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Original Article

Identification, characterization and expression analysis of *SHORT INTERNODES* (*SHI*) gene in *Jatropha curcas* L

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ABSTRACT

Physic nut (*Jatropha curcas*) is a valuable crop whose seeds contain a high percentage of oil and can be converted to high-quality biodiesel. Physic nut can grow in tropical and subtropical areas and has minimal requirements of its environment. Physic nut starts its production 9 mth after planting and its yield peaks in 2–3 yr. The major hurdle in harvesting its seeds is the extreme height of this crop. *J. curcas* plants often reach 1.5–2 m after the first year and continue to grow. Plant height control had been achieved by manipulating levels of gibberellic acid (GA). One possible way to accomplish GA manipulation is through the action of the *SHORT INTERNODES (SHI)* gene since it has been found to repress GA responses. The current study identified the *SHI* gene in *J. curcas*. Further characterization of the expression pattern and structure of the *SHI* gene revealed that *JcSHI* is a single copy gene that encodes 315 amino acid residues and is highly expressed in the shoot meristem and root. The phylogenetic tree showed a close relationship between JcSHI and PtSHI that are both in woody plants. The JcSHI protein contained a specific RING zinc finger (C3HC3H type) and the IGGH domain indicating the possible function of *SHI* as a transcriptional regulator of genes involved in plant growth and elongation.

Introduction

Fossil fuel is a non-reproducible resource for fuel (MacKenzie, 1998). The accelerating energy demand has imposed an urgent need to develop alternative energy sources (Shahzad, 2015). Biodiesel is a good candidate since it can be used in either pure form or blended with petroleum diesel (Demirbas, 2009). It has been reported that biodiesel blends can significantly reduce particulate matter and carbon elements released into the environment (Traviss et al., 2012). There are many plant materials that can be used to produce biodiesel such as algae, pennycress, oil palm, coconut, sun flower, soybean and Jatropha (Hassan and Kalam, 2013). However, many candidate materials have faced technical challenges as well as competing with alternative use for human consumption (Tenenbaum, 2008). Compared with the above resources, *Jatropha curcas* has several advantages such as its fast and adaptable growing conditions as well as presenting no competition to human consumption (Fatemeh and Laimer, 2013).

Jatropha curcas L. or physic nut ($2n = 2x = 22$) is a biofuel plant that originated in South America (Fatemeh and Laimer, 2013). It is widely cultivated in the tropical and subtropical zones around the world because of its ability to tolerate drought and its resistance to several pest and diseases (Openshaw, 2000). Besides being used for oil production, the plant can be used for animal feed, herbal drugs or hedges (Debnath and Bisen, 2008). The physic nut seed contains 27–40% oil by weight (Ginwal et al., 2004; Vyas and Singh, 2007; Achten et al., 2008) which can be easily extracted to give a high-quality biodiesel (Achten et al., 2007). Major concerns in growing *J. curcas* on a large scale is the low seed yield and the difficulty in harvesting (Rivero et al., 2016). Normally, a one-year-old *J. curcas* plant often reaches 1.5–2 m in height which becomes problematic for hand picking which is the most common method of harvesting (Yang et al., 2010).

Controlling the crop height and breeding semi-dwarf crops have been applied to multiple species including wheat, sorghum and rice (Hirano et al., 2017; Peng et al., 1999; Sasaki et al., 2002). Most of semi-dwarf phenotypes have been achieved by a gibberellic acid (GA) deficiency. The *SHI* (*SHORT INTERNODES*) gene is an excellent candidate to breed a shorter variety of *J. curcas* as the *SRS* (*SHI-RELATED SEQUENCE*) gene family includes *SHI, LATERAL ROOT PRIMORDIA 1* (*LPR1*), *STYLISH1* (*STY1*), *STYLISH2* (*STY2*) and

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SRS3–SRS8 genes (Kuusk et al., 2002; Fridborg et al., 2001). This gene family is involved in the regulation of plant growth and development (Fridborg et al., 1999, 2001). The *SHI* gene is a negative regulator of gibberellin (GA) hormones that are involved in promoting the growth and elongation of plant cells (Itoh et al., 1999; Zawaski et al., 2011). In *Arabidopsis,* overexpressing the *SHI* gene caused a dwarf phenotype as well as altering the endogenous levels of multiple GAs (Fridborg et al., 1999). In *Kalanchoe*, *Populus* and poinsettia, the presence of the *Arabidopsis SHI* (*AtSHI*) overexpression cassette resulted in dwarf growth without abnormal morphology. The dwarf phenotype of *AtSHI* mildly overexpressed *Populus* because the decrease in internode length was compensated by the increase in the number of internodes, while the decrease in height was more pronounced in *AtSHI* overexpressed *Kalanchoe* (Lütken et al., 2010; Zawaski et al., 2011). In poinsettia, overexpression of *AtSHI* led to a more compact shape without any flowering delay and the level of *AtSHI* expression correlated well with the level of compact stature of the plant (Islam et al., 2013).

