

## Treatment of 5-Azacytidine as DNA Demethylating Agent in *Jatropha curcas* L.

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### ABSTRACT

The role of DNA methylation (the most well-known epigenetic regulation mechanism found in many plant species) was investigated in the development of *Jatropha curcas* L. using the DNA demethylating agent, 5-azacytidine (AzaC). The treatments were performed in the greenhouse and as a separate embryo culture experiment. The results showed that plants responded to AzaC by both accelerating and inhibiting growth and development. Some plants exhibited observable morphological abnormalities, such as stem bending, reduced plant height and increased stem branching. The most severe effect in the treated plants was the significant failure of root development, which was lethal. The efficiency of AzaC was confirmed by methylation sensitive amplification polymorphism (MSAP) analysis of the treated plants. The MSAP fingerprints showed changes in DNA methylation at the nucleotide level. The cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis revealed differential gene expression in the treated plants compared to the untreated control plants in both the greenhouse and embryo culture experiments. The differential sequences matched with some known genes. However, the majority of differential sequences were found to be retroelement derivatives. Reverse transcription polymerase chain reaction (RT-PCR) analysis of four major DNA methyltransferase genes indicated that only the *DRM* and *Dnmt2* genes were up-regulated in AzaC-treated *J. curcas* plants. This study demonstrated the important role of DNA methylation in the normal development of *J. curcas*. The cDNA-AFLP and RT-PCR results led to the hypothesis that AzaC inhibits DNA methylation in particular regions during the first stage of plant development and is involved in the movement of the transposable element in the genome which in turn causes phenotypic abnormalities and activates RNA-dependent DNA methylation pathways. However, this hypothesis requires further intensive study.

**Keywords:** *Jatropha curcas*, DNA methylation, 5-azacytidine, methylation sensitive amplification polymorphism, cDNA-AFLP, transposable element

### INTRODUCTION

*Jatropha curcas* L. (*J. curcas*) is a flowering plant which is cultivated in tropical

and subtropical regions around the world (Gubitz *et al.*, 1999). Seeds of *J. curcas* contain 30–50% non-edible oil which can be processed to produce a high quality of biodiesel fuel (Openshaw, 2000).

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*J. curcas* has been widely cultivated in Thailand and although some cultivars have been selected and regarded as high yield production, the genetic analysis using commonly used DNA markers cannot identify the specific markers for the high yield production traits (Rattanamanee *et al.*, 2009). In addition, many genetic diversity studies of *J. curcas* have resulted in very low polymorphism among the samples and thus, it has been proposed that *J. curcas* has a narrow genetic basis (Yi *et al.*, 2010). Recently, the role of DNA methylation in many plant species in relation to phenotypic expression has been widely discussed. Although *J. curcas* was proposed to have a narrow genetic basis, variation in DNA methylation has been detected (Yi *et al.*, 2010; Kanchanaketu *et al.*, 2012). DNA methylation is the process whereby a methyl group is added to the nucleotide which could affect gene expression without nucleotide changes (Chan *et al.*, 2005; Vaughn *et al.*, 2007) and has been known to play important roles in many processes such as the control of gene expression, regulation of transposable elements, plant development and plant adaptation to environments (He *et al.*, 2011). Methylation of DNA is responsible for the action of DNA methyltransferases which accomplishes two functions—recognition of a specific DNA sequence and catalysis of the transfer of a methyl group from co-factor S-adenosyl-L-methionine (AdoMet) to carbon 5 in the pyrimidine ring of cytosine residues (Pavlopoulou and Kossida, 2007). The plant DNA methyltransferases are classified into four main families—maintenance DNA methyltransferase (*MET1*), chromomethyltransferase (*CMT*), domains-rearranged methyltransferase (*DRM*) and DNA methyltransferase homologue 2 (*Dnmt2*; Pavlopoulou and Kossida, 2007). Study on the role of DNA methylation in plants has been carried out using two main methods comprising disruption of the DNA methyltransferase gene and treatment with DNA demethylating agents. Alteration of DNA methylation by the transformation of the *MET1* gene antisense in *Arabidopsis* resulted in

