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RESEARCH PAPER

A methylation status analysis of the apomixis-specific region in *Paspalum* spp. suggests an epigenetic control of parthenogenesis

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Abstract

Apomixis, a clonal plant reproduction by seeds, is controlled in *Paspalum* spp. by a single locus which is blocked in terms of recombination. Partial sequence analysis of the apomixis locus revealed structural features of heterochromatin, namely the presence of repetitive elements, gene degeneration, and de-regulation. To test the epigenetic control of apomixis, a study on the distribution of cytosine methylation at the apomixis locus and the effect of artificial DNA demethylation on the mode of reproduction was undertaken in two apomictic *Paspalum* species. The 5-methylcytosine distribution in the apomixis-controlling genomic region was studied in *P. simplex* by methylation-sensitive restriction fragment length polymorphism (RFLP) analysis and in *P. notatum* by fluorescene *in situ* hybridization (FISH). The effect of DNA demethylation was studied on the mode of reproduction of *P. simplex* by progeny test analysis of apomictic plants treated with the demethylating agent 5'-azacytidine. A high level of cytosine methylation was detected at the apomixis-controlling genomic region in both species. By analysing a total of 374 open pollination progeny, it was found that artificial demethylation had little or no effect on apospory, whereas it induced a significant depression of parthenogenesis. The results suggested that factors controlling repression of parthenogenesis might be inactivated in apomictic *Paspalum* by DNA methylation.

Key words: Apomixis, 5′-azacytidine, B_{III} hybrids, DNA methylation, epigenetics, parthenogenesis.

Introduction

Apomictic reproduction allows for the production of viable clonal seeds by circumventing meiosis and fertilization (Nogler, 1984). Among the several variants of apomictic development, gametophytic apomixis involves the formation of non-reduced embryo sacs from somatic nucellar cells (apospory) or from a megaspore mother cell (MMC) itself, after a suppressed or modified meiosis (diplospory). Both aposporous and diplosporous non-reduced embryo sacs carry egg cells that develop parthenogenetically into embryos without fertilization, whereas for endosperm development fertilization of the central cell is usually required (pseudogamous apomixis) (Nogler, 1984; Asker and Jerling, 1992; Crane, 2001).

Apomixis is a desirable trait to be introduced in those crops commercialized as hybrid seeds, since it allows the fixation of heterosis without loss of its beneficial effect on vigour and yield (Hanna, 1995). Although the phenomenon of apomixis is well known at the cytological level, its genetic basis is still poorly understood. Attempts to transfer the trait to crops from wild apomictic relative species have resulted to date in partially fertile, cytogenetically unstable, and agronomically unsuitable lines. In order to develop an artificial apomixis system to be introgressed into major crops, efforts are focused on the identification of the genetic determinants of apomixis in: (i) sexual model species (Ravi *et al.*, 2008; Olmedo-Monfil *et al.*, 2010); (ii) wild apomictic species (Rodrigues *et al.*, 2003; Laspina *et al.*, 2008; Sharbel *et al.*, 2009; Polegri *et al.*, 2010; Schallau *et al.*, 2010; Vijverberg *et al.*, 2010; Koltunow *et al.*, 2011; Zeng *et al.*, 2011); and (iii) potential target crops (Nonomura *et al.*, 2003; Zhao *et al.*, 2008; García-Aguilar *et al.*, 2010; Singh *et al.*, 2011). Furthermore, plants producing partial clonal progeny have been obtained in *Arabidopsis* (Marimuthu *et al.*, 2011), constituting the first proof of principle of the possibility of developing an artificial apomictic system in a diploid sexual species. However, despite the impressive progress made in the last few years, no genuine apomictic plants have been obtained neither have any of the genetic determinants of apomixis (i.e. genes able to shift the sexual to the apomictic pathway or vice versa) been identified to date. The reason for this delay is due to the fact that apomixis loci in natural apomictic species are often large-sized regions, recalcitrant to recombination-based genetic mapping, and belonging to non-model species for which the common tools of molecular biology are difficult to apply (Pupilli and Barcaccia, 2012).

Among the multiple natural apomictic systems used as models to study apomixis and identify the possible genetic determinants of the trait, the grass genus *Paspalum* as a whole presents a number of interesting characteristics that make it amenable for mining the apomixis genes: chief among these are: (i) the reduced genome size; (ii) the existence of sexual and apomictic cytotypes within the same species and ploidy level; (iii) the capacity to produce a large seed set; and (iv) the availability of genetic transformation methodologies (Ortiz *et al.*, 2013; Mancini *et al.*, 2014). Within the *Paspalum* genus, *P. simplex* and *P. notatum* are the best studied species for apomixis. In both of them, apomictic reproduction is of the apospory type and controlled by a single locus characterized by a strong repression of recombination and synteny with a subtelomeric region of the long arm of rice chromosome 12 (Pupilli *et al.*, 2001, 2004; Stein *et al.*, 2007; Podio *et al.*, 2012). The block of recombination seems to have induced sequence isolation, accumulation of transposable elements (TEs), and partial hemizygosity (Labombarda *et al.*, 2002; Calderini *et al.*, 2006). Therefore, the apomixis-controlling region (ACR), although inherited as a single dominant genetic unit, may consist of a supergene rather than a unique genetic determinant, that controls the fundamental and diverse components of apomictic reproduction, namely apospory, parthenogenesis, and development of endosperm in which the parental genome contribution deviated from the canonical 2(maternal):1(paternal) (Lin, 1984) to a 4(m):1(p) ratio.