In the current study, SHI from *J. curcas* was identified based on the protein similarity and the critical domains were characterized. From the protein structure prediction, the *SHI* protein seemed to function in the nucleus and involved in transcription regulation. The analysis of gene expression was done in several tissue types of *J. curcas* by both semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (PCR).

Material and methods

Plant material

Mature seeds of *J. curcas* cultivar KUBP 78-9 were obtained from Miss Anna Saimaneerat, the National Corn and Sorghum Research Center, Thailand under collaboration through the Kasetsart University Biodiesel Project.

Identification of J. curcas SHI gene

The coding sequence (CDS) of the *Arabidopsis thaliana SHI* (*AtSHI*) gene (NM_126033.1) was used to search for the *J. curcas SHI* (*JcSHI*) gene from the *J. curcas* complete genome in the GENBANK database using a nucleotide blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained *J. curcas* sequence was used to design *JcSHI* specific primers.

Cloning and sequencing of JcSHI gene

Total RNA was extracted from 21-day-old *Jatropha* seedlings using the GF-1 Total RNA Extraction Kit (Vivantis; Chino, CA, USA) that included DNase treatment and RNA purification steps. Then, 3 μg of total RNA was used to synthesize cDNA with Viva-2 Step RT PCR (Vivantis; Chino, CA, USA). The coding sequence of the *JcSHI* gene was amplified with the primer pair JcSHI_F and JcSHI_R (Table 1) that were designed based on the selected sequence from the BlastN result. The PCR reactions (10 μL) were performed using 1 μL of cDNA, 1X of ViBuffer A, 0.2 μM of each primer, 1.5 mM of $MgCl_2$, 1 mM of dNTPs Mix and 1 U of Taq DNA polymerase (Vivantis; Chino, CA, USA) using the following PCR conditions: 94 °C for 10 min; then 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and a final extension step of 72 °C for 10 min. The PCR product was analyzed using gel electrophoresis and the remaining product was purified using the GF-1 AmbiClean Kit (Gel & PCR) (Vivantis; Chino, CA, USA) and ligated with pGEM-T easy vector (Promega; Madison, WI, USA) and then transformed into *Escherichia coli*. Sequencing of the putative *JcSHI* gene on the plasmid was done by Macrogen (City, Republic of Korea).

Table 1
Primer names and sequences.

No.	Name	Sequence (5′–3′)
1	SHI_JC_F	CACCATGGCTGGGTGGTTCTATC
2	SHI_JC_R	CTAGGATCTTGGGGGTGG
3	SHI_582JC_R	TGACGTGGTAGTGCCTAAAC
4	qJcKASIII_F	TCTCAATCTCAAATACCCAGG
5	qJcKASIII_R	ACTAAAAACCATTACCAATCCAT
6	qSHI_F	CAACAACATCAAGAACAGCAAC
7	qSHI_R	GCCTCTAAACATATGACCTCC
8	qJcActin_F[a]	AAACGCGTATGGCTGATGCTGAGGATATCC
9	qJcActin_R[a]	AATCCAGCACGATACCAGTTGTACG

[a] Primers selected from Juntawong et al. (2014).

Protein sequence comparison and phylogenetic analysis

The *JcSHI* amino acid sequence (JcSHI, BAJ53180.1) was used to construct phylogenetic trees with protein sequences from *Arabidopsis thaliana* (AtSHI, NP_190675.4), *Populus trichocarpa* (PtSHI, XP_006383148.1), *Zea mays* (ZmSHI, NP_001150905.1) and *Physcomitrella patens* (PpSHI, AAX53173.1). The phylogenetic trees were constructed using the MEGA 6 program (Tamura et al., 2013). For comparison, phylogenetic trees were separately constructed from five models—maximum likelihood (ML), neighbor-joining (NJ), unweighted pair group method with arithmetic mean (UPGMA), minimum evolution (ME) and maximum parsimony (MP)—with 1000 bootstrap replicates.

