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Short communication

Modifying DNA methylation pattern in papaya embryos to harness useful and stable variants

K. Chaturvedi*, M. Manmohan and L. Sahijram
Indian Institute of Horticultural Research, Bangalore 560089, Karnataka

ABSTRACT

The purpose of this study was to investigate the effect of various DNA methylation modifying agents on genome wide global DNA methylation status and to develop papaya cv. Surya plants with useful and stable horticulturally important variations. DNA methylation-status modifying agents (Zebularine, 5-azacytidine, 5-aza deoxycytidine (DNA hypomethylating agents), 3'-azido-deoxythymidine (DNA hypermethylating agent) were used at two concentrations (0.3mM and 0.5mM) on papaya cv. Surya zygotic embryos for incubation periods ranging from 0-120 hrs. The technique of MS-RAPD-PCR which resolves global DNA methylation pattern was employed. A total of 87 primers were tested on the control samples, 55 primers showed PCR amplification, of which 11 showed polymorphic bands. Six primers showing clear and reproducible bands were selected further for analysing methylation-modifying-agent treated samples. This technique was found to be reliable and efficient for detecting changes in DNA methylation at both the scale and pattern in a genome wide standpoint.

Key words: *Carica papaya*, MS-RAPD-PCR, zygotic embryo

DNA methylation refers to addition of a methyl (CH_3) group to the fifth carbon atom of cytosine or guanine nucleotides. In plants, cytosine at CpG, CpHpG, and CpHpH sites, where H stands for any nucleotide but guanine, can be methylated. In many eukaryotes, including plants, DNA methylation serves to provide a heritable mark that guides formation of transcriptionally silent heterochromatin. DNA methylation is an epigenetic phenomenon defined as the meiotically and mitotically heritable changes in gene expression that cannot be accounted for by changes in the DNA sequence and is thought to be of potential value for breeding applications (Peng and Zhang, 6).

Changes in DNA methylation pattern resemble mutations in DNA sequence, and can be inherited stably just as in classical Mendelian genetics. Linnaeus, Father of binomial nomenclature in biology, discovered a mutant of *Linaria vulgaris* about a quarter of millennium ago, in which floral symmetry was found to be fundamentally altered. Subsequently, it was found that Lcyc- a gene connected to flower development – in this mutant was heavily methylated and consequently silenced. DNA methylation variations can impact traits that are agriculturally important, for eg, yield (Yoshiki *et al*., 10), flowering time (Brown *et al*., 2) and resistance to biotic stress (Boyko *et al.,* 1). At high plating density, high yielding hybrid lines in maize had lower methylation levels than in the low yielding lines. Among other traits, heritable dwarfism has been induced in plants using chemical agents that alter/ modify DNA methylation status, especially when applied at developmental stages. Epigenetic mechanism helps somatic cells of plants 'memorize' an experience several of which are coordinated by methylation of GC-rich sequences, thus, ensuring passage of this memory to progeny faithfully.

A number of DNA methylation modifying agents are available for the researchers to bring about both hypo and hyper methylation of targeted DNA. Zebularine, 5-azacytidine, 5-aza deoxycytidine (DNA hypomethylating agents), 3'-azido-deoxythymidine (DNA hypermethylating agent) have been used extensively in several studies. Zebularine and 5-azacytidine, which cause DNA hypomethelation were studied extensively in a short-day (SD) plant *Pharbitis nil* (syn. *Ipomoea nil*), var. Violet to induce flowering and dwarfism. Dwarfism has been reported to be correlated with DNA methylation in maize (*Zea mays*) (Sano *et al*., 7) elucidating that the genes regulating plant height are influenced by DNA methylation. In this study, removal of the inhibitor of DNA methylation by repeated washing, resulted in a 28% reduction in the total stem length of treated plants at maturity in comparison with untreated controls implying the involvement of DNA methylation in the control of genes regulating plant height. In rice, it has been observed that DNA methylation modifiers induce heritable dwarfness (Sano *et al.,* 8). In a study involving effect of demethylating

*Corresponding author's Email: kp.kanu@gmail.com

reagent 5-azacytidine on growth, flowering time and sexual phenotype of spinach it was reported that low concentration (30 µM) promoted seed germination. Negative correlation was observed between the germination percentage, root length, and plant height with 5-azaC at 100–1000 µM. Significant reduction in flowering time and remarkable increase in percentage of monoecious individuals was also observed implying that both vegetative and reproductive growth are epigenetically regulated in spinach (Li *et al*., 5).

