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Evaluation of DNA methylation using methylation-sensitive amplification polymorphism in plant tissues grown in vivo and in vitro

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Abstract In present study, methylation-sensitive AFLP (MSAP) markers were employed to assess DNA methylation, degree of alterations in DNA methylation and methylation polymorphism in plant tissues growing in vivo and in vitro. The leaf tissues of six plants growing in vivo and in vitro were subjected to MSAP profiling. A total of 717 MSAP markers in *Salvadora persica*, 801 in *Commiphora wightii*, 874 in male (M) and 845 in female (F) genotype of *Simmondsia chinensis*, 719 in *Jatropha curcas* and 880 in *Withania coagulans* were obtained with seventeen MSAP primer combinations. Percentage methylation in genome obtained was higher in in vivo-grown tissues of *S. persica* (39.47 %), *S. chinensis*—M (61.71 %) and *W. coagulans* (71.59 %); and in in vitro-grown tissues of *C. wightii* (65.17 %), *S. chinensis*—F (60.83 %) and *J. curcas* (68.29 %). The percentage polymorphism in methylated DNA obtained was 8.71 % in *S. persica*, 9.81 % in *J. curcas*, 10.10 % in *S. chinensis*—F, 10.26 % in *W. coagulans*, 10.66 % in *S. chinensis*—M and 13.98 % in *C. wightii*. The difference in DNA methylation and polymorphism in genomes reflect the plasticity in genomes of the plants growing under two different environments. Different pattern of DNA methylation of the homologous nucleotide sequences and polymorphism in the methylated DNA in tissues under in vitro and in vivo conditions suggest possibility of involvement of these fragments in the dynamic processes regulating plant growth and development under prevailing growth conditions.

Keywords Epigenetic · In vitro · In vivo · Methylation · MSAP · Polymorphism

Introduction

Plant genetic resources are the basis of global security for food and health. Overexploitation from wild, hostile environmental factors and habitat disturbances affect the survival of a number of plant species. In vitro multiplication strategies have been recognized as a key component of biotechnological approaches and have several benefits with continuous supply of plant material making significant contributions to the exploitation of plant species and eliminating the need for harvest from wild (Rathore et al. 2012). Under in vitro conditions different from natural conditions, plants grow under unique environment. After optimization of culture and growth conditions, the microenvironment of culture vessel is the main stress for plants; to which plant has to adapt at various stages of growth and culture durations (viz. 0, n/2 and nth day of transfer; n is number of days after which tissues are sub-cultured). Environmental stresses affect plants by inducing oxidative stress and plants respond by differential expressions of hundreds of genes and protein functions in response to different stresses. Different plant species tolerate stresses to a varying degree depending on reprogramming the gene

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expression to modify their physiology, metabolism, and growth. One of the molecular mechanism by which plants could silence or super-activate the selected DNA templates is epigenetic modifications that change the gene expression without changing the DNA sequences (Habu et al. 2001).

Methylation of cytosine in DNA strand is the most important epigenetic mechanism, which plays a central role in epigenetic control of spatial and temporal patterns of gene expression (Rapp and Wendel 2005). DNA methylation is one of epigenetic changes occurring in plants growing under different environmental conditions. Evidence suggests that the level of DNA methylation is known to be modulated during plant development and organ/tissue differentiation (Berdasco et al. 2008; Bottley et al. 2008; Riddle and Richards 2002; Zhang et al. 2010). Valledor et al. (2007) reviewed involvement of DNA methylation in tree development and micropropagation. In callus, evidence has indicated that there is cell-to-cell methylation diversity (Krizova et al. 2009). DNA methylation has been suggested as an important mechanistic basis of somaclonal variation in plants (Kaeppler et al. 2000) and leading factor for genetic variation (Golyasnaya and Tsvetkova 2006). Thus, the epigenetic variations accumulated during regeneration process are important and should not be overlooked due to their diverse roles.

