NOTE / NOTE

Search for methylation-sensitive amplification polymorphisms associated with the "mantled" variant phenotype in oil palm (*Elaeis guineensis* Jacq.)

E. Jaligot, T. Beulé, F.-C. Baurens, N. Billotte, and A. Rival

Abstract: The methylation-sensitive amplification polymorphism (MSAP) technique has been employed on somatic embryo-derived oil palms (*Elaeis guineensis* Jacq.) to identify methylation polymorphisms correlated with the "mantled" somaclonal variation. The variant phenotype displays an unstable feminization of male organs in both male and female flowers. Using MSAP, the methylation status of CCGG sites was compared in three normal versus three mantled regenerants sampled in clonal populations obtained through somatic embryogenesis from four genotypically distinct mother palms. Overall, 64 selective primer combinations were used and they have amplified 23 markers exhibiting a differential methylation pattern between the two phenotypes. Our results indicate that CCGG sites are poorly affected by the considerable decrease in global DNA methylation that has been previously associated with the mantled phenotype. Each of the 23 markers isolated in the present study could discriminate between the two phenotypes only when they were from the same genetic origin. This result hampers at the moment the direct use of MSAP markers for the early detection of variants, even though valuable information on putative target sequences will be obtained from a further characterization of these polymorphic markers.

Key words: DNA methylation, epigenetics, MSAP, oil palm, somaclonal variation.

Résumé : La technique de polymorphisme d'amplification sensible à la méthylation (« methylation-sensitive amplification polymorphism » (MSAP)) a été utilisée sur des palmiers à huile (*Elaeis guineensis* Jacq.) provenant d'embryons somatiques, dans le but d'identifier des polymorphismes de méthylation liés à la variation somaclonale « mantled ». Ce phénotype variant présente une féminisation instable des organes mâles sur les fleurs des deux sexes. Par MSAP, le statut de méthylation des sites CCGG a été comparé dans quatre descendances clonales composées de trois régénérants de chaque phénotype (normal et « mantled »), provenant de quatre plantes-mères génotypiquement distinctes. Un total de 64 amorces d'amplification sélectives a été employé et a révélé 23 marqueurs présentant un profil de méthylation spécifique du phénotype. Chacun de ces 23 marqueurs n'a montré de polymorphisme lié au phénotype qu'au sein de la même origine génétique. Ce résultat empêche pour l'instant l'utilisation de marqueurs MSAP pour la détection précoce de variants, mais la caractérisation prochaine de ces marqueurs fournira des informations très utiles sur de potentielles séquences cibles.

Mots clés : méthylation de l'ADN, épigénétique, MSAP, palmier à huile, variation somaclonale.

Introduction

The "mantled" somaclonal variation of oil palm (*Elaeis guineensis* Jacq.) affects the formation of floral organs in both male and female flowers. In this species, male and female inflorescences are produced alternately in time on the same plant (temporal dioecy). The mantled phenotype displays a transformation of the male floral organs (stamens

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and staminodes) into pseudocarpels, which may lead to fruit abortion in the most severe cases (Corley et al. 1986). This abnormality affects on average 5% of the regenerants obtained through somatic embryogenesis, and both its occurrence and its severity are highly variable between and among clonal progenies. The mantled variation still jeopardizes the development of oil-producing fruits and therefore postpones the commercial production of clonal plantlets (Rival 2000). To monitor the somatic embryogenesis protocol and (or) discard variant lines before field planting, an early detection test of this somaclonal variation is clearly needed.

Neither flow cytometry (Rival et al. 1997) nor RAPD (Rival et al. 1998) analyses carried out in our laboratory have detected any major genetic defect in abnormal tissues. In accordance with the characteristics of the abnormality mentioned above, together with those revealed during extensive field studies (non-Mendelian heredity, time-dependent reversion), the hypothesis of an epigenetic origin for the mantled phenotype has emerged. Alterations in DNA methylation patterns have been widely found in plants displaying phenotypic defects, notably in cases of aberrant floral morphogenesis (Finnegan et al. 2000) and in somaclonal variants (Kaeppler et al. 2000). Thus, we tried to determine if such perturbations could be detected in association with the mantled phenotype in oil palm. The existence of a significant hypomethylation of the genome was demonstrated in calli and leaves originating from abnormal regenerants compared with their normal counterparts (Jaligot et al. 2000). As the determination of genomic methylation rates is not sufficient to discriminate between normal and abnormal tissues, it was necessary to target sequences specifically affected in their methylation pattern in mantled palms. Toward this aim, we used oil palm cDNA sequences as probes in methylation-sensitive restriction fragment length polymorphism – Southern blot studies. Two oil palm cDNAs (namely CPHO62 and CPHO63) were found to display a differential methylation pattern between normal and abnormal embryogenic calli (Jaligot et al. 2002). However, since this technique only allowed the examination of the methylated sites addressed by the probes, a more thorough analysis of sequence-specific methylation status is clearly needed.

