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Inter-individual variation in DNA methylation is largely restricted to tissue-specific differentially methylated regions in maize

Massimiliano Lauria^{1†}, Rodrigo Antonio Echegoyen-Nava^{2†}, Dalia Rodríguez-Ríos², Silvio Zaina³ and Gertrud Lund^{2*}

Abstract

Background: Variation in DNA methylation across distinct genetic populations, or in response to specific biotic or abiotic stimuli, has typically been studied in leaf DNA from pooled individuals using either reduced representation bisulfite sequencing, whole genome bisulfite sequencing (WGBS) or methylation sensitive amplified polymorphism (MSAP). The latter represents a useful alternative when sample size is large, or when analysing methylation changes in genomes that have yet to be sequenced. In this study we compared variation in methylation across ten individual leaf and endosperm samples from maize hybrid and inbred lines using MSAP. We also addressed the methodological implications of analysing methylation variation using pooled versus individual DNA samples, in addition to the validity of MSAP compared to WGBS. Finally, we analysed a subset of variable and non-variable fragments with respect to genomic location, vicinity to repetitive elements and expression patterns across leaf and endosperm tissues.

Results: On average, 30% of individuals showed inter-individual methylation variation, mostly of leaf and endosperm-specific differentially methylated DNA regions. With the exception of low frequency demethylation events, the bulk of inter-individual methylation variation (84 and 80% in leaf and endosperm, respectively) was effectively captured in DNA from pooled individuals. Furthermore, available genome-wide methylation data largely confirmed MSAP leaf methylation profiles. Most variable methylation that mapped within genes was associated with CG methylation, and many of such genes showed tissue-specific expression profiles. Finally, we found that the *hAT* DNA transposon was the most common class II transposable element found in close proximity to variable DNA regions.

Conclusions: The relevance of our results with respect to future studies of methylation variation is the following: firstly, the finding that inter-individual methylation variation is largely restricted to tissue-specific differentially methylated DNA regions, underlines the importance of tissue-type when analysing the methylation response to a defined stimulus. Secondly, we show that pooled sample-based MSAP studies are methodologically appropriate to study methylation variation. Thirdly, we confirm that MSAP is a powerful tool when WGBS is not required or feasible, for example in plant species that have yet to be sequenced.

Keywords: Inter-individual variation in methylation, Tissue-specific, CG and non-CG methylation, Class II transposable elements, Endosperm

* Correspondence: gertrud.lund@cinvestav.mx

†Equal contributors

²Gertrud Lund, Department of Genetic Engineering, CINVESTAV - Unidad Irapuato, Km. 9.6 Libramiento Norte Carretera Irapuato-Leon, Apdo. Postal 629, C. P. 36500 Irapuato, GTO, Mexico

Full list of author information is available at the end of the article



Background

In plants cytosine methylation occurs at symmetric 5'-CpG-3' dyads (CG) and 5'-CpHpG-3' (CHG; H is A, C or T) triads, in addition to asymmetric 5'-CpHpH-3' (CHH) triads [1–3]. In each case, methylation is controlled by distinct DNA methyltransferases. In *Arabidopsis thaliana*, the main CG, CHG and CHH methylases are METHYLTRANSFERASE1 (MET1), CHROMOMETHYLASE 3 (CMT3) and CHROMOMETHYLASE 2 (CMT2) or DOMAINS-REARRANGED METHYLTRANSFERASE 2 (DRM2), respectively. In maize, the corresponding homologs are ZMET1 and ZMET2 or 5, CMT2 is absent and ZMET3 [4–10]. In addition, the DOMAINS-REARRANGED METHYLTRANSFERASE 2 (or ZMET3 in maize) plays an important role in the RNA-directed DNA methylation pathway [4, 9, 11], first discovered in tobacco plants [12], which culminates with de novo methylation of cytosine in CG, CHG and CHH contexts in response to small RNA signals (reviewed in [13]).

The genome-wide distribution of DNA methylation has been detailed both in *Arabidopsis* [14, 15] and agronomically important plants such as rice, maize, soybean, cassava, soybean, common bean, wheat and cotton [16–23]. Collectively, these studies show that the bulk of DNA methylation is located within transposable elements (TEs), underlining its important and well-characterized function - proposed several years ago - in regulating TE activity [24, 25]. In addition, those data also uncovered the prevalence of CG methylation within the gene-body.

To date, nearly all genome-wide methylation studies of natural variation in DNA methylation, either within a genetically identical population following several generations, or across distinct genetic populations or tissue-types, compare average DNA methylation states of pooled individuals or less than 2 individuals per generation [18, 25–36]. However, the few studies that take inter-individual variation into account, show that both natural and stress-induced methylation responses are heterogeneous across individuals and can vary between developmental stages [37–42]. All the aforementioned studies were performed using the methylation sensitive amplified polymorphism (MSAP) technique. Although this technique only surveys the methylation state of a defined restriction enzyme site that is sensitive to DNA methylation (e.g. *HpaII*), it does give a reliable readout of the genome-wide methylation state. As an example, we demonstrated that the 13% reduction in DNA methylation in maize endosperm relative to leaf and embryo tissues largely resulted from maternal hypomethylation [42], results that were subsequently confirmed by high-throughput bisulfite sequencing of *Arabidopsis*, rice, sorghum, maize and castor bean genomes [34, 43–47].

Currently scarce information is available regarding inter-individual methylation variation (ii-MV) across genetically identical progeny and whether such variation

differs across plant tissues. Given the lack of such studies, we analysed methylation profiles of ten individual leaf and endosperm tissues derived from single cobs of two hybrid and one inbred line by MSAP. Furthermore, since pooled samples have been used in the majority of DNA methylation variation studies, we addressed the important methodological issue of whether individual samples better reflect methylation variation compared to pooled samples. Our data reveal that ii-MV is readily detected in both leaf and endosperm tissue, but largely restricted to tissue-specific differentially methylated regions (tDMRs). We find that the majority of such variation is detectable by analysis of pooled samples and show that MSAP represents a reliable alternative to WGBS for analysing methylation variation.