Different kind of environmental stresses has also been suggested to alter DNA methylation pattern (Mastan et al. 2012). Vanyushin and Ashapkin (2011) have established that DNA methylation in plants is not only species but also tissue, organ, and age specific. Functional differentiations of cells suggest variations in DNA methylation status of different cells in an organism. Arnholdt-Schmitt et al. (1995) found that total DNA methylation levels varied with developmental stage in carrot plants regenerated from tissue culture. During rejuvenation, levels and tissue distributions of DNA methylation may vary significantly. The methylation re-patterning might play role in genome plasticity by facilitating somatic recombination. Methylation-sensitive amplification polymorphism (MSAP) profiling is an AFLP based method for detection of DNA methylation (Xu et al. 2000). Using MSAP markers, cytosine methylation status of somatic embryo derived *Secale cereale* L. regenerates (Gonzalez et al. 2013) and long-term proliferating embryogenic suspension cultures of oil palm (Rival et al. 2013) were assessed. Wang et al. (2012) assessed methylation profiles of petal, primary and secondary leaf and shoot tip-derived plantlets using MSAP. With these significances and generalities of DNA methylation variations, present study was carried out to assess the DNA methylation pattern, degree of methylation alterations and polymorphism in methylated DNA in in vivo and in vitro-grown tissues from six plants (*Salvadora persica*, *Commiphora wightii*, male and female genotype of *Simmondsia chinensis*, *Jatropha curcas* and *Withania coagulans*) using MSAP markers.

Materials and methods

Plant material and genomic DNA extraction

In present study in vivo and in vitro-grown tissues from *S. persica* Linn. (Salvadoraceae), *C. wightii* (Arn.) Bhandari (Burseraceae), male (M) and female (F) genotype of *S. chinensis* (Link) Schneider. (Simmondsiaceae), *J. curcas* L. (Euphorbiaceae) and *W. coagulans* (Stocks) Dunal (Solanaceae) were processed for MSAP profiling. Leaf tissue samples were taken from field/in vivo-grown mature plants and in vitro-grown shoot cultures. Third and fourth leaves from the in vitro established shoot cultures were harvested and used for present study. The proliferating cultures were maintained in vitro by repeated subculture on 0.75 % agar-gelled Murashige and Skoog's medium (Murashige and Skoog 1962) supplemented with optimized concentrations of plant growth regulators (PGRs) for respective plant species. The in vitro cultures were maintained in a culture room at 26 ± 2 °C, 55–60 % relative humidity (RH), under 12 h per day (h day^{-1}) photoperiod with a light intensity (provided by white florescent tubes Philips, Mumbai, India) of 35–40 µmol m^{-2} s^{-1} spectral flux photon (SFP) of photosynthetically active (460–700 nm) radiations (PAR). Leaf tissues from in vivo and in vitro-grown plants were harvested and immediately processed for genomic DNA extraction. Genomic DNA was extracted using cetrimonium bromide (CTAB) protocol (Doyle and Doyle 1990) with slight modifications (Mastan et al. 2012). DNA samples were quantified spectrophotometrically using Epoch micro-volume spectrophotometer (BioTek Instruments Inc., USA). The aliquots were diluted to the final concentration of 10–15 ng μl^{-1}.

Methylation-sensitive amplification polymorphism fingerprinting

The genomic DNA (200 ng) of in vivo and in vitro-grown tissues from each plant was digested with *EcoR* I/*Msp* I and *EcoR* I/*Hpa* II restriction enzymes at 37 °C for 2 h. The digested aliquot were ligated to *EcoR* I and *Msp* I or *Hpa* II specific adaptors (Xu et al. 2000) to avoid reconstruction of restriction sites one for *EcoR* I sticky ends and other for *Msp* I or *Hpa* II sticky ends, at 20 °C for 90 min. The ligated DNA was diluted for 1:10 and pre-amplified using *EcoR* I and *Msp* I or *Hpa* II primer with one selective nucleotide at the 3′ end each. The pre-amplified product was diluted 1:10 with sterile tris–EDTA (TE) buffer. These

diluted products were amplified using different combinations of *EcoR* I and *Msp* I or *Hpa* II primer each with three selective nucleotides at the 5′ and 3′, respectively. Selective amplifications were performed using 65 °C as the initial annealing temperature for the first cycle and for subsequent 11 cycles the annealing temperature was successively reduced by 0.7 °C. Twenty-three cycles were run at 56 °C annealing temperature. A total of 25 pairs of primers (combinations of *EcoR* I and *Hpa* I–*Msp* I primers) were evaluated for this analysis. Formamide dye was added to PCR product in 1:5 ratio (one volume of dye to five volumes of sample) and subjected to electrophoretic separation on 6 % denaturing polyacrylamide gel (PAGE) in 1X TBE buffer in a sequencing gel system (LKB, Sweden) at 300 V for 5–6 h at room temperature (26–28 °C). The gels were stained with silver nitrate and scanned for further data recording. To verify reproducibility and confirm accuracy of MSAP profiles, reaction with each primer was repeated at least twice.

