *JcKASIII*, *AtKASIII*, *AtKASII*, and *AtKASI* sequences and chose *AtKASIII* specific fragments for knockdown of only *AtKASIII* in *Arabidopsis*. Transcript analysis of two representative *KASIII-RNAi* lines at 28 DAS revealed that the expression of *KASIII* was reduced to 20 % of that in the WT (Fig. 3*A*). The content of C16:0 fatty acids was unchanged in the *KASIII-RNAi* seeds, but there was an increased content of C18:0 fatty acids when compared to that of the WT (Fig. 4*B*). In the seeds of the *KASIII-RNAi* plants with *JcKASIII* expression, the content of C18:0 fatty acids was largely restored to that of the WT. The content of C16:0 fatty acids was elevated nearly two-fold when compared to that of the WT. The ratio of C16 to C18 fatty acids increased about two-fold (Table 1). Taken together, these results suggest that *Jatropha* JcKASIII could function well in the *Arabidopsis* FAS system and substitute for *Arabidopsis* KASIII to a certain degree, resulting in an elevated content of C16:0 fatty acids and a higher C16/C18 ratio.

To further explore the influence of interference of *KASIII* expression on seedling development, seeds from the T3 homozygous populations of the *KASIII-RNAi* and *P35S:JcKASIII/KASIII-RNAi* transgenic lines were germinated on the MS medium, and root lengths were measured. We found that the *KASIII-RNAi* seedlings showed fewer and shorter roots than the WT plants when

they were grown in the absence of sucrose and glucose (Fig. 5*A,E*). The addition of 1 % (m/v) sucrose or glucose mostly recovered root growth (Fig. 5*F,G*). The roots of the *P35S:JcKASIII/KASIII-RNAi* seedlings resembled those of the WT when the plants were grown in the absence and presence of sucrose or glucose (Fig. 5*I,J,K*). The same results were obtained with the addition of mannitol which served as positive control (Fig. 5*D,H,L*).

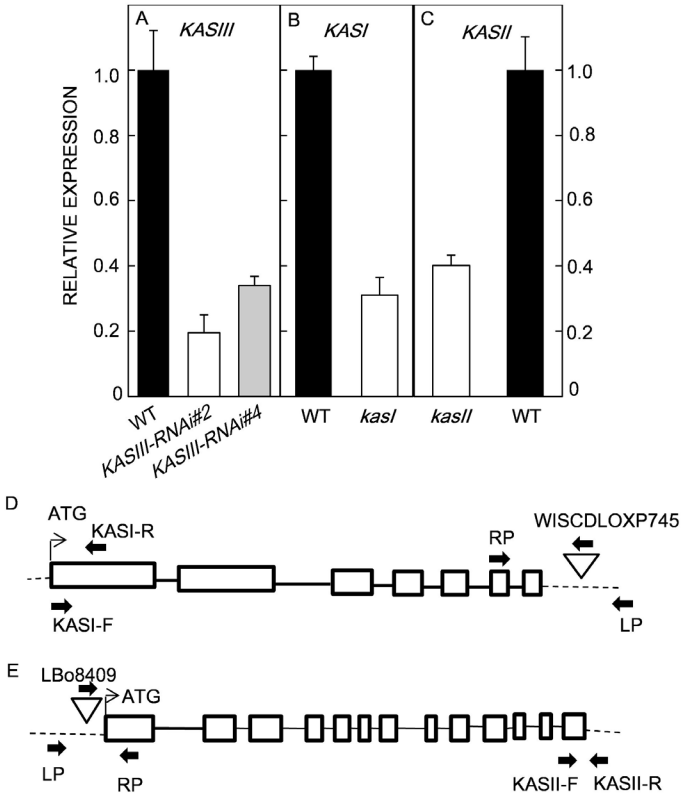

Fig. 3. The relative expression of *KASIII*, *KASI*, and *KASII* in *KASIII-RNAi, kasI*, and *kasII* seedlings, respectively. *A* - the qRT-PCR analysis of *KASIII* expression in the *KASIII-RNAi* seedlings. *B* - the qRT-PCR analysis of *KASI* expression in the *kasI* seedlings. *C* - the qRT-PCR analysis of *KASII* expression in the *kasII* seedlings. The relative expression was calculated by comparison with that of the WT (set as 1.0). Means ± SE, $n = 3$. *D* - a schematic representation of the *KASI* gene. A primer pair KASI-F/R (located close to the start codon ATG) was used for the qRT-PCR analysis of *KASI* expression. *E* - a schematic representation of the *KASII* gene. A primer pair KASII-F/R (located close to the stop codon TGA) was used for the qRT-PCR analysis of *KASII* expression. The *open boxes*, *lines*, and *dotted lines* indicate exons, introns, promoter regions (*left*) and 3'-UTRs (*right*), respectively. The *triangles* indicate T-DNA insertion sites.