Results

Characterization of inter-individual methylation variation (ii-MV) in maize endosperm and leaf using MSAP

MSAP was employed to characterize ii-MV. This technique is a modification of AFLP (Amplified Fragment Length Polymorphism), which is based on random amplification of restriction fragments typically generated by digestion of genomic DNA with *EcoRI* and *MseI* restriction enzymes [48]. In MSAP, *MseI* is replaced by *HpaII*, which cleaves CCGG sites, unless one or both cytosines are methylated on both strands [49]. Adaptors are ligated to digested restriction sites and resulting fragments are subsequently amplified in two consecutive PCR reactions with primers complementary to core sequence of adaptors and recognition sites of restriction enzymes. Typically, the number of selective nucleotides added to the primers at 3' ends is increased in the second amplification reaction. In addition, one primer is radioactively labelled to enable visualization of restriction fragments by autoradiography.

Ten individual endosperms harvested 15 days after pollination (DAP) and ten 14-day-old leaves (**W23/A69Y**, the seed donor is in bold) were analysed by MSAP and AFLP using 12 and 10 selective primer combinations, respectively. In either case, tissue samples were derived from a single hybrid cob. A total of 13 (69/526) and 3% (14/440) of endosperm and leaf MSAP fragments, respectively, showed variation across individuals (Additional file 1: Table S1). The higher number of total MSAP fragments in endosperm versus leaf (i.e. 526 and 440, respectively) was expected given that the former is hypomethylated relative to leaf [42]. Conversely, no variation was detected by AFLP (results not shown). Further MSAP analyses of individual endosperms and leaves from the **Mo17/B73** hybrid, in addition to individual endosperms from the A69Y inbred line, revealed that ii-MV did not differ significantly, neither between genetic background, nor between inbred and hybrid lines

(Additional file 1: Table S1). Subsequently, we scored ii-MV of tissue-specific or common MSAP fragments of the two hybrid crosses; i.e. of MSAP bands detected in one or both tissues, respectively (Fig. 1a and Table 1). In either hybrid, ii-MV was significantly increased of tissue-specific compared to common MSAP band ($p < 0.0005$ and $p < 0.0001$, respectively). However, neither common nor tissue-specific MSAP fragments showed significant differences in ii-MV between tissues.

Next, we assessed whether ii-MV occurred preferentially of cytosines in a CG or CHG context by comparing MSAP profiles of individual endosperms from the W23/A69Y hybrid using either *HpaII* or its isoschizomer *MspI* in the initial restriction digest. These restriction enzymes differ in their sensitivity to methylation of the CCGG recognition site: *HpaII* is sensitive to methylation of either cytosine residues, but is insensitive to hemi-methylation of the external cytosine residue, whereas *MspI* is sensitive to hemi- or complete methylation of the external cytosine. We found that 89% of ii-MV occurred in a CG context; i.e. variation was only detected following *HpaII*, but not *MspI* digestion (Fig. 1b, panels i and ii). In 37% (28/75) of such cases, no fragment was detected with *MspI* (panel ii). This suggested either that the external cytosine residue of

the CCGG recognition site was hemi-methylated, or the presence of an internal *HpaII* site that was methylated in a CG context only. The latter explanation is likely given that ~20% of MSAP fragments have internal CCGG sites [42]. In contrast, only 11% (8/75) of ii-MV occurred exclusively in a CHG context, or in both a CG and CHG context; i.e. band absence following both *HpaII* and *MspI* digestion (Fig. 1b, panel iii).

To understand whether these profiles reflected context-dependent endosperm-specific hypomethylation, we performed an in vitro methyl-accepting assay on 15 DAP endosperms and 14-day-old leaf tissues from the W23 inbred line. This assay exploits the ability of bacterial DNA *HpaII* and *MspI* methylases to methylate the internal and external cytosine of unmethylated CCGG sequences, respectively [50]. In addition, total CG methylation was measured with the *SssI* methylase, which methylates cytosines in CpG dinucleotides independent of sequence context. Although CG methylation levels were increased twofold relative to CHG methylation, the methylation level of either was reduced fivefold in endosperm compared to leaf (Table 2). This contrasts with a comparative Methyl-seq analysis of 12 DAP endosperm and leaf tissue from the B73 inbred line where

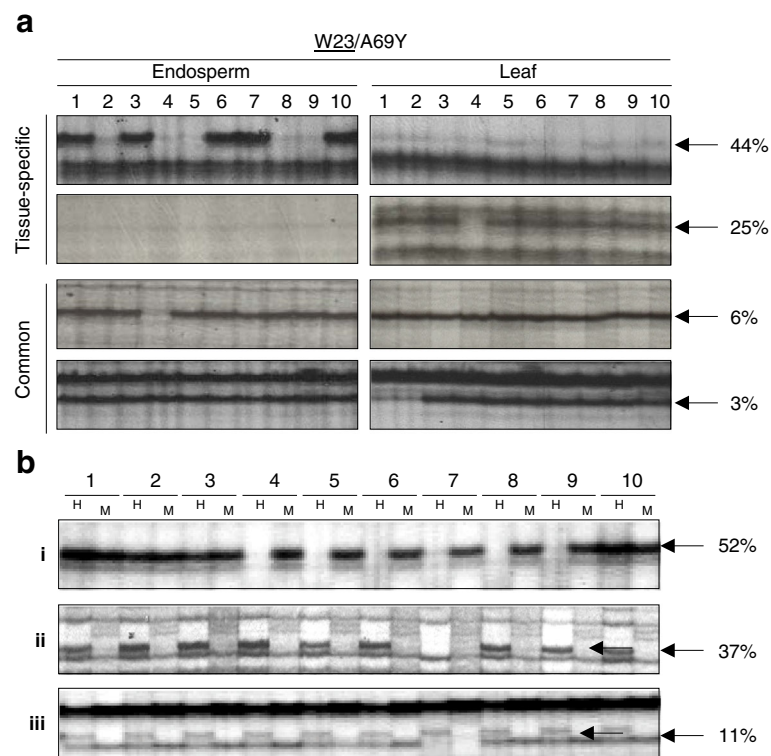


Fig. 1 MSAP analysis of individual tissues derived from a single cob of W23/A69Y hybrid (the egg donor of the cross is underlined). **a** MSAP analysis of 10 individual two-week-old leaves and 15 DAP endosperms; arrows indicate tissue-specific and common MSAP fragments that vary across individual endosperm and leaves (the two upper and two lower panels, respectively); **b** MSAP analysis of 10 individual endosperms using either *HpaII* (H) or *MspI* (M) in the initial restriction digest; panels shows examples of inter-individual variation in CG methylation (i and ii) and CHG methylation, or CG and CHG methylation (iii)