phenotypic abnormalities or even mortality (Wada, 2005). The best known DNA methylation inhibitors are the nucleotide analogues, 5-azacytidine (AzaC). These compounds are ring analogs of the cytosine nucleoside that can incorporate into newly synthesized DNA and once incorporated into the DNA, they can form a covalent complex with the major DNA methyltransferase and trap the enzyme resulting in a passive demethylation process in which newly synthesized DNA strands remained hypomethylated and the loss of methylation patterns is propagated during replication (Cheng *et al.*, 2005). Study on the role of DNA methylation in *J. curcas* has been very limited. The objective of the present study was to study the role of DNA methylation in the development of *J. curcas* using the DNA demethylating agent, AzaC. Abnormalities in morphological traits as well as differentially expressed genes resulting from AzaC treatment were examined. In addition, expression levels of DNA methyltransferase genes were also investigated. The results from this study will provide basic understanding of the biology of DNA methylation in *J. curcas* which could be useful for plant improvement.

## MATERIALS AND METHODS

### **Treatment of 5-azacytidine and methylation sensitive amplification polymorphism analysis**

In the first experiment, seeds of *J. curcas* (KUBP78-9) were treated with AzaC at concentrations of 0, 50, 100, 200, 400 and 600  $\mu$ M for 18 and 24 hr. Then, the seeds were washed with water and grown in germination pots. The germination percentage at 14 d after planting (DAP), the survival percentage at 21 DAP and plant characters (such as plant height and other morphological abnormalities) were recorded at 21 DAP. The second experiment was performed to study the AzaC effects on root development at concentrations of 0, 100, 250, 500 and 1,000  $\mu$ M for 24 hr. Data from the experiment were analyzed to determine the effects of AzaC on plant

development. Some of the treated plants were selected for methylation sensitive amplification polymorphism (MSAP) analysis as described by Kanchanaketu *et al.* (2012). The primer pairs used in this procedure were E+AAC/HM+TAA, E+ACG/HM+TTC, E+AAG/HM+TTC, E+AAC/HM+TAC and E+ACT/HM+TTC.

### Plant material preparation and total RNA extraction

Plant materials used in the gene expression analysis were prepared for the treatment of AzaC using two methods. In the first method, seeds of *J. curcas* (KUBP78-9) were presoaked in water overnight. Then, seeds were treated with AzaC at 0, 50, 100, 250 and 500  $\mu$ M for 24 hr and grown in germination pots in the greenhouse. Seven-day-old seedlings of *J. curcas* were used for total RNA extraction individually using Trizol reagent (Invitrogen; Oslo, Norway). The RNA extraction protocol followed the manufacturer's directions. In the second method, embryos were removed from seeds and cultured in MS medium (Murashige and Skoog, 1962) supplemented with 100 and 250  $\mu$ M of AzaC for 1 wk. Then, the treated embryos were subcultured in standard MS medium. The 3 wk-old seedlings were used for RNA extraction as described above.

### Identification of differentially expressed genes in *J. curcas* after treatment with 5-azacytidine

First strand cDNA was synthesized using a First strand cDNA synthesis kit (Fermentas; Vilnius, Lithuania) and was used as the template for the second strand synthesis. The cDNA-amplified