The methylation-sensitive amplification polymorphism (MSAP) procedure (Reyna-López et al. 1997) is a powerful tool that is adapted to this purpose. Indeed, it provides the high resolution of amplified fragment length polymorphism, while the use of isoschizomeric enzymes (*Msp*I/*Hpa*II) allows the evaluation of the methylation status within the corresponding target site (CCGG).

Material and methods

Plant material

Mature oil palm leaves of F_{+1} order have been sampled from adult oil palm somaclones originating from hybrid *tenera* origin (Deli × LaMé) in SOCFINDO plantations in Indonesia and at the CNRA research station in LaMé, Côte d'Ivoire. Each set of three normal and three abnormal regenerant palms was obtained through in vitro somatic embryogenesis (Pannetier et al. 1981) from a single mother palm. Within each set, only individual palms originating from the same production batch (and therefore with the same in vitro and ex vitro background) were used. The four clonal lines studied here (LMC3, LMC51, SOC2104, and SOC2804) originated from the cloning of four genotypically distinct mother palms. Within the same lines, a significant difference in genomic 5mdC content between the normal and mantled phenotypes has previously been demonstrated (Jaligot et al. 2000). Thus, this population was likely to represent a test panel for the identification of markers displaying changes in methylation patterns associated with the mantled phenotype. Oil palm genomic DNA was isolated from leaf material as described in Rival et al. (1998).

MSAP procedure

The protocol was adapted from Reyna-López et al. (1997) and Xiong et al. (1999). Samples of oil palm genomic DNA were double-digested with one of the methylation-sensitive enzymes *Hpa*II or *Msp*I and then with the methylation-insensitive *Eco*RI. The resulting fragments were ligated with the corresponding double-stranded adapters and a first preselective amplification was carried out (see the sequences of adaptaters and primers in Table 1). The selective amplification used one of the 64 possible 3′-variant primer pairs (Table 1). To ensure that only fragments generated by methylation-sensitive digestion (i.e., those delimited by one or two CCGG sites) would be visible, only the Hpa/Msp primer was end-labeled with [γ^{33}P]ATP. Amplification products were separated on a denaturing gel (5% polyacrylamide, 8.3 M urea) before autoradiography.

Two independent visual scorings of the bands were performed. A band was considered as phenotype specific if it was present in all three regenerants bearing one phenotype and absent in the other three.

Results and discussion

The present work involved the scoring of a total of 21 449 different amplification products.

When comparing the different clonal lines, the banding pattern obtained with one given primer pair appeared variable (Fig. 1). This result is in accordance with the conclusions made by Matthes et al. (2001) on oil palm leaves from different genotypes. It further confirms the strong influence exerted by the genetic background of the regenerants on both global genomic methylation rates (Jaligot et al. 2000) and sequence-specific methylation (Jaligot et al. 2002) revealed in our previous studies.

Of the 64 primer pairs tested, 15 (23%) revealed one or two differential bands discriminating between the two phenotypes (Table 1; Fig. 1). Overall, 23 amplification markers showing a phenotype-specific methylation pattern were found, and each of them was strictly restricted to one given clonal line.