Papaya (*Carica papaya* L.) is one of the leading fruit crops of India. Induction of any variation, for eg., dwarfing, using DNA demethylating agents would result in interesting and potential applications of the technique. The objectives of the study were to (i) develop papaya cv. Surya plants with useful and stable horticulturally-important variations using various DNA methylation modifying agents (ii) to detect the genome wide global DNA methylation changes

DNA methylation-status modifying agents (Zebularine, 5-azacytidine, 5-aza deoxycytidine (DNA hypomethylating agents), 3'-azido-deoxythymidine (DNA hypermethylating agent) were used at two concentrations (0.3mM and 0.5mM) on papaya cv. Surya zygotic embryos for incubation periods ranging from 0-120 hrs. At shorter exposure-time, survival rate of plantlets from hypermethylating treatments was almost equal (74%) to that in control (76%). Longer exposure to hypermethylating resulted in 52% and 58% plantlet survival at concentrations 0.3mM and 0.5mM respectively. Although the trend was similar in hypomethylating treatments, survival rate was higher at shorter exposure (68.5% average at the two concentrations), and 65% at longer duration of exposure. Thus, prolonged incubation period in methylation modifying treatments was found to be more critical in determining survival rate than concentration of the chemicals. Plantlets developed from these embryos were initially transferred to protrays with vermiculite under laboratory conditions and, finally, transferred to plastic cups in soil mix and shifted to the glasshouse under ambient temperature.

Advancement in molecular techniques have made detection of changes in DNA methylation status in plants and animals possible. The technique of MS-RAPD-PCR works by recruiting a pair of isoschizomers, *MspI* vs *HpaII, TfiI* vs *PfeI*, in regular RAPD. This is a reliable and efficient method for detecting changes in DNA methylation at both the scale and pattern in a genome wide perspective (Singh, 9). Both the isoschizomers stated above recognize the same restriction site (5'-CCGG) albeit with differential sensitivity to particular methylation states of cytosines. For instance, if either of the two cytosines is fully methylated (double strand), *HpaII* will not be able to cut. On the other hand, *MspI* is unable to cut if the external cytosine is methylated fully or hemi-methylated (single strand) (Fig. 1). In

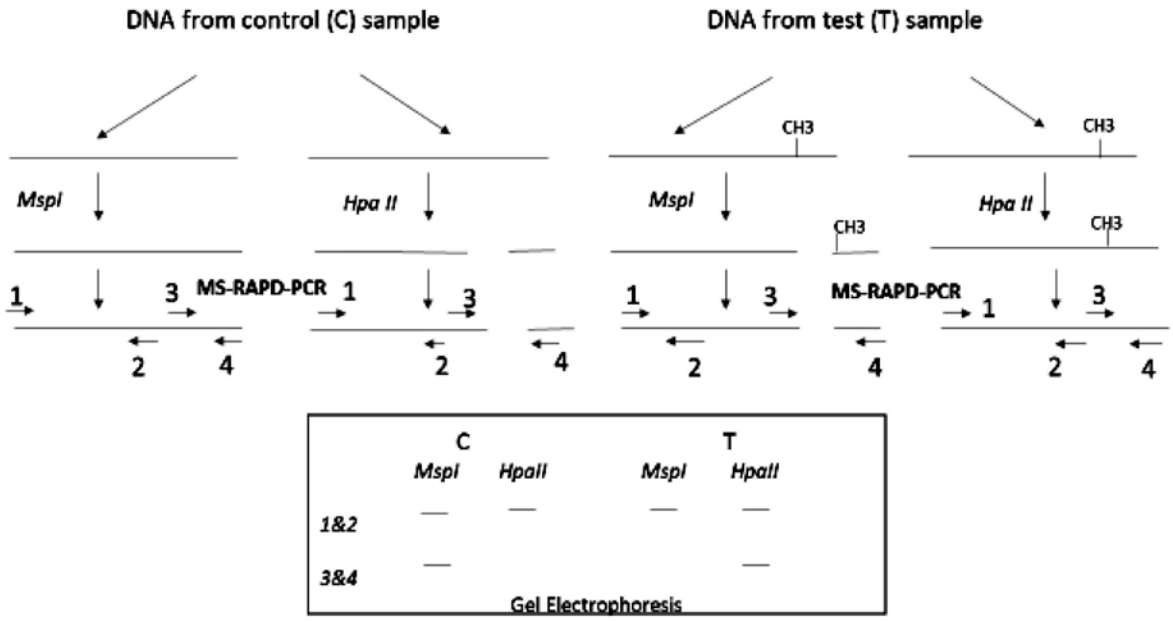

Fig. 1. Schematic diagram for DNA methylation analysis using MS-RAPD-PCR. CH3 indicates methylated sites in DNA and numbers 1,2,3,4 indicate primer binding sites (Singh,9).