fragment length polymorphism (cDNA-AFLP) was carried out following the protocol of Bachem *et al.* (1998) with some modifications. Briefly, double stranded cDNAs were digested with restriction enzymes, *Mse*I + *Msp*I, and the digestion products were ligated with the corresponding adapters. *Mse*I recognizes the sequence 5'-TTAA-3' whereas *Msp*I recognizes the sequence 5'-CCGG-3'. The pre-selective amplification reaction was performed using *Mse*I and *Msp*I adapter-directed primers (*Mse*I+C: GATGAGTCCTGAGTAAC; *Msp*I+T: ATCATGAGTCCTGCTCGGT). Selective amplification of the diluted pre-amplification products was carried out using three selective base primer combinations (*Mse*I+3, *Msp*I+3). A total of 14 and 12 primer combinations were used for the analysis of differential expression in the greenhouse samples and the embryo culture samples, respectively (Table 1). Some polymorphic bands were excised from polyacrylamide gel, re-amplified and cloned to the pTG-19t vector (Vivantis; Subang Jaya, Malaysia) for sequencing. DNA sequences were edited and searched for homologies using the BLAST ([www.ncbi.nlm.gov/BLAST](http://www.ncbi.nlm.gov/BLAST)) and *Jatropha* genome (<http://www.kazusa.or.jp/jatropha/>) databases and also analyzed for the presence of a transposable element (TE) using the TE Class program (Abrusan *et al.*, 2009; <http://www.compgen.uni-muenster.de/tools/teclas/s/?lang=en&mscl=0&cscl=0>).

### Expression level analysis of *MET1*, *CMT*, *DRM* and *Dnmt2* genes in *J. curcas* after treatment with 5-azacytidine

Specific primers of four DNA

**Table 1** Primer combinations used in cDNA-amplified fragment length polymorphism analysis.

Primer pairs	M+CAT	M+CTA	M+CTC	M+CTG	M+CTT
HM+TAA	GS	GS	GS	GS	GS
HM+TCC	GS, ES				
HM+TTC	GS, ES	GS, ES	GS, ES	GS	ES
HM+TTG	ES	ES	ES		

GS = Greenhouse sample; ES = Embryo culture sample.

methyltransferase genes—*MET1*, *CMT3*, *DRM* and *Dnmt2*—were designed based on the *Jatropha* genome database (Table 2). Reverse transcription polymerase chain reaction (RT-PCR) was carried out using a Superscript III one step RT-PCR kit with platinum *Taq* (Invitrogen; Oslo, Norway). The PCR amplification was performed following the manufacturer's protocol. The PCR conditions were: 60 °C for 30 min followed by 94 °C for 2 min, 35 cycles of denaturation at 92 °C for 15 s, annealing at the appropriate temperature (Table 2) for 30 s and extension at 68 °C for 60 s with a final extension at 68 °C for 5 min. The PCR products were checked for the presence of bands on 1% agarose, using actin as the control.

## RESULTS

### Study on the role of DNA methylation in *J. curcas* using 5-azacytidine

*J. curcas* seeds were treated with AzaC at concentrations of 50, 100, 200, 400 and 600 µM for 18 and 24 hr. The results showed that treatment of AzaC affected the survival percentage in *J. curcas* seedlings. The average survival percentage was reduced from 100% in the untreated (control) to 41.67% in the treatment using an 18 hr

exposure time and to 47.22% in the treatment using 24 hr. The observed effects of AzaC on plant development were the reduction in plant height and delayed seed germination (Figure 1a). Some seedlings exhibited abnormal development; for example, stem bending during preliminary development (Figure 1b) and some grew faster than the control plants. Only some of the abnormal plants survived after treatment. Interestingly, it was found that more than 30% of the germinated seeds from the experiment had died after 3 wk of treatment. Similar results were reported by Bossdorf *et al.* (2010) with *Arabidopsis* treated with AzaC which had a greater than 40% rate of mortality than the untreated *Arabidopsis*. Some of the treated *J. curcas* seedlings had limited root development and some had no root development (Figure 1c) compared to control seedlings (Figure 1d). It is suggested that the undeveloped root was one of the possible causes of mortality in AzaC-treated plants.