Profiling scoring and data analysis

The fingerprints showing reproducible results between replicas were scored for MSAP data analysis. In PAGE profiles the bands present in both *EcoR* I/*Hpa* II and *EcoR* I/*Msp* I lane were considered as Type I (non-methylated); in *EcoR* I/*Msp* I lanes, but not in *EcoR* I/*Hpa* II were considered as Type II (methylated), in *EcoR* I/*Hpa* II, but not in *EcoR* I/*Msp* I lane as Type III (methylated) and absent in both the lanes as type IV (unknown). The absence of bands in both the *Msp* I and *Hpa* II lanes could be due to either genetic polymorphism or hyper-methylation. A site was considered "methylation polymorphic" (MP) if there was at least one sample in which the site was methylated and at least one sample for which the site was not methylated. The scored MSAP bands were transformed into a binary character matrix, using "0" and "1" to indicate the absence and presence, respectively, of particular loci. Loci that showed changes in one pair of iso-schizomer were taken into account for detection of methylation polymorphism, confounding the effect due to nucleotide changes at CCGG sites. Percentage of methylation was calculated as number of methylated bands × 100/total number of bands. Percentage of methylation polymorphism was calculated using the formula (=number of polymorphic methylated bands × 100/number of methylated bands).

Results

Out of 25, 17 selective combinations of MSAP primers were used to generate MSAP fingerprints (Fig. 1) and data analysis. Seventeen combinations of primers produced a total 717 bands in *S. persica*, 801 in *C. wightii*, 874 and 845 in *S. chinensis*—M and *S. chinensis*—F genotypes respectively, 719 in *J. curcas* and 880 in *W. coagulans*. The highest and least number of methylated and polymorphic bands in in vivo and in vitro-grown tissues was recorded in *W. coagulans* and *S. persica*, respectively (Table 1; Fig. 2a). The percentage methylation obtained in in vivo-grown tissues ranged from 39.47 % in *S. persica* to 71.59 % in *W. coagulans*. While in in vitro-grown tissues the percentage methylation ranged from found 37.8 % in *S. persica* to 68.29 % in *J. curcas*. The percentage polymorphism in methylated DNA ranged from 8.71 % in *S. persica* to 13.98 % in *C. wightii* (Fig. 2b).

In overall analysis irrespective of growth conditions, the highest percentage of methylation was detected with primer MSAP-1 in *S. persica* (75 %), MSAP-21 in *C. wightii* (88.46 %), MSAP-12 in *W. coagulans* (88.89 %) and *S. chinensis*—F (88 %), MSAP-4 in *S. chinensis*—M (92.31 %) and MSAP-2 in *J. curcas* (92.31 %). While MSAP-15 in *S. persica* (30.95 %) and *S. chinensis*—F (66.67 %), MSAP-16 in *S. chinensis*—M (53.19 %), MSAP-11 in *C. wightii* (30.58 %), MSAP-9 in *J. curcas* (54.05 %) and MSAP-3 in *W. coagulans* (34.29 %) showed the least percentage of methylation. Similarly the MSAP-15 primer in *Salvadora* (30.77 %), MSAP-21 primer in *S. chinensis*—M (50 %) and F (28.57), MSAP-4 in *C. wightii* (27.78 %), MSAP-19 in *J. curcas* (48.57 %) and *W. coagulans* (28.57 %) showed highest polymorphism in methylated DNA. MSAP-2, 11, 12, 13 and 16 in *Salvadora*; MSAP-13 in *S. chinensis*—M; MSAP-2 in *J. curcas*; MSAP-1 and 3 in *C. wightii*; MSAP-3 and 11 in *W. coagulans* fail to detect polymorphism while MSAP-11 showed least polymorphism (1.85 %) in *S. chinensis*—F (Table 2; Fig. 3) in overall analysis irrespective of growth conditions. When compared in vivo and in vitro-derived tissues of plant species under investigations, the percentage methylation in genome obtained was higher in in vivo-grown tissues of *S. persica* (39.47 %), *S. chinensis*—M (61.71 %) and *W. coagulans* (71.59 %); and in in vitro-grown tissues of *C. wightii* (65.17), *S. chinensis*—F (60.83 %) and *J. curcas* (68.29 %). The average percentage of methylation in tissues of plants studied was found in the order of *J. curcas* (79.3 %), *S. chinensis*—F (76.6 %), *W. coagulans* (71.8 %), *S. chinensis*—M (70 %), *C. wightii* (68.7 %) and *S. persica* (49.9 %). While the order of average polymorphism in tissues of plants studied was found in the order of *S. chinensis*—M (14.8 %), *S. chinensis*—F (11.5 %), *J. curcas* (10.9 %), *C. wightii* (10.8 %), *W. coagulans* (10.3 %) and *S. persica* (8.5 %). The ratio of methylated to non-methylated bands in in vivo tissue was found 0.74 in *S. persica*, 2.07 in *C. wightii*, 2.41 in *S. chinensis*—M, 2.17 in *S. chinensis*—F, 4.67 in *W. coagulans* and 2.51 in *J. curcas*. Similarly in in vitro-grown tissues it was found 0.75 in *S. persica*, 2.57 in *C. wightii*, 2.86 in *S.*