Protein structure analysis

In total, five SHI protein sequences (AtSHI, JcSHI, PtSHI, ZmSHI and PpSHI) were analyzed by using Jpred 4 (Drozdetskiy et al., 2015; http://www.compbio.dundee.ac.uk/jpred/), InterPro (Mitchell et al., 2015; http://www.ebi.ac.uk/interpro/), Motif Scan (Pagni et al., 2004; http://myhits.isb-sib.ch/cgi-bin/motif_scan) and PROSITE (Sigrist et al., 2013; http://prosite.expasy.org/).

Determination of JcSHI copy number using quantitative real-time PCR

Quantitative real-time PCR was used to determine the *JcSHI* gene copy number using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). As a reference, a single copy *KAS*III gene (NCBI accession number DQ987701.1) was used (Li et al., 2004). Primers were designed for the *JcSHI* gene's exon 1 (SHI_JC_F and SHI_JC_582R) and for the *JcKASIII* gene's exon 1 and 4 (qJcKASIII_F and qJcKASIII_R) that amplify the 582 bp and 412 bp fragments, respectively (Table 1). Genomic DNA extracted from young leaves using the CTAB method (Doyle and Doyle, 1990) was used as a DNA template to determine the efficiency of the primers. The 10-fold gDNA dilution series (500 ng/μL, 50 ng/μL, 5 ng/μL and 0.5 ng/μL) was amplified in triplicate with the *JcSHI* and *JcKAS*III specific real-time primers separately using a KAPA SYBR FAST qPCR kit Master Mix (2X) Universal (KAPA Biosystems; Wilmington, MA, USA) with Master Cycler Realplex 4 (Eppendorf; Hauppauge, NY, U.S.A.). The PCR reactions (10 μL) were performed using 1 μL of each gDNA dilution, 1X of KAPA SYBR FAST qPCR kit Master Mix and 0.8 μM of each primer with the following PCR conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 25 s. Melting curve analysis was done as the final step according to the instrument guidelines. The obtained C_T values and the log of fold gDNA series dilution were used to examine Δn and the correlation coefficients (R^2), and the standard curve was constructed using the

linear regression method. The slope value of standard means Δn was used to calculate the efficiency value using the equation: % $E = [10^{(-1/slope)}-1] \times 100$.

To determine the gene copy number, 5 ng/μL of gDNA was amplified in triplicate with *JcSHI* and *JcKAS*III quantitative real-time PCR primers separately using the same reactions and conditions as the efficiency test for three biological replicates. The copy number of the *SHI* gene was calculated using equation: *SHI* gene copy number $= 2^{-\Delta CT}/\Delta C_T = C_T$ (mean *SHI*)$-C_T$ (mean *KAS*III).

SHI expression analysis in J. curcas

The three tissue types from 7-day-old seedlings (cotyledon, stem and root) and five tissue types from 21-day-old seedlings (cotyledon, mature leaf, stem, root and shoot meristem) were used for total RNA extraction and subsequently for cDNA synthesis. The primer pair of *J. curcas*'s *Actin* (qActin_F and qActin_R) were used according to Juntawong et al. (2014). It amplified a 477 bp fragment from cDNA. The *JcSHI* primer pair (qSHI_F and qSHI_R) was designed to flank an intron and amplified a 218 bp fragment from cDNA and a 700 bp fragment from gDNA (Table 1).

Semi-quantitative RT-PCR was carried out to evaluate tissue-specific expression analysis. The *Actin* gene was used as the cDNA loading control. Each 10 μL reaction contained 2 μL of cDNA, 1X of ViBuffer A, 0.2 μM of each primers, 1.5 mM of $MgCl_2$, 1 mM of dNTPs Mix and 1 U of Taq DNA polymerase (Vivantis; Chino, CA, USA). The following PCR conditions were used: 94 °C for 10 min; 28 cycles of 94 °C for 10 s, 60 °C for 10 s and 72 °C for 20s. For the *SHI* gene, the PCR reactions were performed using 2 μL of adjusted cDNA with the same PCR reaction and conditions as for the *Actin* gene. The PCR products were collected at 20 cycles, 25 cycles, 30 cycles, 35 cycles and 40 cycles and were analyzed using agarose gel electrophoresis.

To confirm the tissue-specific expression results from the semi-quantitative RT-PCR, the quantitative real-time PCR was carried out for three biological replicates. The primer pairs of the *Actin* and *SHI* genes were tested for efficiency separately. Root cDNA from 21-day-old seedlings was used as templates for the efficiency test using the five concentrations of the 10-fold dilution series. The PCR reactions (10 μL) were performed using 2 μL of each cDNA dilution, 1X of KAPA SYBR FAST qPCR kit Master Mix and 0.2 μM of each primer using the following PCR conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. The melting curve application was added as the final step according to the instrument guidelines. Master Cycler Realplex 4 started from 95 °C for 15 s and th temperature increased from 60 °C to 95 °C for 15s per temperature unit. Each reaction was done as three replicates. Next, to determine the expression of the *SHI* gene, cDNA of each sample was assayed with the same quantitative real-time PCR reaction and conditions as the efficiency test. C_T values were obtained and the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to evaluate the relative expression level. The ΔC_T values were analyzed using analysis of variance followed by Tukey's HSD post hoc test.