A T-DNA insertion line (CS853792) of *KASI*, named *kasI*, was identified from ABRC. The T-DNA was inserted in the 3'-UTR of *KASI* (202 nucleotides upstream of the TGA stop codon) (Fig. 3*D*). Analysis using T-DNA border primers (WISCDLOXP745) and a KASI-RP primer confirmed the insertion of T-DNA in the *KASI* gene (Fig. 2 Suppl.). The qRT-PCR analysis using primers KASI-F/R (located in *KASI* upstream regions) revealed that the expression of *KASI* in the *kasI* mutants were reduced to 31 % of that in the WT (Fig. 3*B*). The content of C16:0 and C18:1 fatty acids in the *kasI* mutants were reduced to 6.6 and 13.5 % of total fatty acids compared to that of the WT (to 8.6 and 17.3 %), respectively (Fig. 4*C*). In the seeds of the *kasI* mutant with expression of *JcKASIII*, the content of C16:0 fatty acids was elevated up to 11.1 % of total fatty acids (Fig. 4*C*). The C16/C18 ratios among the WT, *kasI*, and *P35S:JcKASIII/kasI* seeds were not significantly different (Table 1).

A T-DNA insertion line (CS350573) of *KASII*, named *kasII*, was identified from ABRC. The T-DNA was inserted in the 5'-UTR of *KASII* (64 nucleotides upstream of the ATG start codon) (Fig. 3*E*). Analysis using T-DNA border primers (GABILBo8409) and a KASII-RP primer confirmed the insertion of T-DNA in the *KASII* gene (Fig. 2*B* Suppl.). The qRT-PCR analysis using primers KASII-F/R (located in *KASII* downstream regions) revealed that the expression of *KASII* in the *kasII* mutants was reduced to 40 % of that in the WT (Fig. 3*C*). In the *kasII* mutant, there were no great differences in the content of C16 and C18 fatty acids compared to the WT (Fig. 4*D*). In the *P35S:JcKASIII/kasII* seeds, the content of C16:0 fatty acids was elevated to 10.5 % of total fatty acids whereas the content of C20:1 fatty acid decreased to

17.8 % when compared to that of WT, respectively (Fig. 4*D*). The C16/C18 ratios of the WT, *kasII*, and *P35S:JcKASIII/kasII* seeds were not significantly different (Table 1). Taken together, these results suggest that JcKASIII had a general role in increasing content of C16:0 fatty acids at the expense of long chain C18 and C20 fatty acids.