this way, full methylation of the internal cytosine, or hemi-methylation of the external cytosine at the CCGG sites assayed in a given sample of DNA, can be distinguished unequivocally (Dong *et al*., 3). In several plant and animal tissues, variations induced in cytosine methylation have been successfully detected by this method. For testing the efficiency of this technique, pooled samples from each treatment were used. Five leaves each wherein the youngest, fully-expanded leaf of similar size was harvested from each plantlet, stacked (i.e., 5 leaves, one from each plantlet under each treatment) and cut in a specified sector for extracting plant DNA by CTAB method for methylation-status analysis. The DNA concentration and purity were determined spectrophotometrically and on 0.8% agarose gel. DNA methylation modification in mature zygotic embryos of papaya cv. Surya induced changes in methylation patterns in M1 individuals. These variants were evaluated by their MSAP profiles (Methylation Sensitive Amplification Polymorphism) using MS-RAPD-PCR. For each sample to be tested for DNA methylation changes, a constant quantity of genomic DNA was added to 0.5 mL PCR tubes. Each sample was digested individually with *MspI, HpaII, TfiI, PfeI* in separate tubes using 2.5 uL of 10x restriction enzyme buffer, X uL of water to make the reaction volume to 23 uL. X uL of restriction enzyme (5 U of enzyme/ ug of DNA). The order of addition was water-DNA-enzyme buffer-enzyme. The mixture was mixed and centrifuged briefly to 30s, and incubated at 37°C overnight for complete digestion. It was heat denatured by keeping at 70°C for 15min. The digested DNA was stored at -20°C for further use.

The DNA samples were diluted (undigested, *MspI* (M), *HpaII* (H), *TfiI*(T*), PfeI* (P)digested DNA from each DNA sample) to 20 ng/uL. A master mix was prepared for each primer using dNTPs (2mM), Primer (5uM), MgCl2 (25 mM), Taq Polymerase (5U/uL), 10x enzyme buffer, sterile water made up to 25 uL. The temperature profile program used was denaturation at 94°C for 4 min, 45 cycles of 94°C 30s, 34°C for 1 min, 72°C for 2 min, elongation at 72°C for 5 min followed by hold temperature. The amplification was analysed on 1.5% agarose gel with ethidium bromide staining in 1x TBE (Tris-borate-EDTA) buffer by running for about 2-3 h at 150V. A 100bp DNA ladder as was used as the fragment size marker. The gels were visualized and documented by placing under UV transilluminator. Scoring was done by comparing the presence/absence of MS-RAPD bands in test samples and control samples. Hypermethylation at a site was confirmed by absence of a band in *MspI* digested test sample but presence in *MspI* digested control sample. Similarly, hypomethylation was indicated at a site by presence of a band in test sample *MspI* digest and its absence in control *MspI* digest (Fig. 1)

A total of 87 primers were tested on the control samples, 55 primers showed PCR amplification, of which 11 showed polymorphic bands. Six primers showing clear and reproducible bands were selected further for analysing methylation-modifying-agent treated samples. With OPK4 primer, no difference was observed in samples digested with M or H, but a difference was observed in samples digested with T and P (O-1, O-2, O-3, O-4, O-5, O-6, N-1, N-2, N-3, N-4, N-5 and N-6). In the case of OPK 11 primer, M and H digested samples showed differences between all the 12 DNA samples tested. With OPK13 primer, no difference was observed in M and H digested samples; however, a difference was observed with T and P digested samples in all the 12 DNA samples. OPK14 primer resulted in no difference in banding pattern between M and H digested samples; yet a difference was observed in T and P digested samples. In the case of OPP07 primer, no difference was observed in M and H digested samples; however, in T and P digested samples, difference was observed in O-1, O-6, N-1, N-2, N-3, N-4, N-5 and N-6 DNA samples. Use of KITA2 14 primer resulted in no difference between M and H digested samples, whereas, difference was observed in O-1, O-2, O-3, O-4, O-5, O-6, N-1, N-2, N-3 and N-6 DNA samples (Fig. 2)

At the genome-wide level MSAP is a reliable technique to detect alterations in cytosine methylation. As inferred from numerous studies in animals, genetic changes brought about by DNA methylation is reported to be earlier, and faster and has a mutagenic effect, (Guo *et al*., 4). Thus, altered DNA methylation can culminate in genetic variation in numerous ways. Involvement of functional genes in producing altered MSAP profiles, implies that expression can change owing to the altered DNA methylation (Zilberman *et al*., 11), bringing about heritable phenotypic changes due to epigenetic variation in M0 or further generations. Such variations need to be tested for stability for commercial use, or, can be exploited for selecting useful mutants.