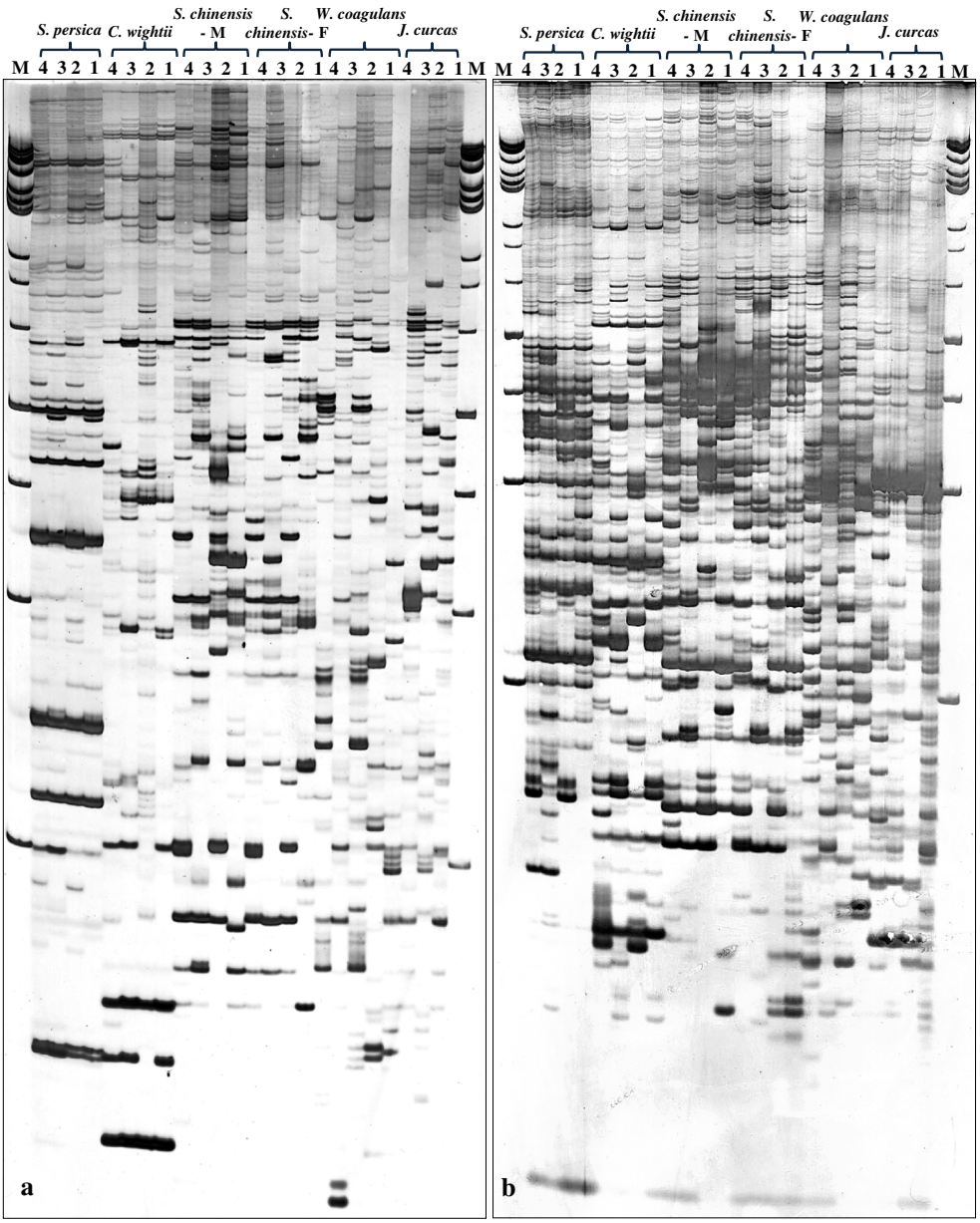

Fig. 1 Methylation-sensitive AFLP fingerprints of *in vivo* (*lane 1* and *2*) and *in vitro* (*lane 3* and *4*) growing tissues using MSAP 10 (**a**) and 17 (**b**) primer. Lane M represent DNA *ladder*; 1–4 methylation-sensitive profile of a plant. Odd number lanes were cut with *Msp*-I and even number lanes were cut with *Hpa*-II

chinensis—M, 2.11 in *S. chinensis*—F, 2.96 in *W. coagulans* and 2.99 in *J. curcas*.

Discussion

The specific interaction between developmental program and external stimuli coordinate the gene expression; which in turn determines the adaptability of a plant species under the prevailing environmental conditions. Global methylcytosine content in DNA varies widely across species, organs, and developmental states. During developmental processes, the cells with same DNA acquire different functions and identity. DNA methylation has been suggested to control the differentiation processes by regulating tissue-specific genes and maintaining cell status stability (Fraga et al. 2002). Under in vitro environment, organogenic processes in plants are reversible and the functional cells become pluripotent; thus the role of DNA methylation cannot be ruled out during this unique event. DNA methylation or epigenetic code has been suggested one among the possible mechanisms involved in the process of