Results

Cloning and sequence analysis of JcSHI gene

The *AtSHI* gene (NM_126033.1) sequence was blasted against the *J. curcas* DNA sequence database (Sato et al., 2011) using the BlastN program and JHL18I08.14 (AP_011966.1) was acquired with 73% identity. The putative *J. curcas SHI* gene cloned from cultivar KUBP 78-9 was 1467 bp long containing 2 exons and 1 intron (Fig. 1). The coding sequence (CDS) was 948 bp encoding 315 amino acids. This gene was addressed as *JcSHI* in the following context.

Fig. 1. Gene structure of putative *JcSHI* gene and locations and directions of primers.

SHI protein sequence comparison

Comparing the SHI protein sequences from *A. thaliana* (AtSHI), *J. curcas* (JcSHI), *P. trichocarpa* (PtSHI), *P. patens* (PpSHI) and *Z. mays* (ZmSHI) showed pairwise percentage identities in the range 38.50–59.74% (lower triangle in Table 2). The highest percentage identity was between JcSHI and PtSHI (59.74%) while the lowest percentage was between JcSHI and PpSHI (38.50%). Upon looking at the conserved domain, the pairwise comparison of 31 amino acid residue sequences of the RING zinc finger domain from the five proteins showed high percentage identity (more than 77%) as seen in the upper triangle in Table 2. For the RING zinc finger domain, the highest percentage identity was between ZmSHI and PtSHI (93.55%) and the lowest percentage was between PpSHI and JcSHI, and between AtSHI and PtSHI (77.42%). High conservation in the number of residues and core residues of the SRS RING zinc finger domain was observed.

Phylogenetic relationship of SRS protein family

The sequence of the JcSHI protein was used to construct phylogenetic trees along with SHI protein sequences from 4 plant species including *A. thaliana*, *P. trichocarpa*, *Z. mays* and *P. patens*. The trees created from using the maximum likelihood, neighbor-joining, UPGMA, and minimum evolution methods were similar, so only the result from the maximum likelihood method is presented here (Fig. 2A). JcSHI was a sister group to PtSHI and they were placed in the same clade as AtSHI with high bootstrap values (more than 85%) forming a dicotyledon clade. ZmSHI was placed closer to the dicotyledon clade, forming an angiosperm group, while the bryophyte PpSHI was placed the furthest. The maximum parsimony method yielded a slightly different tree (Fig. 2B) in which ZmSHI shared the same node as PpSHI; however, this grouping was supported by a very low bootstrap value (lower than 50%).

Secondary structure of SHI protein

The results from the Jpred 4 program showed that the JcSHI, PtSHI and AtSHI proteins have similar structures. They all contain 8 β-sheets and 1 α-helix with the structure β-sheets5 – α-helix1 – β-sheets3 while the ZmSHI protein showed a slightly different structure of β-sheets3 – α-helix1 – β-sheets3 (6 β-sheets and 1 α-helix). The PpSHI protein, however, showed a different structure

Table 2
Pairwise sequence percent identity for five SHI proteins based on complete amino acid sequences (lower triangle) and the RING zinc finger domains (upper triangle) from *J. curcas* (JcSHI), *A. thaliana* (AtSHI), *P. trichocarpa* (PtSHI), *P. patens* (PpSHI) and *Z. mays* (ZmSHI).

Protein	JcSHI	AtSHI	PtSHI	ZmSHI	PpSHI
JcSHI		80.65	90.32	87.10	77.42
AtSHI	51.55		83.87	90.32	77.42
PtSHI	59.74	54.29		93.55	77.42
ZmSHI	40.15	45.96	45.96		80.65
PpSHI	38.50	39.57	39.67	38.66	