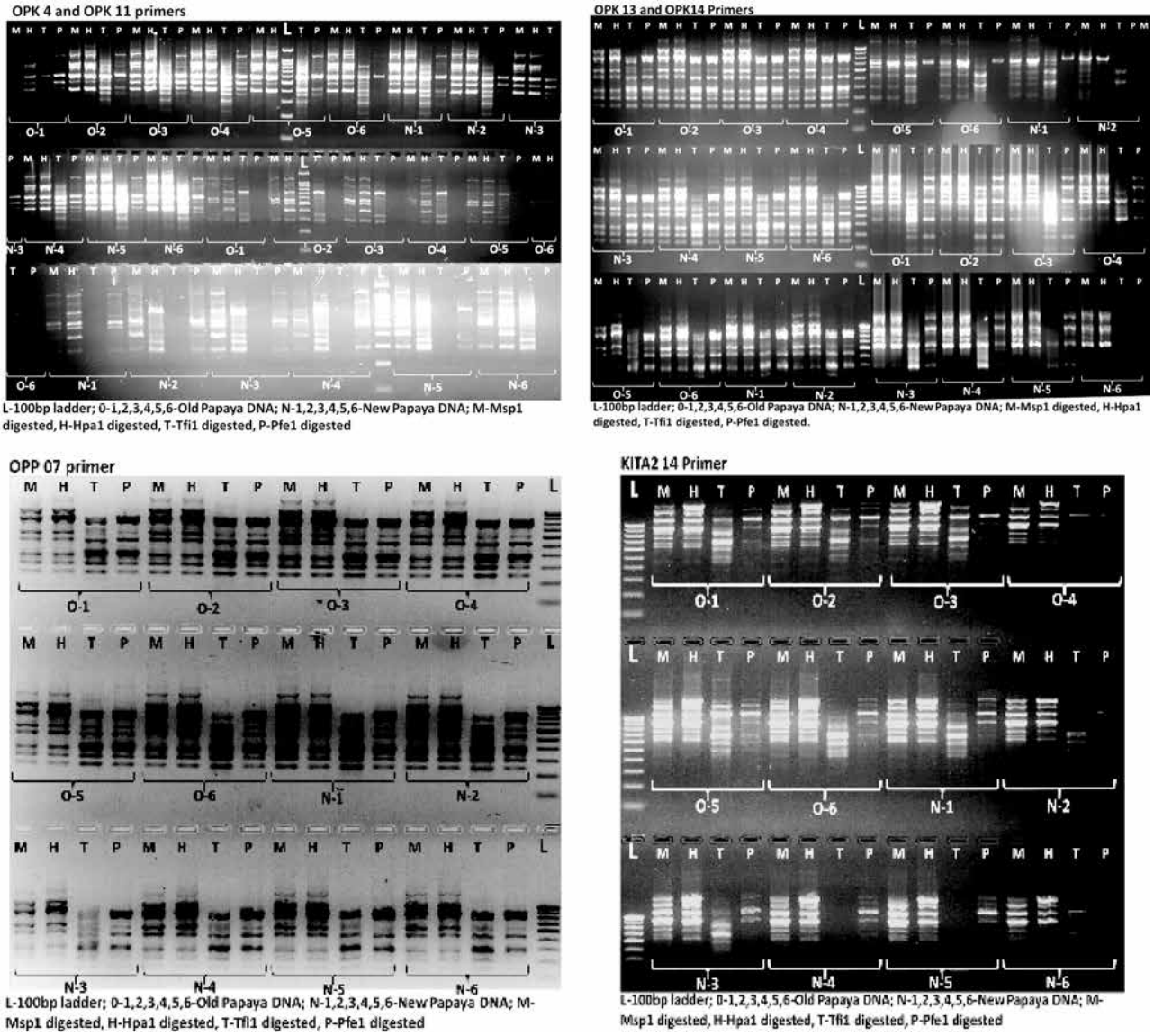

Fig. 2. L-100bp ladder, O-1,2,3,4,5,6 control papaya; N-1,2,3,4,5,6 treated papaya DNA; M-*MspI*, H- *HpaII*, T-*TfiI*, P-*PfeI* digested sample with RAPD primers

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