Table 1 Summary of different types of bands, methylation and polymorphism obtained in MSAP analysis of plant tissue grown under in vivo and in vitro conditions

Results	*S. persica*	*C. wightii*	*S. chinensis*—M	*S. chinensis*—F	*W. coagulans*	*J. curcas*
No. of total bands	717	801	874	845	880	719
No. of methylated bands in in vivo-grown tissues	283	482	533	511	630	462
No. of methylated bands in in vitro-grown tissues	274	522	512	514	565	491
% Methylation in in vivo-grown tissues	39.47	60.17	61.71	60.47	71.59	64.26
% Methylation in in vitro-grown tissues	37.8	65.17	59.17	60.83	64.2	68.29
No. of methylated polymorphic bands	31	78	71	61	72	52
% Methylation polymorphism	8.71	13.98	10.66	10.10	10.26	9.81

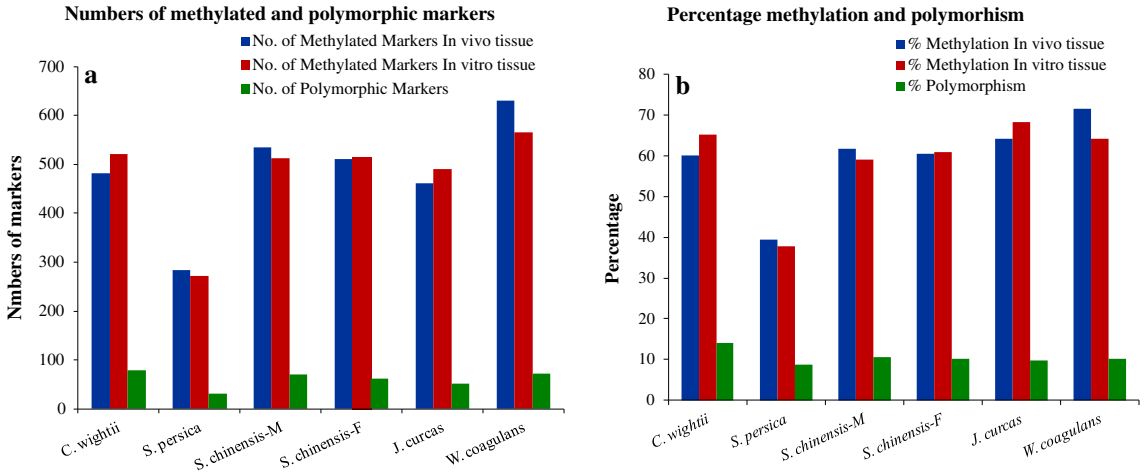

Fig. 2 Numbers of methylated, non-methylated and polymorphic markers (**a**) and percentage methylation and polymorphism (**b**) in in vivo and in vitro-derived tissues

regaining the pluripotent nature (Fraga et al. 2002), however very limited knowledge about epigenetic control during plant cell differentiation or dedifferentiation and organogenesis is available (Goodrich and Tweedie 2002). Several studies dealing with DNA methylation in relation to tree development, micropropogation and somaclonal variation demonstrated DNA methylation levels are hallmarks for growing seasonal periods and are related to open windows of competence in plants (Valledor et al. 2007).