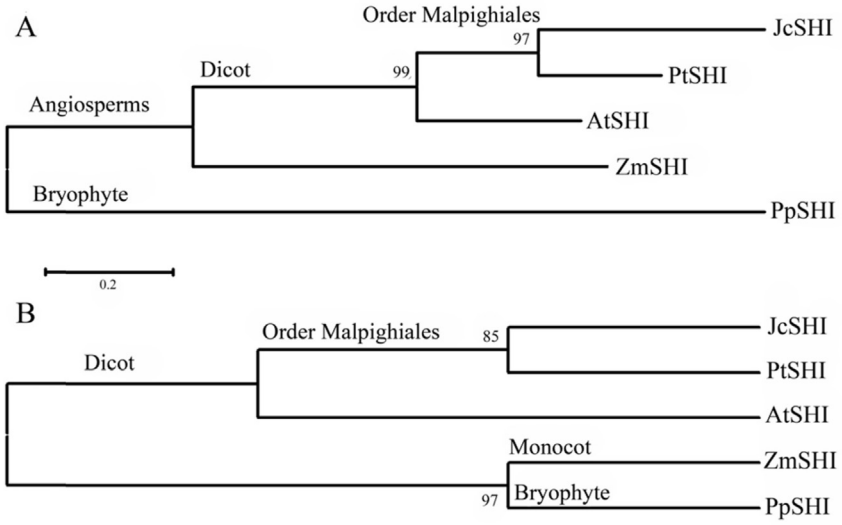

Fig. 2. Phylogenetic tree of five SHI proteins: *J. curcas* (JcSHI), *A. thaliana* (AtSHI), *P. trichocarpa* (PtSHI), *Z. mays* (ZmSHI) and *P. patens* (PpSHI), where trees were constructed using: (A) maximum likelihood; (B) maximum parsimony, with 1000 replicates for the bootstrap test using MEGA 6 program.

of 6 β-sheets and 2 α-helixes with the structure β-sheets2 – α-helix1 – β-sheets1 – α-helix1 – β-sheets3. Despite variations, all these proteins showed the same grouping and equal spacing between the last 3 β-sheets (the approximate structural maps are shown in Fig. 3).

Protein structure of JcSHI protein

Analysis from various programs revealed that the AtSHI, JcSHI, PtSHI, ZmSHI and PpSHI proteins shared a conserved domain of the SRS protein family containing the RING zinc finger domain and the IGGH domain. The RING zinc finger domain in the SRS protein was in the class C3HC4 and could be specifically described as Cys1-X2-Cys2-X7-Cys3-X1-H1-X2-Cys4-X2-Cys5-X7-Cys6-X2-H2 (C3HC3H in short) (Fig. 4), where X represents any amino acid residues (except in the PpSHI protein that lacked the first histidine). The nuclear localization signal (NLS) was found behind the RING zinc finger domain in all SHI proteins. Furthermore, the cysteine rich region and glutamine rich region (except in ZmSHI and PpSHI) were found in all sequences. Finally, all five SHI proteins terminated with a specific C-terminal of the RING zinc finger domain (the approximate protein domain map is shown in Fig. 5).

Gene copy number of SHI in J. curcas

The efficiency test of quantitative real-time PCR primers for the *SHI* gene copy determination showed high efficiencies (86.5% and 100.3% for the *JcKAS*III and *JcSHI* fragments, respectively) and high correlation coefficients (0.994 and 0.993 for the *JcKAS*III and *JcSHI* fragments, respectively). The specific melting temperatures were 79 °C and 84 °C for the *JcKAS*III and *JcSHI* fragments, respectively. The results from quantitative real-time PCR of the *JcSHI* and *JcKAS*III genes when calculated for the *JcSHI* copy number were 0.8, 0.8 and 0.9 for the three biological replicates. Hence the *JcSHI* gene is a single copy gene (see Fig. 6).

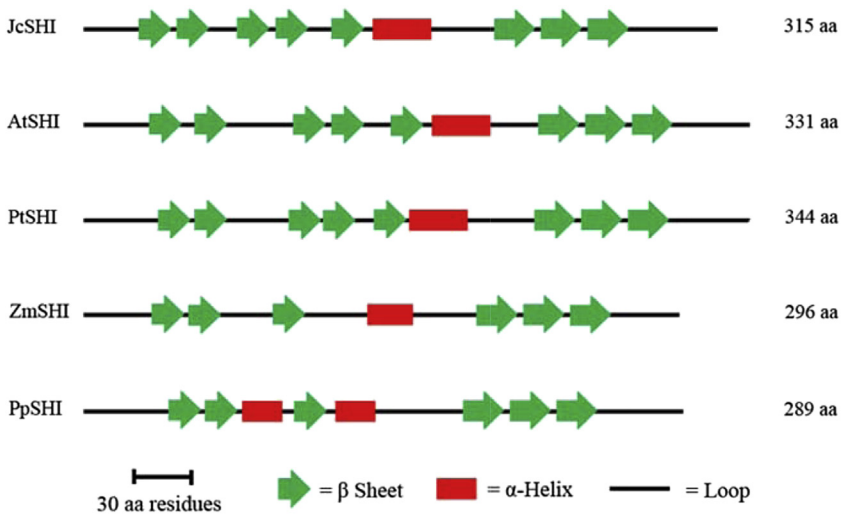

Fig. 3. Approximate secondary protein structure map of SHI protein from *A. thaliana* (AtSHI), *J. curcas* (JcSHI), *P. trichocarpa* (PtSHI), *P. patens* (PpSHI) *and Z. mays* (ZmSHI), where scale bar represents a distance of 30 amino acid residues.