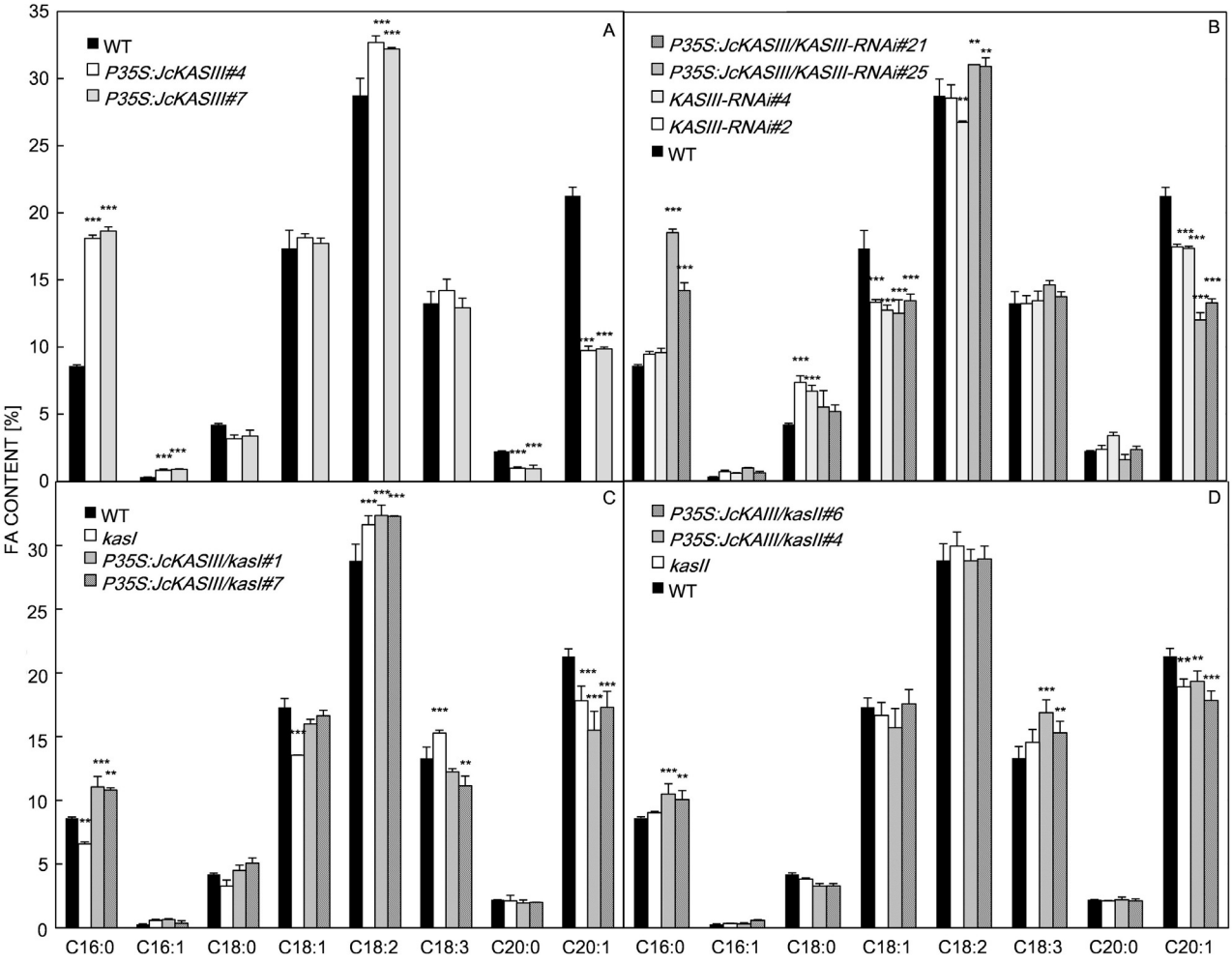

Fig. 4. Heterologous expression of *JcKASIII* modulated fatty acid (FA) composition in *Arabidopsis seeds*. *A* - FA content in WT and *P35S:JcKASIII* seeds. *B* - FA content in seeds of WT, *KASIII-RNAi*, and *P35S:JcKASIII/KASIII-RNAi* transgenic lines. *C* - FA content in seeds of WT, *kasI*, and *P35S:JcKASIII/kasI* transgenic lines. *D* - FA content in seeds of WT, *kasII*, and *P35S:JcKASIII/kasII* transgenic lines. Means ± SE, $n = 3$. Significant differences between transgenic lines and WT at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. FA content is in percentage of total fatty acids.

Discussion

Jatropha curcas is currently one of the highly promoted oilseed crops. The major fatty acids in *Jatropha* seed are C18:1, C18:2, C16:0, and C18:0 fatty acids (Gubitz *et al*. 1999). The fatty acid profile of seed oil directly determines the properties of biodiesel such as the cetane number (CN) value (Bamgboye *et al*. 2008). The CN value of *Jatropha* seed oil is within the range of 43.4 to 51, which is lower than that of the conventional premium diesel fuel (60) in Europe (Qu *et al*. 2012). To increase the CN value of *Jatropha* seed oil, it is necessary to increase the ratio of C16 to C18 fatty acids. In this study, we utilized the model plant *Arabidopsis* to investigate the function of *Jatropha* KASIII in regulation of C16 and C18 fatty acid biosyntheses. We overexpressed *JcKASIII* in the *Arabidopsis* WT, *KASIII-RNAi*, *kasI*, and *kasII* plants to examine the effect of *JcKASIII* on fatty acid content. The results reveal that overexpression of heterologous *JcKASIII* led to an increased C16:0 fatty acid content along with a compensatory decrease in long chain fatty acid content in the *Arabidopsis* seeds. The

ratio of C16 to C18 fatty acids increased two-fold in the *JcKASIII* overexpression seeds. Functional characterization of *JcKASIII* provides useful information for understanding fatty acid metabolism of *Jatropha* and may be useful in genetic engineering of this plant.

Overexpression of heterologous *JcKASIII* in the *Arabidopsis* WT plants increased expression of *KASI* and led to an increase in content of C16:0 and C18:2 fatty acids, which was compensated by a decrease in content of C20:1 fatty acid. In an earlier study, overexpression of *E. coli KASIII* in rapeseed also led to an increase in content of C18:2 fatty acid and an accompanied decrease in content of C18:1 fatty acid (Verwoert *et al*. 1995). In the plastid, KASIII mainly catalyzes a condensation reaction of malonyl-ACP with acetyl-CoA to yield ketobutyryl-ACP which also serves as one of the substrates used by KASI to catalyze a subsequent condensation reaction. Therefore, introduction of heterologous KASIII into *Arabidopsis* may result in an elevated amount of butyryl-ACP substrate for KASI and consequently lead to an increased KASI activity, which is in accordance with the findings of a previous immunoblot analysis study revealing an increased content of ACP in plants over-expressing *KASIII* (Dehesh *et al.* 2001).