In higher plants, cytosine bases are often extensively methylated i.e. 5-methylcytosine (m^5C) and often its content is comparable to that of cytosine (Gonzalez et al. 2013). The results presented here suggest that the cytosine methylation levels vary and the plant tissues under in vivo and in vitro exhibit polymorphism in methylated DNA (Li et al. 2000). DNA methylation is not static and has unique dynamics during specific developmental stages (Valledor et al. 2007). DNA methylation patterns have been shown to vary among regenerated plants. The present study provides basic evidence that methylation changes occur at a sufficiently high frequency, which might be one of the sources of tissue culture-induced variations (Gonzalez et al. 2013). Study showed both decrease and increase in methylation depending on plant species, thereby altering gene expression pattern by affecting access to the DNA. Methylation in the promoter region might influence the gene expression through regulatory mechanism. Beside changes in gene expression, this could lead to changes in recombination rates, and changes in the timing of DNA replication, perhaps leading to chromosome breakage (Phillips et al. 1994; Gonzalez et al. 2013) during long term culture maintenance leading somaclonal variations. DNA methylation has also been reported to play an important role in the formation of polyploids. Rapid adjustments of DNA methylation levels and patterns have been revealed in the studies on characteristics of DNA methylation in the polyplodization of *Arabidopsis* (Madlung et al. 2005), cotton (Keyte et al. 2006) and Cucumis (Chen and Chen 2008). Cytosine methylation is one of epigenetic modification in plants occurring mainly in the repetitive elements

Table 2 Details of total number of methylated (MB), polymorphic bands (PB), % methylation (%M) and polymorphism (%P) obtained with different MSAP primers