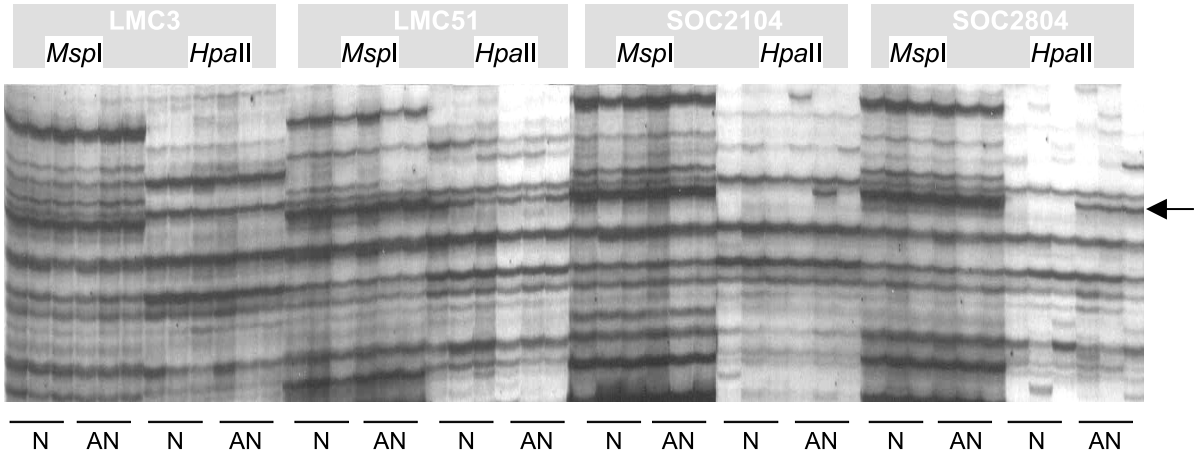
On average, unmethylated CCGG sites (bands common to both digests), $C^{m}CGG$ symmetrical methylation (bands present in *Msp*I but not in *Hpa*II lanes), and hemimethylated $^{hm}CCGG$ sites (bands present in *Hpa*II but not in *Msp*I lanes) accounted respectively for 81.2%, 14.7%, and 4.1% of the total number amplification products, respectively (Table 2). Similar proportions of methylated CCGG sites were found with the same technique by Xiong et al. (1999) and

Table 1. Primers (three variable nucleotides) used for MSAP amplification.

	H+AAC	H+AAG	H+ATG	H+ATT	H+CCG	H+CGC	H+CTG	H+CTT
E+AAC				+				
E+AAG					+			
E+ACA	+	+					+	
E+ACC	+			+	+	+	+	
E+ACG	+					+		
E+ACT								
E+AGC								
E+AGG		+	+		+			

Note: *Eco*RI adapter (E): 5′-CTCGTAGACTGCGTACC-3′, 3′-CATCTGACGCATGGTTAA-5′; *Hpa*II–*Msp*I adapter (H): 5′-GATCATGAGTCCTGCT-3′, 3′-AGTACTCAGGACGAGCC-5′; preamplification primers (one variable nucleotide): E+A: 5′-GACTGCGTACCAATTC**A**-3′, H+A: 5′-ATCATGAGTCCTGCTCGG**A**-3′, H+C: 5′-ATCATGAGTCCTGCTCGG**C**-3′. Combinations of primers revealing a phenotype-dependent methylation polymorphism in at least one of the two digestion products (*Eco*RI–*Msp*I and *Eco*RI–*Hpa*II) are marked "+". The methylation patterns of the 23 detected markers are detailed in Table 2.

Fig. 1. Detail of the MSAP profile generated by the E+ACC/H+CGC primer pair on DNA samples from three normal (N) and three mantled (AN) regenerant palms originating from four different clonal lines. The right-hand arrow indicates the abnormal-specific marker amplified in *Hpa*II digests from SOC2804 regenerants.



Ashikawa (2001) in rice (16.3%) and by Peraza-Echeverria et al. (2001) in micropropagated banana plants (23%).

It is interesting to note that for 87.0% (20/23) of the phenotype-specific markers (Table 2), the observed methylation polymorphisms involved the appearance of a supplementary band in one or both of the digests obtained from mantled palms. Such patterns reflect the preferential occurrence of a loss of methylation, affecting one or both cytosines, in the CCGG sites of the abnormal genome (McClelland et al. 1994).

Our previous study, carried out on the same regenerant palms, has shown that genomic methylation was decreased by 1.2% on average in mantled regenerants compared with their normal counterparts (Jaligot et al. 2000). Also, according to the importance of the DNA hypomethylation found in the leaves of abnormal regenerants, the clonal lines can be distributed in the order SOC2104 > LMC51 > LMC3 > SOC2804. By contrast, the present work shows that the hypomethylated CCGG sites detected in abnormal palms represented only 0.09% of the sequences amplified (20 out of 21 449), irrespective of the clonal line. Moreover, the ranking given by the percentage of methylation polymorphism calculated for each clone is LMC3 (10 phenotype-specific markers out of an average of 5608 markers per individual sample: 0.18%) > SOC2104 (9/5248: 0.17%) > SOC2804 (3/5244: 0.06%) > LMC51 (1/5349: 0.02%).