Fig. 4. RING zinc finger domain comparison of five SHI proteins from *A. thaliana* (AtSHI), *J. curcas* (JcSHI), *P. trichocarpa* (PtSHI), *P. patens* (PpSHI) and *Z. mays* (ZmSHI), where * represents the core residue of protein motif. Black shade = 100% identical in all species, gray shade <100% identical in all species.

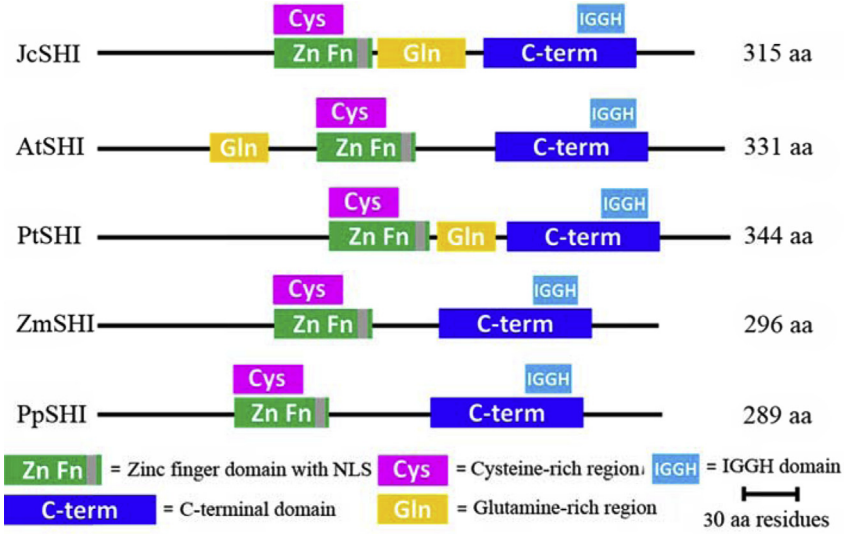

Fig. 5. Approximate protein domain map of SHI proteins from *A. thaliana* (AtSHI), *J. curcas* (JcSHI), *P. trichocarpa* (PtSHI), *P. patens* (PpSHI) *and Z. mays* (ZmSHI), where scale bar represents 30 amino acid residues.

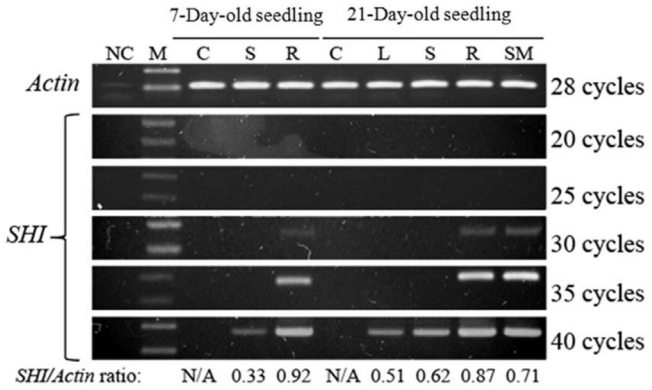

Fig. 6. Semi-quantitative RT-PCR results of *SHI* gene in 3-day-old seedling and 21-day-old seedling, where first row shows *Actin* expression and C = cotyledon, S = stem, R = root, L = leaf, SM = shoot meristem and N/A = not available.

SHI expression analysis in J. curcas

From the semi-quantitative RT-PCR results, no detectable levels of *SHI* expression were found in cotyledons from the 7- and 21-day-old seedlings while high expression levels were observed in the root tissue from the 3-day-old seedlings and in root and shoot meristems from the 21-day-old seedlings. Intermediate *SHI* expression was found in the stem from the 21-day-old seedlings. Low expression levels were observed in the leaf samples from the 21-day-old seedlings. The lowest *SHI* expression was detected in the stem tissue from the 3-day-old seedlings.