Primer	*EcoR* I primer	*Hpa* II/*Msp* I primer	*S. persica*				*S. chinensis*—M				*S. chinensis*—F				*C. wightii*				*J. curcas*				*W. coagulans*			
			MB	PB	%M	%P	MB	PB	%M	%P	MB	PB	%M	%P	MB	PB	%M	%P	MB	PB	%M	%P	MB	PB	%M	%P
MS-1	E-AAC	HM-CAC	24	4	75	16.67	27	3	81.82	11.11	21	4	67.74	19.05	18	0	69.23	0.00	27	5	71.05	18.52	35	1	83.33	2.86
MS-2	E-ACA	HM-CAC	9	0	39.13	0	25	5	62.50	20	30	4	75	13.33	16	2	57.14	12.5	24	0	92.31	0	39	4	84.78	10.26
MS-3	E-ACG	HM-CAC	27	1	51.92	3.70	40	3	70.18	7.5	46	5	73.02	10.87	43	0	74.14	0	44	1	86.27	2.27	12	0	34.29	0
MS-4	E-AGC	HM-CAC	18	1	69.23	5.56	36	1	92.31	2.78	29	2	69.05	6.90	18	5	48.65	27.78	41	1	82.00	2.44	16	2	50.00	12.50
MS-9	E-AGC	HM-CAA	28	6	71.79	21.43	32	4	78.05	12.50	40	3	78.43	7.50	24	1	61.54	4.17	20	1	54.05	5	24	2	68.57	8.33
MS-10	E-ACT	HM-CAA	22	1	53.66	4.55	36	4	76.60	11.11	44	2	86.27	4.55	34	7	79.07	20.59	55	3	91.67	5.45	42	3	87.50	7.14
MS-11	E-AAG	HM-CAG	18	0	35.29	0	28	3	53.85	10.71	54	1	84.38	1.85	37	2	30.58	5.41	47	3	81.03	6.38	27	0	81.82	0
MS-12	E-AAG	HM-CAT	22	0	45.83	0	53	2	85.48	3.77	44	3	88.00	6.82	54	2	78.26	3.70	56	3	81.16	5.36	48	2	88.89	4.17
MS-13	E-AAG	HM-CTA	20	0	45.45	0	33	0	75	0	44	8	69.84	18.18	32	6	62.75	18.75	39	3	75	7.69	30	1	81.08	3.33
MS-14	E-AAG	HM-CTC	12	1	31.58	8.33	30	1	69.77	3.33	35	7	70	20	43	1	81.13	2.33	57	3	86.36	5.26	51	2	85	3.92
MS-15	E-AAG	HM-CTG	13	4	30.95	30.77	21	3	67.74	14.29	42	3	66.67	7.14	50	4	74.63	8	38	6	76	15.79	14	4	46.67	28.57
MS-16	E-AAG	HM-CTT	16	0	33.33	0	25	6	53.19	24	48	5	82.76	10.42	33	6	71.74	18.18	50	3	84.75	6	24	2	64.86	8.33
MS-17	E-ACC	HM-CAG	36	2	64.29	5.56	37	7	63.79	18.92	44	4	78.57	9.09	42	8	76.36	19.05	43	3	84.31	6.98	38	7	79.17	18.42
MS-18	E-ACC	HM-CAT	33	4	66	12.12	32	10	59.26	31.25	35	3	74.47	8.57	36	4	65.45	11.11	44	3	84.62	6.82	30	4	68.18	13.33
MS-19	E-ACC	HM-CTA	26	2	55.32	7.69	46	9	74.19	19.57	41	7	83.67	17.07	40	8	74.07	20	35	17	63.64	48.57	35	10	67.31	28.57
MS-20	E-ACC	HM-CTC	13	1	40.63	7.69	29	3	70.73	10.34	41	2	85.42	4.88	38	2	74.51	5.26	45	6	81.82	13.33	40	4	78.43	10
MS-21	E-ACC	HM-CTG	19	4	39.58	21.05	28	14	56	50	28	8	68.29	28.57	46	3	88.46	6.52	37	11	72.55	29.73	25	4	71.43	16
Total			356	31	–	–	558	78	–	–	666	71	–	–	604	61	–	–	702	72	–	–	530	52	–	–
Avg. (%)			–	–	49.9	8.5	–	–	70.0	14.8	–	–	76.6	11.5	–	–	68.7	10.8	–	–	79.3	10.9	–	–	71.8	10.3