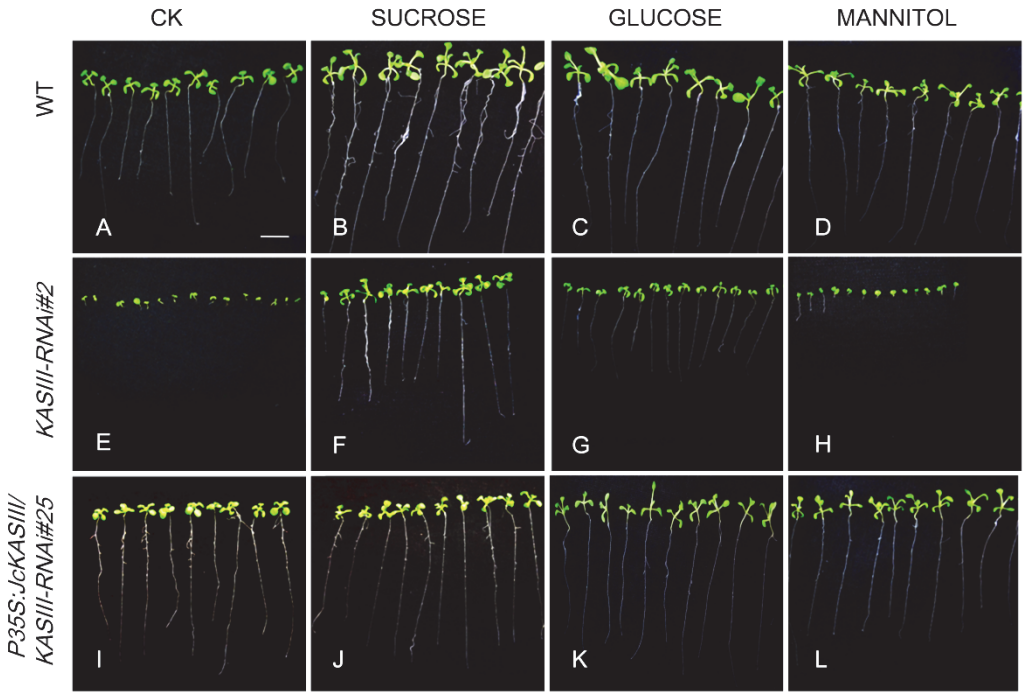

Fig. 5. Roots of 10-d-old WT, *KASIII-RNAi*, and *P35S:JcKASIII/KASIII-RNAi* seedlings grown in a sucrose-free MS medium (*A*,*E*,*I*), 1.0 % sucrose-containing medium (*B*,*F*,*J*), 1.0 % glucose-containing medium (*C*,*G*,*K*), and 1.0 % mannitol-containing medium (*D*,*H*,*L*). For each genotype, at least two independent lines were analyzed. For each line, $n = 30$ seedlings. The experiments were repeated twice with similar results. *Bar* = 1.0 cm.

Recently, functional characterization of several genes in *Jatropha* plants has been reported, including *JcFAD2*, *JcFT*, and *JcSDP1* (Ye *et al*. 2009, 2012, 2014a,b, Qu *et al*. 2012, Kim *et al*. 2014). These works greatly contributed to the genetic engineering of *Jatropha*. Our group has attempted to establish the genetic transformation system in *Jatropha* using kanamycin selection. But rooting efficiency is low, which may be due to the sensitivity of *Jatropha* tissues to the selection agent. Different selection agents including hygromycin, kanamycin, and phosphine-thricin have been tested in *Jatropha*, nevertheless, the transformation and seedling rate are still low (Li *et al*. 2008b, Kumar *et al*. 2010, Pan *et al*. 2010, Zong *et al*. 2010). More work may be done by using a different selection antibiotic marker gene. In addition, the virus-induced gene silencing (VIGS) method has been used in *Jatropha* leaves for testing roles of several genes including *JcKASII* and *JcFATB* (Ye *et al*. 2009). With optimization of VIGS in *Jatropha* fruits and seeds, functional analysis of other genes involved in fatty acid metabolism in *Jatropha* may be suitable. With development of a genetic transformation system (Ye *et al*. 2012) and functional genomics (Maghuly and Laimer 2013), molecular breeding of *Jatropha* with a better quality and higher yield of oil should become feasible.

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