Table 1 Inter-individual variation in methylation of common and tissue-specific MSAP fragments

		Total ^a		Variable ^b		% Variable	
		C ^c	Ts ^d	C ^c	Ts ^d	C ^c	Ts ^d
W23/A69Y	Endosperm	428	98	26	43	6	44
	Leaf	428	12	11	3	3	25
Mo17/B73	Endosperm	507	155	30	55	6	35
	Leaf	507	21	15	5	3	24

^aSum of MSAP fragments that lacked or showed variation in DNA methylation in endosperm or leaf tissue using 12 selective primer combinations

^bMSAP fragments that showed variation in methylation between individual endosperms or leaves

^cC = Common MSAP fragments (*i.e.* fragments detected in both leaf and endosperm)

^dTs = Tissue-specific MSAP fragments (*i.e.* fragments detected either in leaf or endosperm)

only CHG methylation differed significantly between leaf and endosperm tissues [34]. This discrepancy may result either from inbred or developmental-specific differences in methylation, or alternatively from technical issues related to amplification of bisulfite-treated DNA such as selective enrichment of unmethylated alleles [51].

The methylation state of pooled samples largely reflects the predominant methylation profile across individual samples

Given that genome-wide studies are frequently performed on DNA pooled from several individuals, we asked to what extent the methylation state of a DNA sample from pooled individuals captured ii-MV. First, we performed an MSAP experiment to detail the limits of detection of particular methylation profiles. To this end, we analysed the inbred lines Mo17 and B73 and their respective reciprocal crosses by MSAP and identified 20 MSAP fragments that were specific to the B73 inbred line. Subsequently, we scored for the presence or absence of these fragments in an “artificial” B73/Mo17 hybrid where the genomic contribution of B73 ranged between 10 and 100%; *i.e.* ten dilution series at 10% intervals that were generated by spiking B73 DNA with DNA from the Mo17 inbred line. These experiments showed that all B73-specific bands were consistently detected when the genomic contribution

of B73 was higher than 30%. However, at a 10 and 20% B73 genomic contribution, the fidelity of detection dropped to 70 and 85%, respectively. Translating these results to interpreting MSAP variability data, the majority of band absence occurs when methylation levels are >90%, while methylation levels between 30 and 90% are indistinguishable. Furthermore, these data suggest that a discrete portion of fragments that are only present in 10–30% of analysed individuals could represent profiles that cannot be accurately determined by MSAP. This prompted us to analyse the frequency of ii-MV in leaf and endosperm tissues of either hybrid line. This frequency was calculated as number of individuals where a band is present divided by total number of individuals analysed. In either tissue, the bulk of variable fragments were detected in more than 30% of individuals analysed - *i.e.* 88 and 64% of Mo17/B73 leaf and endosperm MSAP fragments, respectively; the corresponding values were 95 and 72% in the W23/A69Y hybrid (Fig. 2a). This indicated that most MSAP ii-MV represented true biological variation. Next, we assessed whether the most abundant methylation state observed across individual leaves and endosperms from the Mo17/B73 hybrid was accurately captured in a pooled sample of either tissue. As expected, the majority of endosperm and leaf MSAP fragments that were present in less than 50% of individuals were associated with band absence in the pooled profile, while MSAP fragments that were present in more than 40% of individuals showed the opposite behaviour (Fig. 2b). In total, these fragments accounted for 84 and 80% of leaf and endosperm variable MSAP fragments, respectively. Of the remaining leaf and endosperm MSAP fragments that deviated from expected profiles, 60 and 90%, respectively, represented low frequency demethylation events (*i.e.* band presence in less than 50% of individuals) (Fig. 2b). Taken together, the data suggest that MSAP analysis of pooled samples is less effective in capturing low, than high-frequency demethylation events across individuals.

Validation of MSAP data

We excised a total of 106 MSAP fragments that either lacked or showed ii-MV in the Mo17/B73 hybrid. Following sequence analysis, 26 non-variable and 28 variable fragments reached our stringent criteria for further analysis (see methods). We also identified six variable and eight non-variable previously isolated MSAP fragments [42] that showed ii-MV in the W23/A69Y hybrid. Using selected fragments as probes in Southern blot analysis we confirmed that endosperm-specific variable MSAP fragments showed reduced levels of both CG and CHG methylation in endosperm relative to leaf tissue, while these profiles were largely identical of a non-variable fragment (Additional file 2: Figure S1a, compare tissue-specific *HpaII* and *MspI* profiles). In addition, we

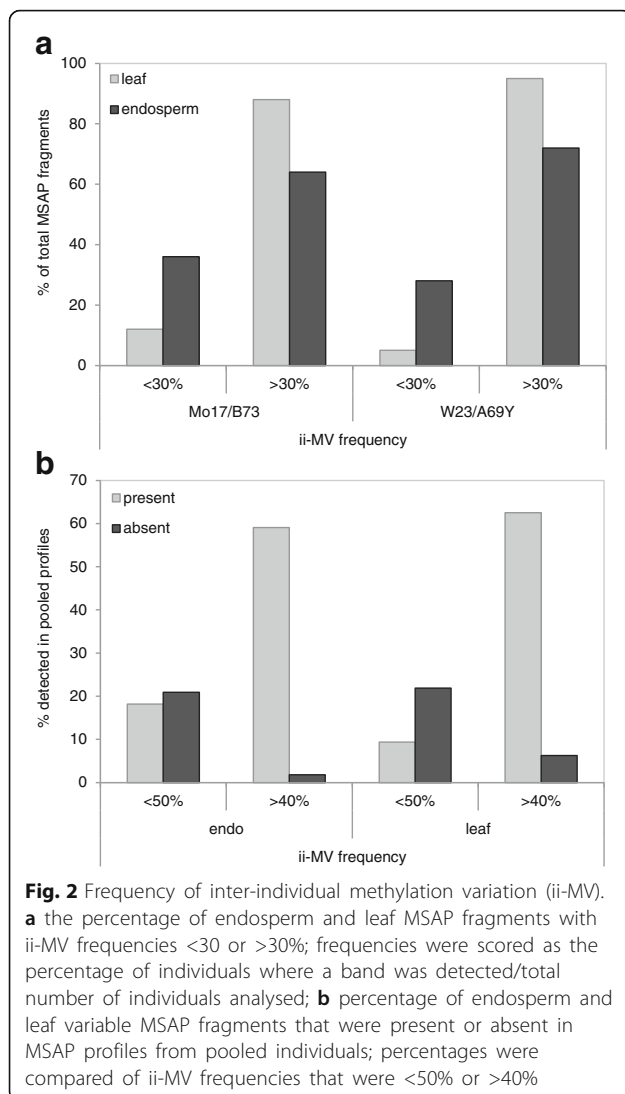
Table 2 Methyl-accepting assay of endosperm and leaf DNA