In order to confirm the effects of AzaC on the root development of *J. curcas*, another experiment was performed at concentrations of 0, 100, 250, 500 and 1,000 µM AzaC for 24 hr, with 20 seeds in each treatment. A decrease in the average root length in the treatments of 250 and

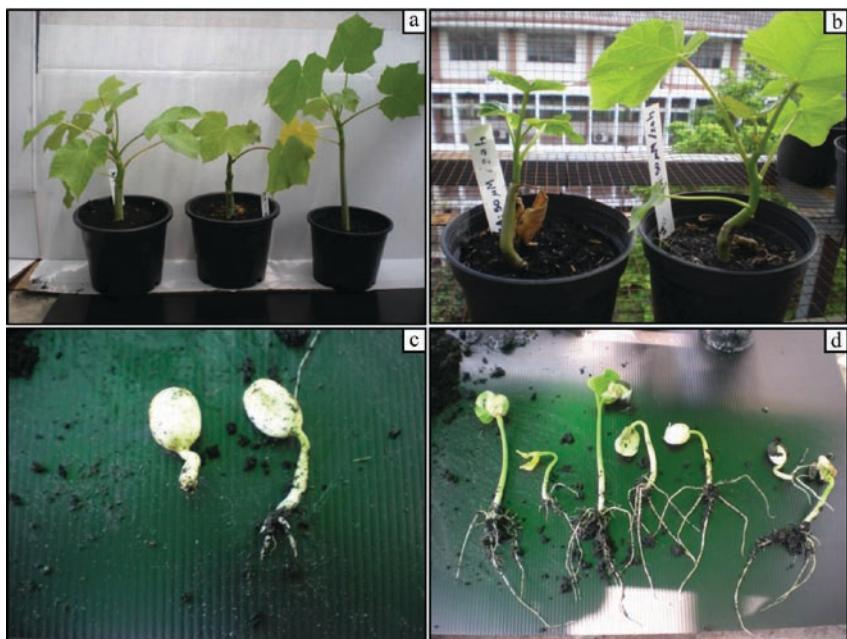
**Table 2** Sequences of DNA methyltransferase specific primers used.

Primer name	Primer sequence (5'-3')	Annealing temp. used in PCR (°C)
MET1-F	TGCTGCCAATGARAGAGAGGTT	57
MET1-R	CTCCGAAYCTAACCTGYGTGGAT	58
CMT-F	GATGAGACTGTGCCGACAG	55
CMT-R	TCAAGCATTGTCTTGACGATGC	57
DRM-F	TGGAATACGGATGATGAGCTT	50
DRM-R	CTAGGACCACTGTATGGCT	51
Dnmt2-F	GGAAACAAAGACGGTGCCTT	51
Dnmt2-R	AATAGCGTGGCCTGGAGTA	51
Actin-F	CAAGTCATCACCATTGGAGCA	52
Actin-R	GCCTCTTAATTCGGCTTAACA	51

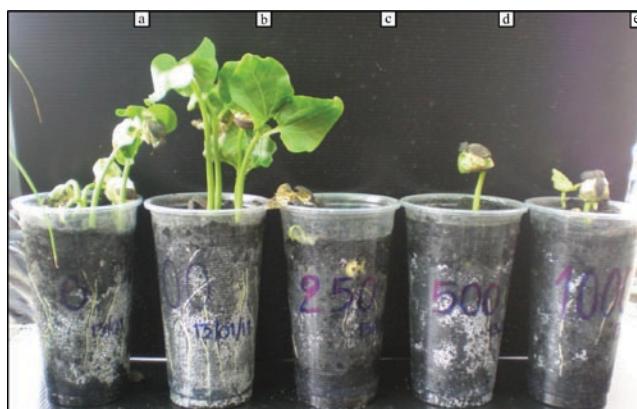
PCR = Polymerase chain reaction; A = Adenine; C = Cytosine; G = Guanine; T = Thymine; Y = C or T; R = G or A.

