Comparison of DNA methylation in developing seeds of *Ricinus communis* L. using MSAP analysis

Fenglan Huang**1**, **2**, **3**, **4**, Mu Peng**1**, **4**, Xiaofeng Chen**1**, Guorui Li**1**, **2**, **3**, Jianjun Di**1**, **2**, **3**, Yong Zhao**1**, Tongtong Jiang**1**, Ruihui Chang**1**, Lifeng Yang**1** and Yongshen Chen**1**, **2**

**1**Department of Biotechnology, College of Life Science, Inner Mongolia University for the Nationalities, Tongliao, PR China; **2**Inner Mongolia Industrial Engineering Research Center of Universities for Castor, Tongliao, PR China; **3**Inner Mongolia Key Laboratory of Castor Breeding, Tongliao, PR China; **4**Inner Mongolia Collaborative Innovation Cultivate Center for Castor, Tongliao, PR China; **5**Department of Genetics, College of Life Science, Northeast Forestry University, Harbin, PR China

**ABSTRACT**

DNA methylation plays an important role in endosperm development and is involved in the epigenetic regulation of gene expression. However, the epigenetic regulation of castor seed development has not yet been reported. In this study, methylation-sensitive amplification polymorphism (MSAP) analysis was used to investigate the methylation level in castor. In 20-day-old castor seeds, 41.17% of the fragments were fully methylated and 15.61% were hemimethylated. In 60-day-old seed, the full methylation and hemimethylation rates were 19.77% and 7.56%, respectively, suggesting that some methylation level changes existed during the castor seed development. A total of 647 bands were identified with different methylation or demethylation patterns during the seed maturation. However, only 37 sequences were homologous to well-known proteins, such as phospholipid-transporting ATPase, phosphatases and methyltransferase. Therefore, these results clearly demonstrated that the MSAP technique is very useful for the analysis of the global DNA methylation state in castor and the complexity of DNA methylation changes during seed development. The different methylation levels may be correlated with the specific gene expression in different seed development stages.

**Introduction**

Castor (*Ricinus communis* L., Euphorbiaceae), an important non-edible oilseed crop, is widely grown for its oleaginous seeds which produce a lot of valuable oil [1]. Castor bean has been used as a medicinal material in over fifty countries [2]. Although its oil has high commercial value in industry, many countries import castor oil stocks due to the lack of domestic production for several years [3]. Therefore, how to increase the yield and oil content of castor bean is a key point of research for breeders. Ribeiro et al. [4] studied the gene expression in castor seeds in the germination and seedling stage. A paper demonstrated that the seed yield of castor bean was significantly reduced by the exposure to cool temperatures during the seed-filling stage, and the oil yield was significantly dependent on the seed yield [5]. Severino and Auld [6] also analysed the influence of air temperature on the growth rate of castor bean. Recently, an important potential use of castor has been tested in the phytoremediation of metalliferous waste or soils [7,8].

DNA methylation, as an epigenetic regulatory mechanism, is a crucial epigenetic process that controls gene transcription activity in eukaryotes [9,10]. The information regarding the methylation state in organisms provides important knowledge of the gene transcription control. Methyl-Sensitive Amplification Polymorphism (MSAP) is a technique used to study large-scale DNA methylation and distinguish individuals characterized by a differential digestion pattern [9]. Therefore, this technique is a useful tool for DNA methylation mapping and the positional cloning of differentially methylated genes. Until now, genomic DNA methylation has been investigated in the seeds of many plants, such as Arabidopsis [11], *Oryza sativa* L. [12], *Pyrus communis* [13], suggesting that DNA hypomethylation is mainly detected in angiosperm. However, no sufficient proof has been reported to explain the mechanism of how this low methylation level is formed and maintained in plants [14].

Seed development, which is a prolonged and complicated process, consists of embryo and endosperm development. It has been reported previously that the
epigenetic regulation affects the seed development and seed size [15]. The systemic characterization of the dynamic DNA methylome will help us understand the effects and mechanism of epigenetic regulation during seed development [16]. Xing et al. [12] determined and investigated DNA methylation during the rice seed development, indicating that a complex methylation pattern was closely related with seed development. Castor oil, as a renewable resource for the chemical industry, is mainly found in the seeds. However, the epigenetic regulation of castor bean has not been reported yet. Therefore, the aim of this work was to study the relationship between DNA methylation and seed development to gain better understand of the underlying mechanisms of castor seed development and improve oil yield.