Methylase	Target sequence ^b	Target/haploid genome (×10 ⁶) ^a	
		Endosperm ^c	Leaf ^c
<i>HpaII</i>	<u>C</u> CGG	0.34 ± 0.12	0.071 ± 0.016
<i>MspI</i>	<u>C</u> CGG	0.64 ± 0.24	0.16 ± 0.024
<i>SssI</i>	<u>C</u> G	1.94 ± 0.51	0.41 ± 0.25

^aMean ± SD; *n* = 6 and *n* = 5 of endosperm and leaf, respectively

^bUnderlined cytosine indicates target of methylase

^cAll differences between groups (*i.e.* between tissues with same enzyme treatment and between enzyme treatments within tissues) were statistically significant (*p* < 0.05)



compared ii-MV in CG and CHG context of two variable fragments and one non-variable MSAP fragment using the methylation-sensitive restriction enzymes *HpaII* and *MspI*, respectively (Additional file 2: Figure S1b). Both variable MSAP fragments showed ii-MV in a CG context, while variation in CHG context was restricted to the variable PMP2 band. Conversely, no evidence of inter-individual variation was observed following digestion with the *EcoRI* and *EcoRV* restriction enzymes that are generally considered methylation-insensitive (Additional file 2: Figure S1b). Likewise, no inter-individual variation was detected of the non-variable fragment, neither with methylation-sensitive nor methylation-insensitive restriction enzymes.

We also validated the methylation states of variable and non-variable *HpaII* sites predicted by MSAP using the publicly available WGBS data from leaf tissue of B73 and Mo17 inbred lines [32]. To this end, we successfully

mapped 17/26 non-variable and 24/28 variable MSAP fragments to unique regions of the B73 inbred line genome (B73 RefGen_V4) (Additional file 3) and recovered the methylation state of each *HpaII* sites. The remaining MSAP fragments, showed partial or ambiguous overlap to the reference genome and/or lacked methylation data. Next, we predicted the methylation state of mapped variable and non-variable *HpaII* sites in leaf based on the presence or absence of MSAP fragments across endosperm and leaf individuals (Additional file 4: Figure S2a). Most non-variable fragments (94%) were detected in both tissues - and in all individuals - and were thus predicted to be unmethylated in both leaf and endosperm. Conversely, the non-variable fragment that was specifically detected in endosperm samples was considered methylated in leaf. Using similar arguments, 79% of variable fragments (*i.e.* 67 + 8 + 4%) were expected to show some degree of methylation in leaf, while the remaining 21% were predicted to lack methylation in this tissue (Additional file 4: Figure S2a). Overall, 85% of the predicted methylation states of individual *HpaII* sites were confirmed in WGBS data of cytosine methylation in a CG context (Additional file 4: Figure S2b). In particular, the predicted unmethylated state was confirmed - in at least one of the two inbred lines- of 88 (15/17) and 100% (5/5) of non-variable and variable fragments, respectively; the corresponding percentages for the predicted methylated state were 100 (1/1) and 79% (15/19), respectively. With respect to CHG methylation, we found that fewer non-variable and variable *HpaII* sites were associated with CHG methylation. In addition, CHG methylation levels were significantly lower than CG methylation levels in both the B73 and Mo17 inbred line ($p < 0.0028$ and 0.0003, respectively) (Additional file 4: Figure S2c).

Collectively, these data confirmed that ii-MV was largely restricted to dDMRs and preferentially associated with CG methylation. Conversely the analysed non-variable regions were largely unmethylated in either tissue.

Characteristics of isolated variable and non-variable fragments

Most variable and non-variable *HpaII* sites mapped within genic regions - *i.e.* <2 kb up or downstream of transcriptional start and termination sites annotated in the maize B73 RefGen_V4 (TSS and TTS, respectively) - in particular within the gene-body, defined as the region between TSS and TTS (Fig. 3a). With respect to CG and CHG methylation, the latter was more abundant within intergenic regions, while CG methylation was largely restricted to genic regions, both of non-variable and variable *HpaII* sites (Fig. 3b). Furthermore, analysis of DNA regions that extended 10 kb up- and downstream of non-variable and variable *HpaII* sites showed that their generally unmethylated and methylated state, respectively was characteristic