500  $\mu\text{M}$  was clearly observed. However, from this experiment, the treatment at 100  $\mu\text{M}$  AzaC seemed to accelerate plant growth (Figure 2) and produced the highest average root length at 21 DAP. Although slow shoot development and abnormal root development were detected in the treatment

of 250  $\mu\text{M}$  AzaC, treatment with 500  $\mu\text{M}$  seemed to cause the highest level of injuries with *J. curcas* seedlings. At 500  $\mu\text{M}$  AzaC, only one plant was found to develop both shoots and roots and more than 50% of plants showed no root development. When the AzaC level was increased to 1,000  $\mu\text{M}$ ,



**Figure 1** Morphology of *Jatropha curcas* plants treated with 5-azacytidine: (a) 5-Azacytidine (AzaC)-treated plants showing reduction in plant height compared to the untreated (control) plant; (b) Some AzaC-treated plants with abnormal shape during development; (c) No root development in *J. curcas* seedlings treated with 600  $\mu\text{M}$  of AzaC; (d) Control with normal root development.



**Figure 2** Effect on seed germination in *Jatropha curcas* of 5-azacytidine concentration at: (a) 0  $\mu\text{M}$ ; (b) 100  $\mu\text{M}$ ; (c) 250  $\mu\text{M}$ ; (d) 500  $\mu\text{M}$ ; (e) 1,000  $\mu\text{M}$ .

effects on shoot and root development were also found but at a lower level compared to 500  $\mu$ M. The average root length of the treatments with 0, 100, 250, 500 and 1,000  $\mu$ M were 7.63, 9.29, 4.29, 5.40 and 6.86 cm, respectively. The results confirmed that AzaC caused severe effects on root development in *J. curcas* seedlings. Similar results were reported by Heras *et al.* (2001) in that AzaC induced chromosomal breakage in the root

tips of wheat. Similar results were also found in the treatment with AzaC using the embryo culture technique. Slow growth, delayed root development and stem bending were observed in the treated plants (Figure 3).

Some treated plants were selected to study the DNA methylation changes at the DNA level using the MSAP technique. Five MSAP primer pairs were used and the results showed that



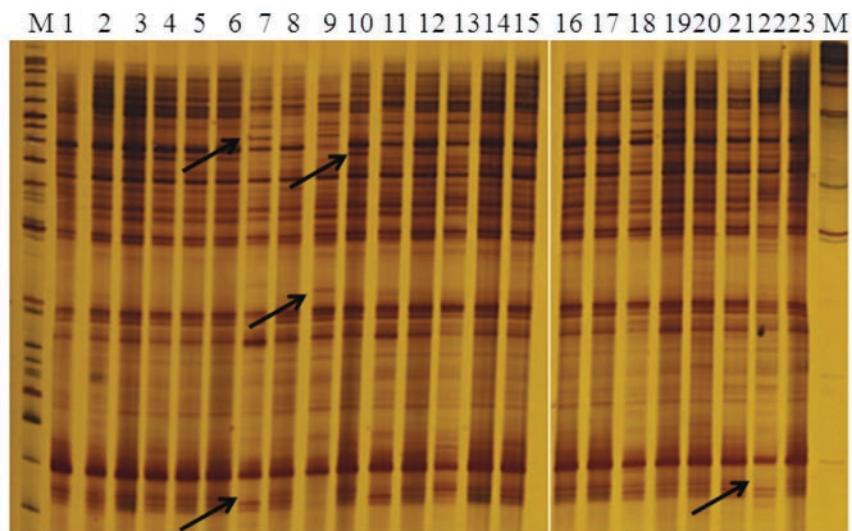
**Figure 3** *Jatropha curcas* treated with 5-azacytidine (AzaC) using the embryo culture technique: (a) at age 2 wk for control; (b) at age 2 wk after 100  $\mu$ M AzaC for 24 hr; (c) at age 2 wk after 250  $\mu$ M AzaC for 24 hr; (d) at age 3 wk for control; (e) at age 3 wk after 100  $\mu$ M AzaC for 24 hr; (f) at age 3 wk after 250  $\mu$ M AzaC for 24 hr.

the majority of bands obtained were monomorphic. However, some differentially methylated DNA bands were detected and most of these bands were not found in any of the control plants (Figure 4). In total, there were 9 and 44 polymorphic bands in the treatments of 18 and 24 hr exposure time, respectively. The results indicated that the longer the plants were exposed to AzaC, the higher the frequency of DNA methylation change obtained.