The efficiency test of the quantitative real-time PCR primers used in the *JcSHI* expression analysis showed high efficiencies (110.7% and 98.2% for the *JcActin* and *JcSHI* fragments, respectively) with high correlation coefficients (0.990 and 0.998% for the *JcActin* and *JcSHI* fragments, respectively). The specific melting temperatures of *J. curcas*'s *Actin* and *SHI* PCR products were 83 °C. The sequences of both fragments were determined using the Oligo Calc program. (Oligonucleotide Properties Calculator; http://biotools.nubic.northwestern.edu/OligoCalc.html). The results showed similar values for both the guanine-cytosine content and melting temperature between the amplified fragments from the two genes (48%, 84 °C for *Actin* and 47%, 81.5 °C for *SHI*, respectively). To confirm the size and number of bands in the reaction, the quantitative real-time PCR products were separated using agarose gel electrophoresis. A clear DNA band representing specific amplification was obtained for each fragment. The *JcSHI* expression levels from the three biological-replicates showed a consistent pattern with the results from the semi-quantitative RT-PCR and quantitative real-time PCR, indicating good agreement. The cotyledons from 7-day-old seedlings (7C) showed no detectable expression when detected using the semi-quantitative RT-PCR method and showed the lowest expression level when detected using quantitative real-time PCR. The expression level of cotyledons from 7-day-old seedlings (7S) was regarded as 1-fold and was used for comparison with expressions in other tissue types. High expression levels of *JcSHI* were found in the roots of 7-day-old seedlings (7R) and the root and shoot meristems of 21-day-old seedlings (21R and 21SM, respectively) with the level of significance at $p < 0.05$. Intermediate expression was found in the stems of 21-day-old seedling (21S), while l ow expressions were found in the stems of 7- day-old

seedlings (7S) and the cotyledons and the leaves of 21-day-old seedlings (21C and 21L, respectively) (Fig. 7).

Discussion

Specific RING zinc finger domain of SRS protein family

Sequence analysis revealed the similarity of JcSHI to its homologs from other species (*Arabidopsis, Populus*, *Zea mays* and *P. patens*); JcSHI contains the RING zinc finger C3HC3H type, a nuclear localization signal and an IGGH domain. The RING zinc finger domain is a member of the zinc finger domain protein family with a rich cysteine region. It was first identified as a DNA-binding motif in a transcription factor type (TFIIIA) from *Xenopus laevis* (Kaulen et al., 1991) and later showed its capability of binding RNA (Elenbaas et al., 1996), protein and lipid (Berg and Shi, 1996). The orientation of cysteine and histidine allows for attachment to 2 Zn^{2+} ions and to bind with a specific substrate by being cysteine-rich. The function of the zinc finger proteins depends on the residues that form the motif.

The RING zinc finger type C3HC3H identified in the SRS protein family corresponded well with the classical RING type C3HC4 described as Cys1-X_2-Cys2-$X_{(9\text{-}39)}$-Cys3-$X_{(1\text{-}3)}$-H-$X_{(2\text{-}3)}$-Cys4-X_2-Cys5-$X_{(4\text{-}48)}$-Cys6-X_2-Cys7 with some minor differences (Freemont, 1993). The first difference was the number of amino acid residues (X) between the Cys2 and Cys3 residing in the first loop (loop1) of the RING zinc finger domain. The least number of observed X was 9 residues in the classical RING type, but the reported SHI proteins showed only 7 residues (SHI, SRS 1–7, LRP1 of Arabidopsis, tomato SRS and *B. rapa* SRS protein) (Fridborg et al., 2001; Hong et al., 2010) similar to PtSHI, ZmSHI, PpSHI and JcSHI reported here. The second difference was in the last residue that was changed from a cysteine to histidine in JcSHI and also in all identified SRS proteins (Fridborg et al., 2001). In short, the JcSHI protein showed the specific RING zinc finger motif that was specific to the SRS family that could be described as Cys1-X_2-Cys2-X_7-Cys3-X_1-H1-X_2-Cys4-X_2-Cys5-X_5-Cys6-X_7-Cys7-X_2-His2 (C3HC3H).

Relationship of SHI gene

The pairwise comparison of sequence percent identity of SHI proteins and RING zinc finger domains showed corresponding results with the constructed phylogenetic tree, the identified protein secondary structure and protein domains. SHI protein from *J. curcas* and *P. trichocarpa* were closest to each other (order Malpighiales); together, they formed the woody plant group. They showed the highest percent identity in both pairwise comparisons, displayed the same protein structure and contained the same domain arrangement. The AtSHI protein placed next to the woody plant group showed only a slight difference in the domain arrangement (a glutamine-rich region was found upstream of the RING zinc finger domain). This relationship was well supported with the grouping of the dicotyledonous plants (*J. curcas, P. trichocarpa* and *A. thaliana*) in the phylogenetic trees with high bootstrap values. The differences in the ZmSHI and PpSHI proteins in the secondary structure correlated with branch separation in the phylogenetic tree which corresponded well with the fact that *Z. mays* is a monocotyledon while *P. patens* is a bryophyte.