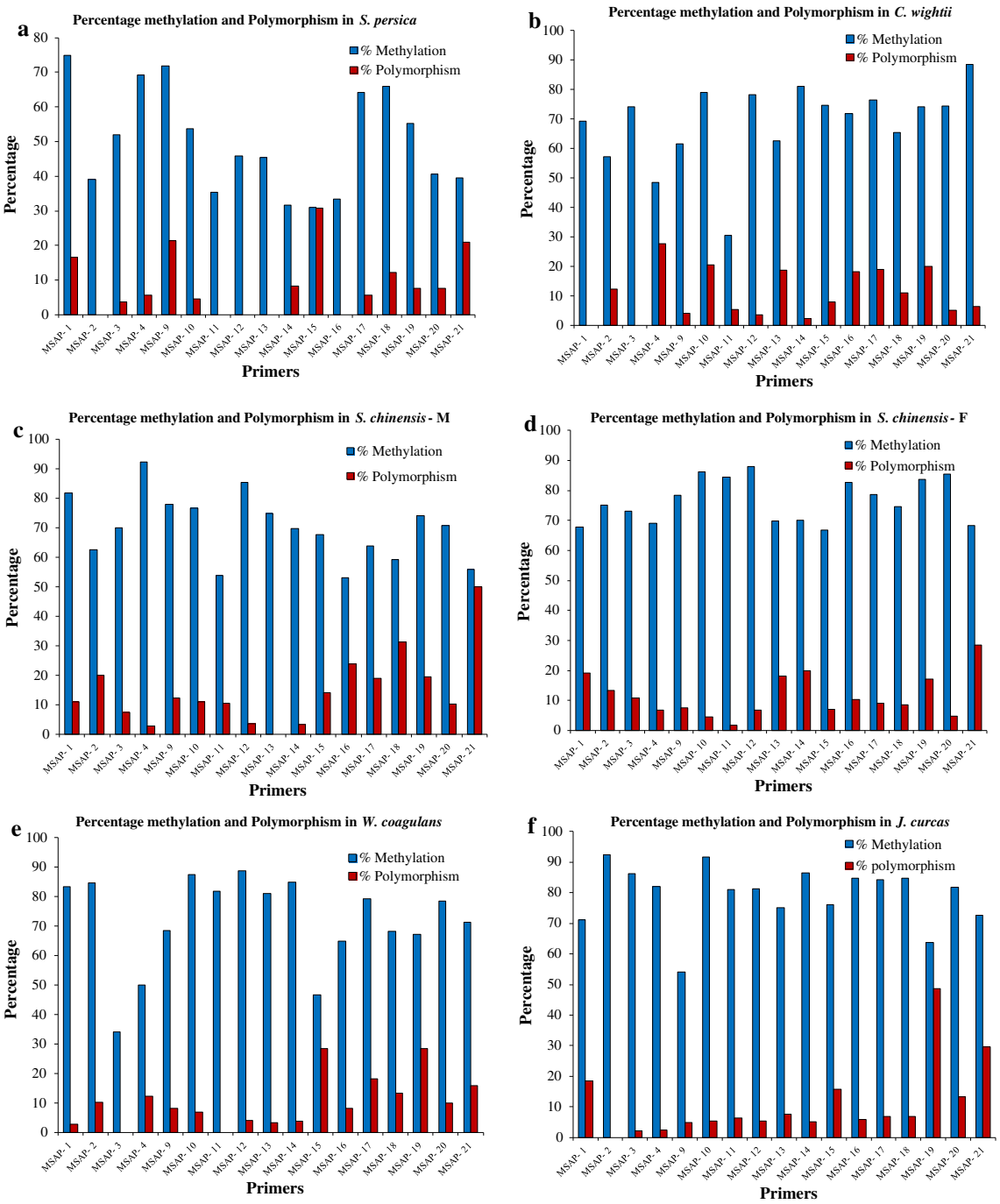

Fig. 3 Percentage methylation and polymorphism obtained with different MSAP primers in *S. persica* (**a**), *C. wightii* (**b**), *S. chinensis*—M and F (**c**, **d**), *W. coagulans* (**e**) and *J. curcas* (**f**)

and transposons. In plant tissue culture processes, genetic stability is certified by DNA markers, however, no uniform regenerates are often obtained (Valledor et al. 2007) and this may be attributed to epigenetic changes. DNA methylation has been suggested reversible, but being heritable their role in tissue culture induced variations can't be ruled out in long-term. These methylation changes may activate transposable elements and may be involved in cytogenetic instability through heterochromatin modification (Kaeppler et al. 2000), thus indicating contribution of such changes in process of somaclonal variations in long-term maintained in vitro cultures.

Environmental conditions influence developmental program and growth conditions; and different environmental conditions may trigger changes in methylation (Wada et al. 2004; Verhoeven et al. 2010; Mastan et al.

2012), which may or may not have evolutionary consequences, as the extent of transmission of environmentally induced methylation changes to offspring is largely unknown (Verhoeven et al. 2010). Environmental stress-induced methylation changes may be targeted specifically to stress-related genes; alternatively, this may generate nonspecific/random differences between individuals, having adaptive significance during stress (Rapp and Wendel 2005) by increasing the range of variation. Environmental induced methylation re-patterning can lead to increased genetic variations by facilitating somatic recombination, which could be adaptive during times of stress (Brautigam et al. 2013) and this is also applicable for apomictic/clonal lineages which grow under different environment. Under in vitro conditions, plants grow under unique environmental conditions and tissue culture-induced methylation variation has been detected by various workers (Rival et al. 2013; Gonzalez et al. 2013; Huang et al. 2012; Wang et al. 2012) and involvement of DNA methylation have been shown in tree development and micropropagation (Valledor et al. 2007). Plant tissue growing under differ environmental conditions like in vivo and in vitro in present case exhibit different degree of methylation. The altered methylation and polymorphism in the methylated DNA contribute phenotypic plasticity in the prevailing environment hence making plant able to grow under particular conditions. Occurrence of tissue culture-induced methylation variation has been reported commonly but long-term maintenance of regenerates in vitro increases chances of mutation or their frequency. In present study methylation changes occur during tissue culture growth conditions thus indicating switching on and off of some genes during tissue culture conditions. The alteration in methylated and hemimethylated sequences suggests that many coding regions may be affected which might play important role in providing adaptive responses to plants under prevailing environment.

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