#### Identification of differentially expressed genes and expression level analysis of four DNA methyltransferase genes in *J. curcas* after treatment with 5-azacytidine

The differential gene expression after treatment with AzaC was studied using the cDNA-AFLP technique. Two sample sets—the greenhouse sample set and the embryo culture sample set—were used. Environmental and external factors in the greenhouse might affect the expression of genes. A total of 14 and 12 *MseI*-*MspI* primer combinations were used to investigate the differential gene expression in the greenhouse

sample set and the embryo culture sample set, respectively. As expected, the difference in band patterns obtained was greater among the samples from greenhouse than from the embryo culture. Differentially detected DNA bands were cut off from the gel and cloned before sequencing. The bands that were absent in the control plant but present in the AzaC treatment were selected. However, one of the band patterns present in the control plant but absent in all the treated plants was also selected. The homology search of these sequences using NCBI BLAST and the *Jatropha* genome database showed interesting results. The majority of the BLAST results showed no significant or reasonable matches to any sequences in the database (Table 3), except for the sequence ES9 which matched the *Ricinus communis* 3'-5' exonuclease and the sequence ES10 which matched the *R. communis* RNA polymerase II mediator complex subunit. More interestingly, these differential sequences were found to be retroelement derivatives based on the TE search program. Expression analysis of the four DNA



**Figure 4** Methylation-sensitive amplification polymorphism fingerprints of 5-azacytidine-treated *Jatropha curcas*. Arrows indicate some polymorphic markers. 1–4 = Control; 5 = 50  $\mu$ M at 18 hr; 6 = 100  $\mu$ M at 18 hr; 7–8 = 200  $\mu$ M at 18 hr; 9–15 = 50  $\mu$ M at 24 hr; 16–20 = 100  $\mu$ M at 24 hr; 21–22 = 200  $\mu$ M at 24 hr; 23 = 400  $\mu$ M at 24 hr.

methyltransferase genes (*MET1*, *CMT3*, *DRM* and *Dnmt2*) in the AzaC treated embryos at 100  $\mu$ M and 250  $\mu$ M for 1 wk was performed using RT-PCR analysis. The results indicated that the expression level of the *MET1* gene was the same between the control and the 100  $\mu$ M-treated plants, but the expression level was slightly decreased in the 250  $\mu$ M-treated plants. Expression of the *CMT3* gene was not detected in the present study. The *DRM* and *Dnmt2* genes were up-regulated in the 100  $\mu$ M-treated plants, but the expression level was slightly decreased in the 250  $\mu$ M-treated plants (Figure 5).

## DISCUSSION

The treatment of *J. curcas* seeds with AzaC demonstrated the important role of DNA methylation in the normal development of *J. curcas*. The experiment showed that alteration of DNA methylation had effects on plant shape, plant development, acceleration or slowing down of plant growth and root development. A study on the treatment of AzaC in *Arabidopsis* showed that a demethylating agent strongly reduced the growth and fitness of plants and delayed their flowering (Bossdorf *et al.*, 2010). The MSAP analysis of

**Table 3** Summary of differential bands detected in cDNA-amplified fragment length polymorphism analysis analysis.

No.	Size (bp)	Concentration of AzaC ( $\mu$ M)			NCBI, <i>Jatropha</i> database search	Transposable element search
		0	100	250		
Greenhouse sample						
GS1	250	----				Unclear retroelements
GS2	450	----	----			LTRs
Embryo culture sample						
ES1	250	----	----	----		Unclear retroelements
ES2	200		----			Unclear retroelements
ES3	100	----	----	----		Unclear retroelements
ES4	250	----	----	----		Unclear retroelements
ES5	200	----	----	----		Unclear retroelements
ES6	350	----				Unclear retroelements
ES7	200		----			LTRs
ES8	200		----			SINEs
ES9	200	----	----		<i>R. communis</i> 3'-5' exonuclease ( $4e^{-33}$ )	Unclear retroelements
ES10	250	----	----		<i>R. communis</i> RNA polymerase II mediator complex subunit (NCBI = $4e^{-19}$ , <i>Jatropha</i> db = $e^{-120}$ )	Unclear retroelements
ES11	400		----			LINEs
ES12	200	----	----			LTRs
ES13	350	----				LTRs

AzaC = 5-Azacytidine; ---- = Band present.