All five SHI proteins shared the specific RING zinc finger domain of the C3HC3H type, even if the fourth core residue of PpSHI protein was not histidine—but this change from cysteine to any amino acid is typical for the SRS family protein (Fridborg et al., 2001)—while three of them showed similar functions. The overexpression of the *SHI* gene in *A. thaliana*, *P. trichocarpa* and *P. patens* affected the plant's hormone levels causing the phenotype change. The *AtSHI* overexpression in *A. thaliana* showed the dwarfism phenotype caused by the reduction of some GA hormones (Fridborg et al., 1999). The heterologous expression of the *AtSHI* gene in *Euphorbia pulcherrima* showed compact growth and a decrease in the indole acetic acid (IAA) hormone level. The *PtSHI* suppression in *P. trichocarpa* caused an increase in plant height while the *PtSHI* overexpression induced the short internode length by a reduction in some GA hormone levels (Zawaski et al., 2011). Homologous disruption of the *PpSHI* gene resulted in a smaller colony diameter with the decrease in the IAA hormone level when compared with the wildtype, while the homologous overexpression did not result in any difference in growth (Eklund et al., 2010).

Possible function of SHI protein from J. curcas

The JcSHI protein was confirmed to be a member of the SRS protein family containing the RING zinc finger domain that is specific for the SRS protein family (C3HC3H type) and the IGGH domain. The presence of the NLS and various glutamine-rich regions in the JcSHI protein indicated that this protein would be transferred from the cytoplasm into the nucleus for protein folding to function properly which corresponded to a protein functioning as a transcriptional regulator (Mitchell and Tjian, 1989). This has been confirmed by homologous expression of a construct containing the *PpSHI* promoter controlling the *GFP* gene (Eklund et al., 2010). The SHI protein is involved in plant growth and development through the action of the RING zinc finger motif that acts in protein-

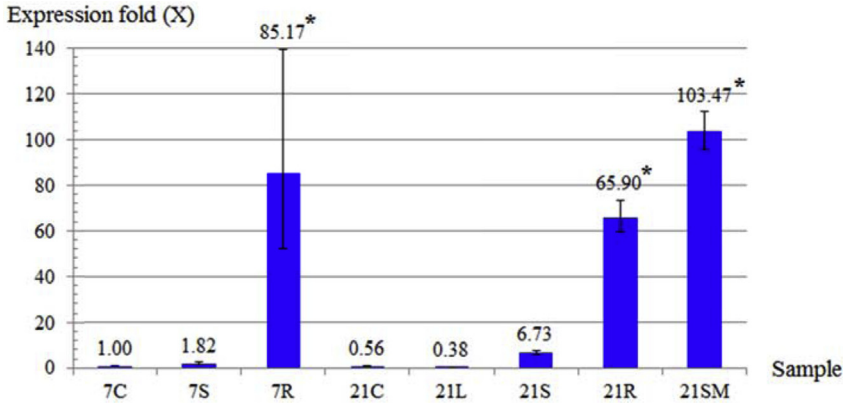

Fig. 7. Relative expression of *SHI* gene in cotyledon, stem and root from 7-day-old seedling (7C, 7S and 7R, respectively) and cotyledon, leaf, stem, root and shoot meristem from 21-day-old seedling (21C, 21L, 21S, 21R and 21SM, respectively) where * = significant difference at $p < 0.05$ and error bars indicate ±SE.

protein interactions. In conclusion, the JcSHI protein could be a transcription factor component that does not bind DNA directly but helps in recruiting other components to form a functional transcription factor to regulate other gene transcription. On the other hand, because the SHI protein is usually found in down regulation processes, the JcSHI protein might attach to a transcription factor to degrade protein by a ubiquitination process.

To confirm the function of this *SHI* gene in *J. curcas*, transgenic plants containing *SHI* silencing and overexpression construct should be produced. GA levels should be assessed along with changes in the phenotype in the transgenic plants in comparison to non-transgenic plants. The conservation of the *SHI* gene from bryophytes up until angiosperms suggested that this gene is crucial for plant development. More information on this gene will be crucial for future manipulation of plant structures.

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