Materials and methods

Plant material

Castor bean (2129 accession) was sampled from the Academy of Agricultural Science in Tongliao, China. Then, mature and healthy seeds were cultivated in a farmland within Inner Mongolia University for the Nationalities, Tongliao, China. For the comparative analysis, seeds at different developing stages were collected at 10, 20, 30, 40, 50 and 60 days after artificial pollination. For the morphological characterization of castor bean at each development stage was done as previously described [17]. The seeds were collected and stored at −80 °C for subsequent experiments.

Measurement of fatty acid compositions

The absolute percentage of fatty acid compositions was calculated according to the corresponding Agilent 1890A gas chromatographic peaks (Agilent Technologies, Wilmington, USA). By applying the computer automatic and manual retrieval with NIST98 and Wiley of Mass Spectral Data, the fatty acid compositions of castor oil were measured and analyzed [18]. The fatty acid compositions and absolute content were measured as previously described [17,18].

MSAP analysis

The genomic DNA was isolated using the CTAB method of Doyle [19]. The MSAP assay was performed according to Xiong et al. [20]. In this technique, genomic DNA (approximately 100 ng) was firstly digested with methylation-sensitive restriction enzymes such as EcoRI, MspI and HpaII. HpaII and MspI recognize CCGG sequences. HpaII can only digest non-methylated CCGG and hemimethylated 5′CCGG sites. MspI can cleave non-methylated CCGG and 5′CGG, but cannot digest 5′CCGG and 5′C′GGG sequences. Then the digested DNA fragments were ligated to adaptors in order to facilitate their amplification. Subsequently, these fragments were selectively amplified using selective primers. The adaptors and the pre-selective and selective primers were the same with Wang et al. [15]. Polymerase chain reaction (PCR) products from the different fragments were compared, once a polymorphic locus was recognised between two different materials. The presence or absence of each single fragment was coded by 1 or 0, respectively. Then, the desired DNA fragment could be isolated from a denaturing polyacrylamide gel.

Isolation and sequencing of TDFs

The polymorphic loci were excised from the gel and soaked in distilled water. Fragments were re-amplified using the same primers as described for the selective amplification and cloned into a pMD18-T vector (TakaRa, Kyoto, Japan). The sequencing of fragments was performed by Sangon Biotech Co., Ltd (Shanghai, China). Sequence homology search was performed with BLAST in the National Center for Biotechnology Information (NCBI) databases.

Data analysis

Data are presented as mean values with standard error of the means (±S.E.M) of three independent experiments. Statistical analysis and correlation analysis were performed using SPSS 17.0 software package (SPSS Inc, Chicago, USA).

Results and discussion

Variations in fatty acid compositions of castor during seed development

The one-hundred-seed weight of castor bean ranged from 36.84 to 48.62 g. The results from the capillary gas chromatography analysis showed that the fatty acid compositions in castor seed included 11 components with large-scale variations (Table 1). The percentage of ricinoleic acid was predominant in all the seeds with large amplitude from 0.02% to 43.66%. The other fatty acids appeared in small concentrations (less than 3%). Interestingly, some correlations were found among the eighteen fatty acid components (Table 2). However, no significant correlations were detected between seed weight and fatty acid compositions. These results suggest that higher-weight seeds could not contain a large
amount of oil. In addition, the content of ricinoleic acid was significantly and positively correlated with the content of stearic, oleic, linoleic, arachidic and arachidonic acids.

Recently, castor bean has gained great economic value from an industrial perspective and has received considerable attention from breeders to improve the oil content. A higher content of ricinoleic acid has already been detected in castor seeds. Therefore, it is difficult to increase the oil content using conventional breeding methods (Tables 1 and 2). Thus, it is considered that molecular techniques can be applied to solve the present situation.

### DNA methylation changes in the developing castor bean

The developing castor seeds were collected at regular intervals after pollination to investigate the epigenetic regulation of the seed development. MSAP analysis was performed from 10-day-old developing seeds to maturation in this study. A total of 64 primer pairs were used for the selective amplification, and 32 of them produced 2090 fragments. These fragments were classified into eleven groups according to different patterns of methylation (Figure 1 and Table 3). Extensive changes in DNA methylation (Types V–IX, 10.81%) were detected at different seed developmental stages. Types I–IV with the greatest value (88.46%) were found with similar fragments at all seed development stages, and the variation was barely observable. These results clearly demonstrated that the MSAP technique is very useful for the genome-wide DNA methylation detection in castor and for the analysis of the complexity of DNA methylation changes during seed maturation.