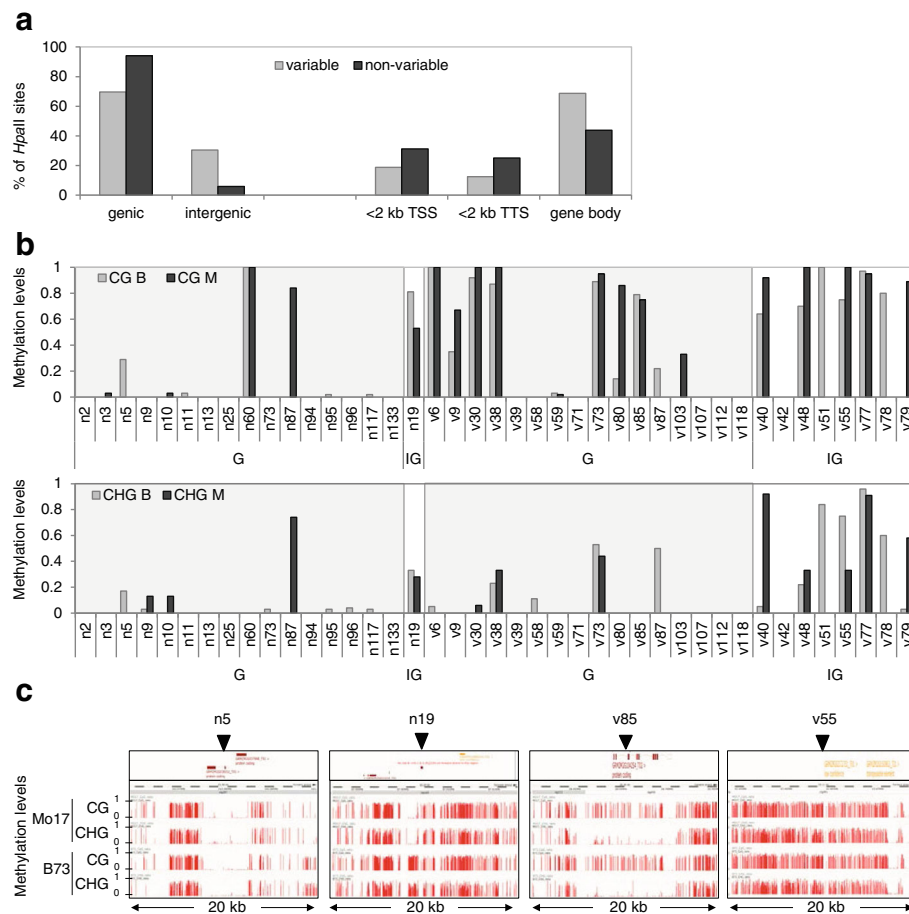


Fig. 3 Characteristics of variable and non-variable *HpaII* sites. **a** genomic distribution of variable and non-variable *HpaII* sites; genic regions were defined as being within 2 kb up or downstream, respectively of transcriptional start (TSS) and termination sites (TTS) annotated in maize B73 RefGen_V4; intergenic regions were defined as being more than 2 kb up or down-stream, respectively of TSS and TTS; **b** levels of CG and CHG methylation of variable (v) and non-variable (n) *HpaII* sites located within genic (G) and intergenic (IG) regions (light grey and no shading, respectively); the Y-axis indicates methylation levels between 0 and 1 (0 and 100% methylation, respectively) derived from WGBS of B73 (B) and Mo17 (M) leaf tissue [32]; **c** illustrative examples of CG and CHG methylation levels of genomic regions located 10 kb up- and downstream of non-variable and variable *HpaII* sites located either within genic (n5 and v85) or intergenic (n19 and v55) regions; arrowheads indicate positions of variable and non-variable *HpaII* sites

of an extended DNA region ranging from ~1–10 kb in size (Fig. 3c and Additional file 5: Figure S3). With respect to genes harbouring variable and non-variable *HpaII* sites, 38 (6/16) and 56% (9/16) respectively, had a paralogue in maize, while 75 and 50%, respectively, had an orthologue in *Arabidopsis thaliana*. Many of the annotated functions of these genes were related to dynamic cellular processes such as transcription and cell signalling (Additional file 6: Table S2).

We also assessed whether *HpaII* sites that showed ii-MV tended to map closer to a repetitive DNA region. As a control group, we included non-variable *HpaII* sites. In each case, we scored the distance to the closest repeat region and annotated both its size and classification. We found that 88% of variable *HpaII* sites were closest to class I or II transposable elements (TEs), while the majority of non-variable *HpaII* sites (53%) were closer to a tandem

repeat (Additional file 7: Figure S4a). With respect to TE, we found that the *hAT* superfamily was the most frequent class II TE found in close proximity to - and exclusively of - variable *HpaII* sites (Additional file 7: Figure S4b). However, neither the average distance to a repeat region, nor its size, differed significantly between variable and non-variable *HpaII* sites ($p < 0.82$ and $p < 0.11$, respectively).

Finally, we analysed whether genes harbouring variable *HpaII* sites were associated with tissue-specific differences in gene expression. As a control group, we included genes harbouring non-variable *HpaII* sites. These genes will subsequently be referred to a v-genes and n-genes. The rationale for comparing expression profiles between these groups was based on their differing methylation profiles across leaf and endosperm tissue; i.e. non-variable *HpaII* sites and their flanking regions were generally unmethylated in both tissues, while most

variable sites showed tissue-specific differences in methylation. For each gene we extracted B73 transcription data of mixed seedling V1 stage and 14 DAP endosperms (ZM37-Plant Expression Database) [52]). These particular developmental stages were comparable to those used for MSAP analysis. Expression data was available for 9 n-genes and 13 v-genes and genes belonging to each group were further subdivided according to the relative methylation state of the *HpaII* site in leaf compared to endosperm, in addition to the genic location of the *HpaII* site (Fig. 4a). In all cases, the relative methylation states were predicted by comparing individual leaf and endosperm MSAP profiles as previously described (see Additional file 4: Figure S2a).

Overall, we found that v-genes were expressed at higher levels in leaf - but not in endosperm tissue - relative to n-genes ($p < 0.008$ and $p < 0.123$, respectively). Furthermore, the majority of v-genes (77%) showed significant (or borderline-significant) differences in expression between tissues compared to only 22% of n-genes (Fig. 4a). However, across tissue-types, we found no specific trend between methylation and expression. For example, of the seven v-genes that were more methylated within the gene-body in leaf compared to endosperm, two (v71 and v9) showed increased levels of transcription in leaf relative to endosperm tissue, while four (v39, v85, v103 and v80) showed the opposite profile (Fig. 4a). The same was true of variable *HpaII* sites located either within 2 kb of TSS and TTS, or within gene-bodies that were more methylated in endosperm relative to leaf. To validate those database-deduced transcriptional profiles by direct expression, we designed primers pairs that spanned the variable *HpaII* sites of MSAP fragments v71, v9 and v59 that were located within GRMZM2G068392, GRMZM2G097109 and GRMZM5G817886 (Fig. 4b). We confirmed the expression profile of the two former - i.e. their increased levels of transcription in leaf relative to endosperm tissue in the B73 inbred line. However, v59 transcription differed from the expected expression profile, since this gene showed increased levels of transcription in endosperm relative to leaf (Fig. 4b). In addition to the B73 inbred line, we also analysed RNA from Mo17, A69Y and W23 inbred lines and found that gene-specific expression profiles were largely conserved across inbred lines (Fig. 4b). The only exception was GRMZM2G068392 that showed higher levels of expression in endosperm tissue from the A69Y inbred line.