LTRs = Long terminal repeats; SINEs = Short intersperse nuclear elements; LINEs = Long intersperse nuclear elements.

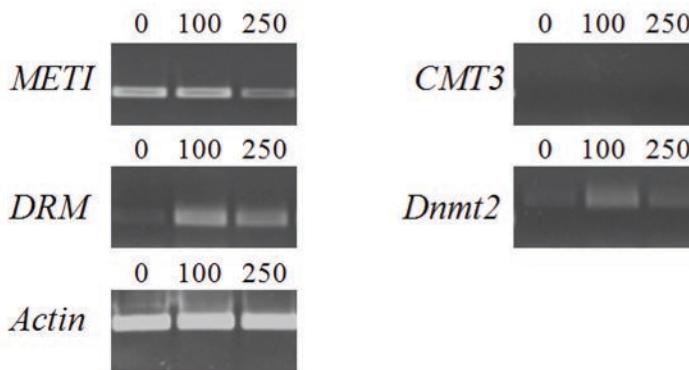
GS = Greenhouse sample; ES = Embryo culture sample.

the treated plants revealed methylation variation among the samples and confirmed that AzaC was a random demethylating DNA and it might not only reduce the overall levels of methylation but also increase the variation in methylation as shown in the MSAP fingerprints.

The expression level of the four DNA methyltransferase genes in *J. curcas* embryos treated with AzaC was investigated using reverse-transcription assay. The results showed that the expression level of the *METI* gene was the same in the control and the 100  $\mu$ M-treated plants, but the expression was slightly decreased in 250  $\mu$ M-treated plants. The *METI* gene has been known to function in maintaining CG methylation after DNA replication (Wada, 2005). The expression of the *CMT3* gene was not detected in this study. Transcripts of *CMT3* have been detected in vegetative tissues and have been found abundantly in flowers (Finnegan and Kovac, 2000). The *DRM* and *Dnmt2* genes were up-regulated in the 100  $\mu$ M-treated plants, but the expression level was slightly decreased in the 250  $\mu$ M-treated plants. These results are interesting because *DRMs* has been known to play an important role in *de novo* methylation by the RNA-directed DNA methylation (RdDM) pathway, which is the pathway that has been reported in the silent movement of transposable elements (TEs) (Chan

*et al.*, 2005; He *et al.*, 2011; Meyer, 2011). The expression patterns could confirm the previous findings in that treatment with AzaC inducing the RdDM pathway. However, the role of the *Dnmt2* gene is still not clear.

Differential expressed bands in the cDNA-AFLP analysis caused by the AzaC treatment could have resulted for three reasons. First, the re-activated genes resulted from DNA demethylation. DNA methylation has been known to suppress gene expression by inhibiting RNA polymerase II activity to transcribe mRNA. Second, some genes might be directly responding in the presence of AzaC to maintain genome stability. Third, different gene expressions were the result of the indirect effects of AzaC. The demethylating agent might interrupt or induce some genes by chance. Most sequences from differentially expressed bands showed no significant match to any known gene sequences, except for three sequences which matched the ribosomal protein L12, the *Ricinus communis* 3'-5' exonuclease and the RNA polymerase II mediator complex subunit. The 3'-5' exonuclease has been reported to play an important and complex role in the RNA interference pathway (Ibrahim *et al.*, 2008), while the RNA polymerase II mediator complex subunit has been recently reported to be involved in *de novo* DNA methylation by the RdDM pathway



**Figure 5** Transcription-polymerase chain reaction analysis of four DNA methyltransferase genes in embryo culture of *Jatropha curcas* treated with 0, 100  $\mu$ M and 250  $\mu$ M AzaC at 1 wk. *METI* = Maintenance DNA methyltransferase, *CMT3* = Chromomethyltransferase 3, *DRM* = Domains-rearranged methyltransferase, *Dnmt2* = DNA methyltransferase homologue 2.