Moreover, we compared the MSAP patterns at several developmental stages (Table 4). In the 20 day-old seeds, 41.17% of the fragments were fully methylated and 15.61% were hemimethylated, whereas the full methylation and hemimethylation rates were 19.77% and 7.56% in the 60-day-old seeds, respectively, indicating that along with seed development, the levels of full methylation and hemimethylation decrease.

DNA methylation is an important process in the epigenetic control of gene expression; this process may also participate in agronomic trait variation. First, we used MSAP analysis to examine the methylation changes of CCGG motifs in castor seeds at different developmental phases. The altered pattern of methylation and polymorphism in the methylated DNA regions could illustrate gene expression variation partly related to fatty acid biosynthesis. In the 10-day-old seeds, a relatively higher percentage (39.77%) of the MSAP fragments
were methylated, whereas this value decreased to 19.77% in the 60-day-old seeds, suggesting that the methylation level was reduced along with the seed maturation (Table 4). Approximately one-third of the fragments exhibited methylation variations and demethylation was found mainly at the later developmental stages. These methylation changes were probably associated with seed coat variation, which gradually matured during the seed development. These results were similar to the degree of methylation reported by Gehring et al. [21], who pointed out that extensive demethylation during the seed development underlies gene imprinting. As cytosine methylation antagonises transcription, Zilberman et al. [22] presented an idea that gene transcription was strongly influenced by DNA methylation. In this study, DNA hypomethylation was found in later developmental stages, and almost all fatty acids decreased in content in the later stages while ricinoleic acid was greatly increased after pollination, suggesting that this could be associated with the changes observed in the patterns of DNA methylation during seed maturation. DNA hypomethylation or demethylation along with seed development seem to be an essential process in the gene expression control.

Table 2. Correlation coefficients for fatty acid composition in developing castor seed.

|                  | One-hundred seed weight | Myristic acid | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid | Linolenic acid | Arachidic acid | Arachidonic acid |
|------------------|--------------------------|---------------|---------------|--------------|------------|---------------|----------------|----------------|-----------------|
| Oleic acid       | -0.06                    | -0.40         | 0.33          | 0.99**       |            |               |                |                |                 |
| Linoleic acid    | 0.10                     | -0.01         | 0.68          | 0.86*        | 0.91**     |               |                |                |                 |
| Linolenic acid   | 0.21                     | 0.70          | 1.00**        | 0.97         | 0.37       | 0.71          |                |                |                 |
| Arachidic acid   | 0.10                     | -0.09         | 0.56          | 0.86*        | 0.89**     | 0.92**        | 0.59           |                |                 |
| Arachidonic acid | 0.01                     | -0.52         | 0.20          | 0.99**       | 0.98**     | 0.85*         | 0.24           | 0.83*          |                 |
| Behenic acid     | 0.16                     | 1.00**        | 0.70          | -0.53        | -0.44      | -0.05         | 0.66           | -0.13          | -0.56           |
| Lignoceric acid  | 0.18                     | 1.00**        | 0.73          | -0.50        | -0.40      | -0.01         | 0.70           | -0.09          | -0.52           |
| Ricinoleic acid  | -0.07                    | -0.55         | 0.17          | 1.00**       | 0.99**     | 0.83*         | 0.21           | 0.84*          | 0.99**          |

Note: *Significant at P < 0.05; **Significant at P < 0.01.
and may contribute to the developmental gene regulation.

**Characterization of the fragments showing methylation changes**

DNA methylation directly modulates gene transcription or even completely silences or activates genes without affecting the DNA sequence [23]. In the process of castor seed development, we detected the co-existence of methylation and demethylation. Our results revealed that the methylation level was mainly higher in the early developmental stages (10–20 days), while demethylation could be found at later developmental stages (40–60 days) (Table 5). Many researches have shown that cytosine methylation is a crucial modification process implicated in gene expression during developing [23,24]. In an internal or an adjacent part of a gene, methylation would repress the expression of a target gene, whereas demethylation could activate these gene expressions. Taken together, combined with the number and BLAST results of polymorphic fragments and variations of fatty acid compositions, the methylation level gradually decreased along with the seed development, thus hypomethylation appeared to be a vital step in the transcriptional activation of the gene expression involved in fatty acid synthesis (Tables 1, 4 and 5), which was consistent with our previous findings [18,25]. As a result, these findings supported the correlation between the gene