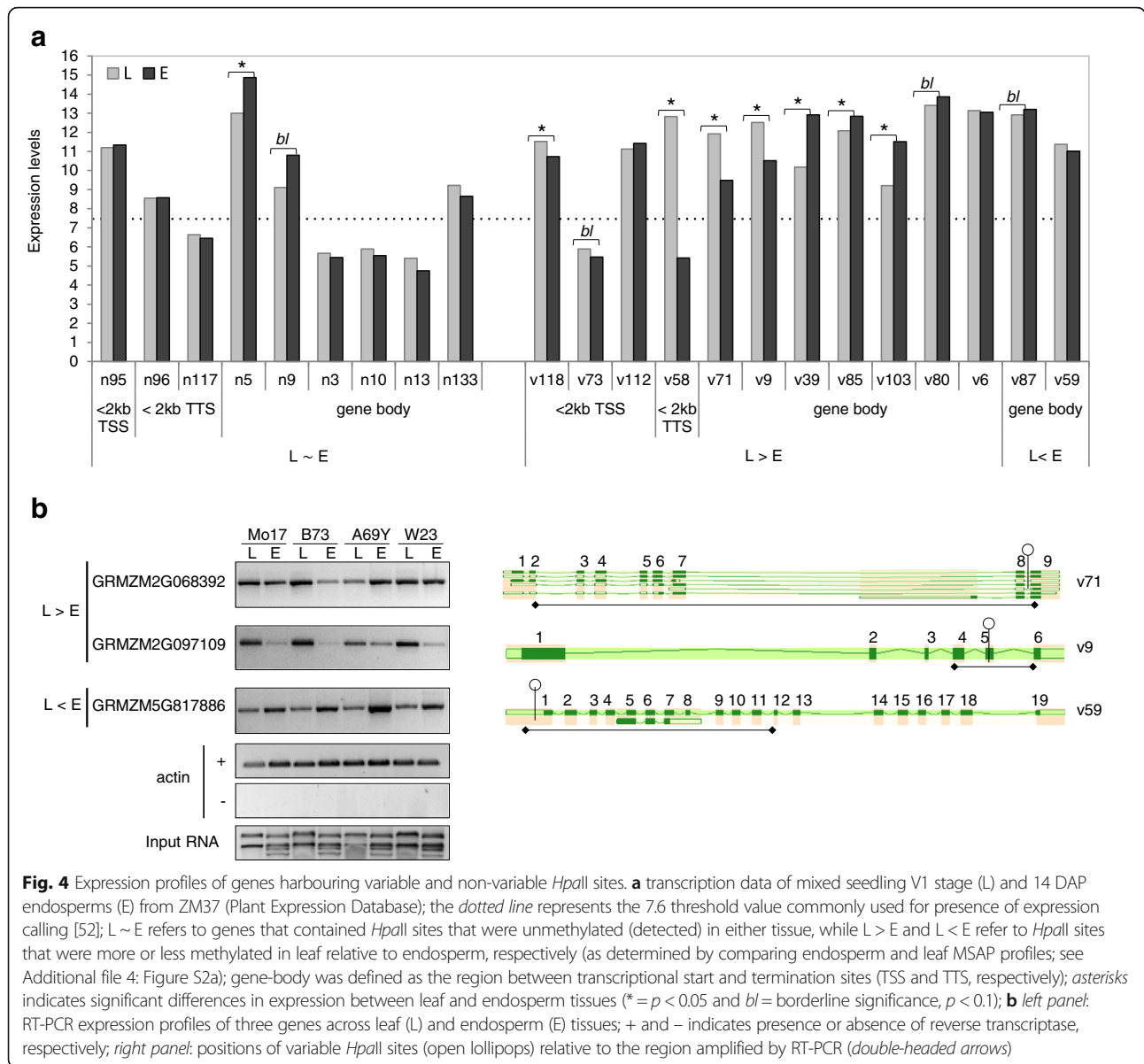
Discussion

Our results reveal that the bulk of methylation at *HpaII* sites (CCGG) in leaf is conserved across maize individuals germinated from single-cob seeds of either inbred or hybrid maize lines. In leaves, only ~3% of MSAP fragments showed ii-MV, which is comparable – albeit

slightly higher – to the less than 1% reported of individual arabidopsis seedling leaf tissue analysed by MSAP [37]. Although we found no significant differences in the frequency of ii-MV between leaf and endosperm, the total number of variable MSAP fragments was increased four to fivefold of the latter. This finding is largely explained by the tight association between ii-MV and tDMRs and the fact that the latter are much more abundant in endosperm relative to leaf [42]. Interestingly, a study of ii-MV across genetically identical mice also found that more than 50% of variable regions overlapped with tDMRs [53].

As a consequence of the above, the bulk of variable MSAP fragments were more methylated in leaf relative to endosperm tissue. By contrast, most non-variable fragments were unmethylated; i.e. detected in both tissues. Using publicly available WGBS data of maize leaf tissue [32], we confirmed that non-variable *HpaII* sites lacked DNA methylation, while most variable *HpaII* sites showed varying levels of CG methylation in genic regions, or CG and CHG methylation in intergenic regions. Furthermore, these particular methylation states were generally representative of extended genomic regions ranging from ~1–10 kb in size. Taken together, the data suggest that ii-MV is preferentially associated with methylated DNA regions. In accordance, a recent analysis of single-cell methylation variation in liver tissue from the Japanese rice fish *Oryzias latipes* found that methylation-variation was increased of hyper rather than hypomethylated DNA regions [54]. Importantly, the convergence between our MSAP methylation and previously generated WGBS from leaf tissue [32], indicates that MSAP represents a reliable and representative read-out of methylation states. This suggests that MSAP represents a valuable alternative for analysing DNA methylation states, either in plant species with incomplete or no genome information, or when the optimal sample size renders WGBS (or any other next generation sequencing technique) not practical.