(He *et al.*, 2011). Most differential sequences were retroelement derivatives. In *Arabidopsis*, siRNA-dependent *de novo* DNA methylation mainly targets transposons and also the end of chromosomes where few genes are distributed. RdDM also functions in gene regulation, especially for those genes flanked by TE and other repetitive DNA sequences (He *et al.*, 2011). The RdDM pathway was also reported to silence movement of TEs. The results were consistent with the idea that morphological abnormalities in AzaC-treated *J. curcas* plants resulted from movement of TE. It is possible that AzaC could inhibit DNA methylation at TEs, allowing them to move at the very first stage of plant development. Movement of TEs could change the expression of genes, block normal development pathways or even cause death. Treatment with AzaC was reported to induce the transposition of *Dart* elements in rice (Tsugane *et al.*, 2006; Eun *et al.*, 2012) and to increase the transposition frequency in *Fusarium oxysporum* (Akiyama *et al.*, 2007). The movement of TEs could be one of the reasonable explanations of the abnormalities in AzaC-treated plants.

However, another explanation for the morphological abnormalities is from the alteration of gene expression. In plants, DNA methylation of promoter regions usually inhibits transcription, but methylation in coding regions does not generally affect gene expression. However, there are some exceptions to this rule. DNA methylation in the transcribed portion of the gene probably causes transcription termination (Chan *et al.*, 2005). In addition, some genes such as ribosomal RNA (rRNA) genes were silenced in the presence of DNA methylation. Treatment with DNA methylation inhibitors can reverse this silencing (Mathieu *et al.*, 2003; Chan *et al.*, 2005). The application of a DNA demethylating agent possibly caused some genes to express at an inappropriate time. Unfortunately, the sequencing results from the present study were not sufficient to identify the appropriate explanation. It could be inferred from the present study that although

AzaC itself is not a mutagen, its possible abilities to re-activate the movement of TEs and alter gene expressions were able to create novel genotypes and phenotypes. With good practice of treatment and selections, AzaC is very useful in a breeding program with specific purposes.

## CONCLUSION

The results from the present study showed the importance of DNA methylation in the normal development of *J. curcas*. The role of DNA methylation in *J. curcas* was studied by treatment with the DNA demethylating agent, AzaC. Plants responded to AzaC by accelerating or inhibiting growth and development. Some plants exhibited observable morphological abnormalities such as stem bending, reduction in plant height and increased numbers of stem branches. The most severe effect in treated plants was significant failure in root development, leading to mortality. Some AzaC-treated *J. curcas* and the untreated control plants were subjected to MSAP analysis and polymorphic bands between treated plants and the control plant were detected. The cDNA-AFLP technique was used to determine the effect of AzaC on various gene expressions. Differential gene expression in AzaC-treated plants and the untreated control plants was detected. The differentially expressed DNA bands were selected and sent for sequencing. The majority of sequences obtained were not of known genes. Most sequences were classified to retroelements and their derivatives. These results were consistent with other study in that AzaC caused movement of transposable elements at the very first stage of development. Expression analysis of four DNA methyltransferase genes (*MET1*, *CMT3*, *DRM* and *Dnmt2*) showed that only the *DRM* and *Dnmt2* genes were up-regulated in the presence of AzaC. The cDNA-AFLP and RT-PCR results led to the hypothesis that AzaC removes DNA methylation during the first stage of plant development and is involved in the movement of TEs in the genome

which in turn causes phenotypic abnormalities and activates RNA-dependent DNA methylation pathways. However, this hypothesis requires further intensive study.

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