**Table 6. Sequences of MSAP fragments homologous to known gene.**

| No. | Accession no. | Coding protein of EST homology | Gene source   | E-value | Similarity/% |
|-----|---------------|--------------------------------|----------------|---------|--------------|
| A0  | XM_002518 552.1 | S-locus-specific glycoprotein S13 precursor | Ricinus communis | 7e-99  | 99           |
| A10 | XM_002532 371.1 | Endo-1,4-beta-xylanase precursor | Ricinus communis | 5e-65  | 99           |
| A19 | XM_002528 652.1 | Phospholipid-transporting ATPase | Ricinus communis | 4e-85  | 99           |
| B02 | XM_002527 219.1 | FAD oxidoreductase | Ricinus communis | 4e-09  | 100          |
| B04 | XM_002512 218.1 | map3k delta-1 protein kinase | Ricinus communis | 2e-16  | 100          |
| C02 | XM_002533 650.1 | Basic helix-loop-helix-containing protein | Ricinus communis | 1e-09  | 98           |
| C15 | XM_002521 509.1 | Pentatricopeptide repeat-containing protein | Ricinus communis | 2e-68  | 99           |
| C17 | XM_002522 776.1 | Actin binding protein | Ricinus communis | 3e-13  | 100          |
| X02 | XM_002510 161.1 | Inositol hexaphosphate kinase | Ricinus communis | 3e-76  | 100          |
| D14 | XM_002530 627.1 | Ferredoxin-2, chloroplast precursor | Ricinus communis | 2e-18  | 98           |
| D16 | XM_002518 963.1 | Alpha-galactosidase/alpha-n-acetylgalactosaminidase | Ricinus communis | 1e-04  | 97           |
| E02 | XM_002521 992.1 | Receptor protein kinase CLAVATA1 precursor | Ricinus communis | 4e-15  | 100          |
| E20 | XM_002526 319.1 | Sumo ligase | Ricinus communis | 1e-20  | 94           |
| F09 | XM_002533 725.1 | Protein C9orf32 | Ricinus communis | 4e-37  | 100          |
| F10 | XM_008227 458.1 | N-terminal protein methyltransferase | Ricinus communis | 4e-07  | 100          |
| F13 | XM_002519 251.1 | Peroxiredoxin | Ricinus communis | 2e-10  | 94           |
| F17 | XM_002519 932.1 | Protein kinase APK1B, chloroplast precursor | Ricinus communis | 8e-06  | 97           |
| H19 | XM_002531 254.1 | Ammonium transporter | Ricinus communis | 3e-12  | 100          |
| J19 | XM_002517 321.1 | Serine-threonine protein kinase, plant-type | Ricinus communis | 2e-41  | 99           |
| J20 | XM_002512 183.1 | Calcium ion binding protein | Ricinus communis | 1e-26  | 97           |
| J30 | XM_002517 601.1 | AMP-activated protein kinase | Ricinus communis | 2e-63  | 99           |
| K01 | XM_002511 434.1 | Ribosomal protein S6 kinase | Ricinus communis | 3e-107 | 99           |
| K29 | XM_002518 473.1 | Tryptophan synthase beta chain | Ricinus communis | 6e-08  | 97           |
| K36 | XM_002518 423.1 | S'-Adenylylsulfate reductase 1, chloroplast precursor | Ricinus communis | 3e-71  | 99           |
| M20 | XM_002526 261.1 | ATP-dependent RNA helicase | Ricinus communis | 5e-64  | 99           |
| M21 | XM_002513 776.1 | Phosphatidylinositol-4-phosphate 5'-kinase | Ricinus communis | 2e-106 | 98           |
| P07 | XM_002510 837.1 | Protein phosphatase 2c | Ricinus communis | 1e-58  | 100          |
| P10 | XM_002301 708.1 | Protein phosphatase 2c | Populus euphratica | 3e-09  | 94           |
| R06 | XM_002532 848.1 | Cyclic nucleotide-gated ion channel | Ricinus communis | 3e-13  | 98           |
| R14 | XM_002514 305.1 | ATP-binding cassette transporter | Ricinus communis | 9e-82  | 100          |
| R24 | XM_0012609 534.1 | CTD nuclear envelope phosphatase 1-like | Gossypium raimondii | 2e-20  | 86           |
| S30 | XM_002513 732.1 | s-Receptor kinase | Ricinus communis | 0.0    | 99           |
| S32 | XM_0012609 534.1 | Naringenin-2-oxoglutarate 3-dioxygenase and flavanone 3-hydroxylase | Ricinus communis | 2e-109 | 99           |
| V12 | XM_002524 611.1 | Stachyose synthase precursor | Ricinus communis | 1e-59  | 99           |
| V15 | XM_002525 181.1 | O-methyltransferase | Ricinus communis | 3e-21  | 95           |
| D11 | XM_002533 940.1 | r23-myb transcription factor | Ricinus communis | 6e-99  | 99           |
| T08 | XM_002513 767.1 | Esterase precursor | Ricinus communis | 9e-48  | 99           |