Similar to variation in methylation between Arabidopsis accessions and maize or soybean inbred lines [26–28, 30, 33], much ii-MV mapped within the gene-body and was largely restricted to CG methylation. A possible explanation for the absence and low levels of CHG ii-MV is that such methylation is only present transiently during transcription due to INCREASED IN BONSAI METHYLATION 1 (IBM1) activity, an H3K9 demethylase that actively prevents CMT3-mediated CHG methylation within gene bodies [55, 56]. Importantly, in this MSAP study CHG methylation was assayed in a CCG context and such methylation is largely MET1-dependent as opposed to CHG methylation in a CTG and CAG context [57]. However, given that actively transcribed genes with a high density of CTG and CAG are preferentially



targeted for gene body methylation [58], we cannot exclude that methylation in those sequence contexts may be more prone to CHG ii-MV. Akin to several other studies we found a complex relationship between gene-body methylation and expression across tissues [14, 15, 18, 43, 44, 59]. Indeed, a recent study shows that the lack of gene body methylation in the angiosperm *Eutrema salsugineum* seemingly has no functional consequences with respect to transcription regulation [58].

Interestingly, a previous study of ii-MV across leaf tissue by MSAP showed that only a minority (17%) of ii-MV was conserved between two leaf developmental stages [60]. Such transient or stochastic ii-MV could have implications with regards to interpreting long-term effects of any particular stress on the epigenome or

identifying epialleles generated across generations. Indeed, stress responses to phosphate starvation, heat, cold, UV or hyperosmosis have been shown to be transient or heterogeneous, both across individuals and generations [61–63]. It follows that differentially methylated regions (or differentially methylated cytosines) identified by studies performed on bulked tissues, or designed with a sub-optimal sample size, detect a combination of methylation variability that can be both transient and stable. This may be particularly relevant of low frequency demethylation events since such variation was less efficiently captured in a pooled sample by MSAP. At any rate, our data demonstrate that sample pooling can faithfully reflect at least a portion of methylation variation.

Several studies in both plants and mammals have shown that TEs exhibit both intra-individual and ii-MV [53, 64–67] and there is ample evidence of methylation variation between and within genotypes resulting from proximity to TEs [30, 32, 62–74]. In this study, we analysed whether variable *HpaII* sites were in closer proximity to a TE compared to non-variable sites. Overall, we found no differences between these two groups, neither with respect to distance, nor size of the TE. However, we did find that variable *HpaII* sites were preferentially located in vicinity of *hAT* superfamily of class II transposons. One obvious *caveat* of the present study is the comparatively small number of fragments yielded by the MSAP platform that showed ii-MV. Nonetheless, analysis of ii-MV in mice by whole genome bisulfite sequencing revealed only a total of 356 loci that showed ii-MV. In that study, ~15% of variable regions were associated with Endogenous retroviruses (ERV), a class I TE. Such data warrant further studies on the relevance of TE on ii-MV following specific environmental or developmental stimuli.

Conclusions

Our data suggest that ii-MV is largely restricted to tDMRs. Importantly, we show that sample pooling is a methodologically appropriate design to study methylation variation in response to a given stimulus. Additionally, comparative analyses to publicly available databases confirm that MSAP is an effective tool for DNA methylation profiling when WGBS is not feasible, either due to lack of genomics/epigenomic data, or because of a large optimal sample size.

Methods

Plant material

A69Y, W23, B73 and Mo17 inbred lines were grown in the field where out-crosses were performed to produce W23/A69Y and Mo17/B73 F1 hybrids (the egg donor of the cross is underlined). B73 and Mo17 seeds were obtained from the Maize Genetics Cooperation Stock Center, while A69Y and W23 seeds were a kind gift from Dr. Angelo Viotti. For each genotype, seeds were harvested either at 15 days after pollination (DAP) or at maturity. Individual endosperms were dissected from immature seeds, whereas mature seeds were germinated for two weeks in the greenhouse to obtain leaf tissue. In both cases, tissue was derived from a single cob.

MSAP and AFLP analysis

MSAP restriction digests, ligations and pre- and selective PCR reactions were performed as previously described [42]. For each sample, three independent MSAP reactions were performed; once reproducible, one sample was used for further analysis. *EcoRI* and *HpaII* preselective primers

were: 5'-AGACTGCGTACCAATTC-3' and 5'-TCATGAGTCCTGCTCGG-3', respectively. Selective primers were identical to preselective primers including additional 3' nucleotides. *EcoRI* selective primers were: *EcoRI*-01 AGT, *EcoRI*-02 ACA, *EcoRI*-03 AGA, *EcoRI*-04 ACC; *HpaII* selective primers were: *HpaII*-02 TAGC, *HpaII*-03 CGAA, *HpaII*-03A CGTT, *HpaII*-04 AATT. An MSAP band was scored at variable if it showed variation between individual endosperms. Only well resolved MSAP bands were scored.

AFLP was conducted as previously described [42]. Preselective primers were complementary to core sequences of *EcoRI* and *MseI* adaptors including one selective nucleotide for both *EcoRI* (5'-GACTGCGTACCAATTC) and *MseI* (5'-GATGAGTCCTGAGTAAC) primers. *EcoRI* selective primers were: E31 AAA and E32 AAC; selective *MseI* primers were M47 CAA, M48 CAC, M49 CAG, M50 CAT, M51 CCA.

Isolation and analysis of MSAP fragments

MSAP bands were isolated from acrylamide gels as previously reported [42]. In maize, a total of 58 and 48 fragments that showed or lacked variation in methylation, respectively were isolated from B73 and Mo17 inbred lines. Following re-amplification, PCR products were cloned and sequenced in triplicate on both strands. Only fragments that were of the expected size, contained the appropriate selective primer sequences and represented a single DNA sequence were selected for further analysis. Blast analysis of variable and non-variable fragments was performed against the updated maize B73 RefGen_V4 (http://ensembl.gramene.org/Zea_mays/); methylation values of variable and non-variable *HpaII* sites were recovered from publicly available methylation data [32].