Our results show that the percentage of methylation polymorphisms at CCGG sites and the extent of the global genomic hypomethylation are not strictly correlated in the mantled palms. A likely explanation is that the different methylatable sites (CG, cytosine – any base – guanine (CNG), asymmetric Cs) are not homogeneously affected by the genomic hypomethylation associated with the mantled phenotype and that CCGG sites contribute poorly to this deficiency. This conclusion was also supported by our previous estimations of CG methylation in the same material using an in vitro saturation assay (Jaligot et al. 2000) as well as by our restriction fragment length polymorphism study using the same isoschyzomeric enzyme pair (Jaligot et al. 2002). Indeed, recent studies indicate that the different methylated sites do not have the same function in the regulation of gene expression and can be affected by different epigenetic events (Fulnecek et al. 2002; Lindroth et al. 2001; Jacobsen et al. 2000; Kato et al. 2003). Besides, the hypermethylation of specific genes can take place even in a severely hypomethylated genomic context (Jacobsen et al. 2000; Jacobsen and Meyerowitz 1997; Kakutani et al. 1995; Kishimoto et al. 2001). Thus, changes in genomic

Table 2. Nature of the phenotype-dependent methylation changes visualized in the studied clonal lines.

Methylation status of the restriction site[a]			
Normal	Mantled	No. of markers showing this pattern	Display by clonal line[b]
$^{m}C^{m}CGG$ or $^{m}CCGG$	$C^{m}CGG$	1	1 (LMC3)
$^{m}C^{m}CGG$ or $^{m}CCGG$	CCGG	3	2 (LMC3)
			1 (SOC2104)
$C^{m}CGG$	CCGG	15	6 (LMC3)
			8 (SOC2104)
			1 (SOC2804)
$^{m}C^{m}CGG$ or $^{m}CCGG$	$^{hm}CCGG$	1	1 (SOC2804)
$C^{m}CGG$	$^{m}C^{m}CGG$	1	1 (LMC3)
CCGG	$^{hm}CCGG$	1	1 (LMC51)
$^{hm}CCGG$	$^{m}C^{m}CGG$ or $^{m}CCGG$	1	1 (SOC2804)

Note: CCGG, unmethylated site; $^{m}C^{m}CGG$, fully methylated site; $C^{m}CGG$, methylation of the inner cytosine; $^{m}CCGG$, methylation of the outer cytosine (both strands); $^{hm}CCGG$, methylation of the outer cytosine (one strand).

[a]The methylation status of the CCGG sites is inferred from the banding pattern according to McClelland et al. (1994).

[b]Distribution of the detected phenotype-specific methylation polymorphisms among the clonal lines.

methylation level do not preclude changes detected at the sequence level. These different results point out the necessity to target specific sites or sequences when DNA methylation is supposed to be involved in a given epigenetic event.

Conclusion

Methylation changes associated with the mantled phenotype were detected at CCGG sites with the MSAP technique among a limited number of samples. Comparatively with the study published by Matthes et al. (2001), which described the comparison of normal–abnormal regenerant pairs from four different mother palms with 10 primer combinations, we explored a larger fraction of the methylation diversity at *Msp*I/*Hpa*II sites. Our results indicate that CCGG sites are poorly affected by the considerable decrease in DNA methylation previously measured in variant tissues (Jaligot et al. 2000). MSAP analyses involving the use of isoschizomeric enzymes targeting trinucleotidic sites (CNG) are currently under way in our group. This might allow us to examine a more significant part of the methylation changes occurring in the "mantled" context and, eventually, to isolate a more important number of discriminant markers.

In the present study, we found that each of the methylation-sensitive markers can only discriminate between the two phenotypes within the same clonal progeny. The most probable explanation for this is that the methylation patterns of the corresponding genes are changed as a consequence of the abnormality. However, since both the mantled phenotype and the associated deficit in DNA methylation are the only recurrent features displayed by all of the clonal progenies of oil palm known to date, it is likely that a common regulating pathway is affected in all of the abnormal plants. With the aim of identifying this mechanism, and designing a detection test, further characterization of all of the markers showing methylation polymorphism is therefore a matter of priority.

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