**Table 5. Statistical results of polymorphic fragments in different seed development stage.**

| Developmental stages (d) | Bands name(size, bp) | Number of bands |
|--------------------------|----------------------|-----------------|
| 10 | X02(185)/P07(332)/M21(295)/F10(96)/F09(90)/B02(41)/A19(204)/P20(334)/K36(182) | 9 |
| 20 | E20(84)/B04(122)/F13(217)/K29(69)/S32(251)/S30(509)/A10(235)/C02(70)/E02(74)/T08(233)/J19(125)/R14(195) | 12 |
| 30 | A01(235)/C15(173)/C17(99)/06R(101)/P10(332)/D16(58) | 6 |
| 40 | V15(497)/R24(278)/D11(229) | 3 |
| 50 | J20(98)/J30(168)/K01(244)/H19(73) | 4 |
| 60 | F17(57)/V12(158)/D14(83) | 3 |
expression changes involving DNA methylation and the fatty acid composition during seed development.

A total of 647 bands were identified with different methylation or demethylation patterns during seed maturation. Among 647 bands, 147 fragments were successfully validated by PCR and sequenced, then these sequences were identified using BLAST. However, only 37 sequences were highly homologous to known genes, including 34 sequences displaying higher similarities to the castor genome (Table 6). No. A19 was highly homologous to phospholipid-transporting ATPase, which is involved in the transport of phospholipids and contributes to transmembrane flipping of lipids in Arabidopsis [26]. Six protein kinases were mapped from the MSAP fragments, including B04, E02, F17, J10, J30 and K01. The homologs X02, M21, P07, P10 and R24 encoded a series of phosphatasess and participated in the fatty acid biosynthesis. F10 and V15 were identified as methyltransferase. Other candidate fragments encoded various proteins, such as FAD oxidoreductase, actin-binding protein, peroxiredoxin, calcium ion binding protein, ATP-binding cassette transporter, MYB transcription factor and ATP-dependent RNA helicase.

Of the 147 fragments cloned for the sequence analysis, 37 loci with known function during seed development exhibited gene induction by methylation. A functional homologue of fragment No. A19 was identified as phospholipid-transporting ATPase (P4-ATPase), which is necessary for the transport of phospholipids and the regulation of the fatty acid biosynthesis [27], and creates lipid asymmetry in terms of ATP hydrolysis [27]. It is thought to participate in ATP biosynthesis and phospholipid transport, which would directly affect the seed oil content [28]. In our study, a homologue of phosphatidylinositol-4-phosphate 5-kinase (PIPK) was detected in 10-day-old seeds with methylation. PIP5K, a novel protein family of liqid kinase, phosphorylates phosphatidylinositol-4-phosphate to produce phosphati-
dylinositol-4,5-bisphosphate, inositol-1,4,5-triphosphate and diacylglycerol, which is a regulator of many cellular proteins involved in signal transduction and cytoskeletal organization, and is activated by phosphatidic acid [29-31]. After abiotic stress in Arabidopsis, the up-regulated expression of AtPIPK provided evidence of the close link between phosphoinositide signaling cascades and stress responses in plants [29]. Two fragments were mapped to the N-terminal methyltransferase and O-
methyltransferase, which are essential for the maintenance of genomic methylation.

Conclusions
These results from our study clearly showed that the MSAP technique is very efficient for the genome-wide DNA methylation detection in castor, and the analysis of DNA methylation changes during the seed development. DNA hypomethylation along with seed development appeared to be an essential step in the gene transcriptional control and might contribute to the gene regulation during development.

Disclosure of conflict of interest
No conflict of interest.

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