DNA extraction and Southern blot analysis

Extraction of genomic DNA, restriction enzyme digests and Southern blotting was performed as described previously [42].

Quantification of CG- and CHG methylation in maize endosperm

The in vitro methyl-accepting assay using S-adenosyl-L-[methyl-³H] methionine ([³H]SAM) was performed exactly as previously described [50]. The rationale of the assay is that when using [³H]SAM as substrate, the amount of incorporated radioactivity is directly proportional to the extent of initial DNA hypomethylation. Reactions were carried out with 0.3–0.5 µg DNA for 3 h. In these conditions, incorporation of radioactivity is linearly proportional to DNA concentration and the reaction is carried out to completeness [50]. Raw data were converted into copies of unmethylated target per haploid genome considering a haploid maize and mouse genome content of ~2.5 and 3.5

pgs, respectively [75] using the formula: $2.5(DN_A)/AS$ where D is total incorporated radioactivity (dpm), N_A is Avogadro's number, A is specific activity (dpm/mole), S is the amount of substrate DNA (pg).

RNA extraction and RT-PCR analysis

RNA was extracted with TRIzol[®] Reagent (Cat. 15596-026 Life Technologies) according to the manufacturer's instructions and treated with DNaseI (Turbo DNA-free kit, Ambion cat. AM1907). RNA quality was assessed by 1% agarose gel electrophoresis and quantified using a Nanodrop-1000 spectrophotometer. For RT-PCR analysis, cDNA was synthesized from 1 µg of total RNA (SuperScript III kit, Invitrogen). Subsequently, 1/20 reaction volume was used in a standard PCR reaction with gene-specific primers. Primer specificity was confirmed by sequence analysis.

Statistical analysis

Multiple comparisons of MSAP data across hybrid and inbred lines were performed with the Kruskal-Wallis test. A student T-test for two independent means was used for comparisons of expression profiles between leaf and endosperm tissues.

Additional files

Additional file 1: Table S1. Extent of ii-MV in hybrid crosses and inbred maize lines. (DOCX 60 kb)

Additional file 2: Figure S1. Southern blot analysis of variable and non-variable fragments in the A69Y inbred line. **a)** DNA pooled from 14 day-old leaves (L) or endosperms (E) harvested 15 DAP were digested and probed with variable (v) and non-variable (n) MSAP fragments as indicated; arrows indicate hybridization to endosperm-specific bands; **b)** individual endosperms were digested and probed as indicated; arrows indicate bands that show ii-MV in a CG and CHG context. (PPTX 381 kb)

Additional file 3: Sequence of variable (v) and non-variable (n) MSAP fragments that mapped to unique regions of the B73 genome. Sequence data of isolated MSAP fragments that showed or lacked ii-MV (v and n, respectively). (TXT 8 kb)

Additional file 4: Figure S2. Comparison of MSAP and WGBS data. **a)** schematic representation of non-variable and variable MSAP profiles and the deduced methylation states of each *HpaII* sites. Black bars and stippled white bars indicate presence and absence of an MSAP fragment, respectively; filled and empty circles indicate a methylated and unmethylated *HpaII* site, respectively. The percentage of MSAP fragments representing each profile and the relative methylation state of *HpaII* sites in leaf compared to endosperm is indicated right and left, respectively, of MSAP profiles; L ~ E indicates a similar methylation state of the *HpaII* site in leaf and endosperm; L > E and L < E indicate *HpaII* sites that are more or less methylated, respectively, in leaf relative to endosperm; **b)** and **c)** comparison of predicted MSAP methylation states of non-variable and variable *HpaII* sites to the CG or CHG methylation values obtained from WGBS of B73 (B) and Mo17 (M) leaf tissue [32]; + and - indicates a predicted methylated or unmethylated state, respectively; Y-axis indicates methylation levels between 0 and 1 (0 and 100% methylation, respectively). (PPTX 97 kb)

Additional file 5: Figure S3. CG and CHG methylation in leaf tissue of the 20 kb genomic regions surrounding non-variable or variable *HpaII* sites. The Y-axis indicates leaf DNA methylation levels between 0 and 1 (0 and 100% methylation, respectively) obtained from WGBS of B73 and Mo17 leaf tissue [32]; arrowheads indicate positions of variable and non-variable *HpaII* sites. (PPTX 1259 kb)

Additional file 6: Table S2. Blast analysis of variable and non-variable fragments. (PDF 55 kb)

Additional file 7: Figure S4. Classification of repetitive elements that were closest to a variable and non-variable *HpaII* site. **a)** percentage of tandem repeats, class I and class II TEs; **b)** percentage of class I and class II TE superfamilies. (PPTX 52 kb)

Abbreviations

(³H)SAM: S-adenosyl-L-[methyl-³H] methionine; AFLP: Amplified Fragment Length Polymorphism; DAP: Days after pollination; ii-MV: Inter-individual methylation variation; MSAP: Methylation Sensitive Amplified Polymorphism; n: Non-variable; tDMRs: Tissue-specific differentially methylated regions; TEs: Transposable elements; v: Variable

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional files.

Authors' contributions

ML generated and analysed MSAP and AFLP data, RE-N and DR-R cloned variable and non-variable MSAP fragments and performed RT-PCR analysis. SZ performed the in-vitro methyl-accepting assay, RE-N and GL performed the bioinformatics analysis, GL designed and supervised the study and wrote the final manuscript version. All authors have read and approved this manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Author details

¹Consiglio Nazionale delle Ricerche, Istituto di Biologia e Biotecnologia Agraria, I-20133 Milan, Italy. ²Gertrud Lund, Department of Genetic Engineering, CINVESTAV - Unidad Irapuato, Km. 9.6 Libramiento Norte Carretera Irapuato-Leon, Apdo. Postal 629, C. P. 36500 Irapuato, GTO, Mexico. ³Department of Medical Sciences, Division of Health Sciences, León Campus, University of Guanajuato, Guanajuato, Mexico.

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