The ACR of *P. simplex* shows a number of similarities to the extensively studied Y chromosome of animals and dioecious plants, including repression of recombination, accumulation of TEs, and gene degeneration (Pupilli and Barcaccia, 2012; Ortiz *et al.*, 2013). Epigenetic control of sex determination in dioecious plants has been reported in the most studied XY sex-determining system of *Silene latifolia*. This plant exists in nature mainly as female and male individuals with XX and XY chromosome constitutions, respectively (Winge, 1931), although rare hermaphroditic individuals with XY chromosome sets have been recognized (Prithman *et al.*, 2003). Demethylation of the 5-methylcytosine (5mC) residues on genomic DNA by treatment with 5′-azacytidine (5-Aza) causes sex reversal and formation of bisexual flowers in male (XY) *S. latifolia* genotypes, indicating that heterochromatic gene silencing might be involved in control of sex-determining genes (Janousek *et al.*, 1996). The authors suggested that the male phenotype (XY) is superimposed over the female phenotype (XX) by the silencing action of Y-bearing genes, and this silencing is under epigenetic control.

The scheme proposed to control sex in *S. latifolia* is strikingly similar to that hypothesized by Koltunow *et al.* (2011) for the apomixis control in *Hieracium*, according to which sexuality is a default state and apomixis is superimposed epigenetically over sexuality by the silencing action of two independent loci, LOA and LOP. As a matter of fact, there is mounting evidence that some aspects of apomixis, mainly related to the production of unreduced egg cells, are under epigenetic control. In *Arabidopsis*, lesions in the genes involved in the non-cell-autonomous sRNA pathway induce the formation of multiple non-reduced embryo sacs within the nucellus, a phenotype strongly resembling apospory (Olmedo-Monfil *et al.*, 2010); similarly, in maize, inactivation of genes involved in RNA-directed DNA methylation induce an apospory-like phenotype (Garcia-Aguilar *et al.*, 2010). Furthermore, artificial parthenogenesis can be obtained by manipulating the centromere-specific CENH3 protein (Ravi and Chan, 2010), indicating that complex mechanisms of chromatin remodelling including the heterochronic loading of CENH3 coupled with variation on DNA methylation can affect natural parthenogenesis (Grimanelli, 2012). Finally, in diplosporous *Eragrostis curvula*, an increased apomixis expression was associated with an increment in 5mC probably involving TEs (Zappacosta *et al.*, 2014).

Therefore, if apomictic reproduction is superimposed over sexuality through epigenetic silencing, then artificial DNA demethylation might reverse apomixis to partial or complete sexuality, as observed for the reversion of the male phenotype to hermaphroditism in the XY sex-determining system in *S. latifolia*. In both cases, the presence of a well-defined genomic portion (an entire Y chromosome in the case of dioecism and the ACR in apomixis) is necessary to express the phenotypes (male flowers and apomixis, respectively) and their control over the respective counterpart phenotypes might be of epigenetic nature.

The aim of the present work was to study the DNA methylation state of the ACR in two representative *Paspalum* species (*P. simplex* and *P. notatum*), in order to hypothesize its possible role on the epigenetic regulation of apomixis. Specific objectives were: (i) to explore the DNA methylation landscape of the ACR by both methylation-sensitive restriction and fluorescence *in situ* hybridization (FISH) analyses and (ii) to evaluate the effect of 5-Aza on both apospory and parthenogenesis.

Materials and methods

Plant material

Apomictic and sexual BC_1 plants belonging to the *P. simplex* mapping population described in Pupilli *et al.* (2001), together with a mapping subpopulation of 34 F_1 plants (17 apomictic and 17 sexual) of *P. notatum* along with their apomictic (Q4117) and sexual (Q4188) parental lines (Martínez *et al.*, 2001) were used in this study. Five plants were selected among the apomictic BC_1s of *P. simplex* and used as seed source for treatment with the demethylating agent. These plants (hereinafter called 'families') were selected on the basis of two criteria: (i) presence of single-dose restriction fragement length polymorphism (RFLP) markers to detect segregation events that provide evidence of a repression of apospory and (ii) absence of markers, which were abundant in the BC_1 population, to enhance the probabilities of detecting rare fertilization events diagnostic of repression of parthenogenesis (an example of such a marker–family combination is given in Supplementary Fig. S1 available at *JXB* online). Five apomictic families were then selected together with two homologous RFLP probes as diagnostic markers (Ps71 and Ps96; Pupilli *et al.*, 1997). The plants originating from open pollinated seeds, derived from selected families, were identified as mother plants 'MPs'. After demethylation treatment of MPs, several open pollination progeny were generated, which were identified as 'test progeny'. These plants were maintained in greenhouses and manually cross-pollinated at the time of blooming.

Methylation-sensitive RFLP analysis

Genomic DNA (8–9 μg) was digested overnight with 20 U of the isoschizomers *Hpa*II and *Msp*I or with *Eco*RI (New England Biolabs, NEB). The RFLP procedure reported by Pupilli *et al.* (2001) was used. Both rice anchor markers and *Paspalum* homologous sequences used as probes are shown in Table 1.

Cytophotometric determinations and pachytene chromosome preparations

Cytophotometric determination of DNA content in leaf nuclei was carried out following the protocol described by Caceres *et al.* (1999). Young leaves were fixed in acetic acid:ethanol 1:3 (v:v) and stored at 4 °C until used. Fixed materials were treated with a 5% (w/v) aqueous solution of Pectinase (Sigma) for 25 min at 40 °C and squashes were made in gelatinized slides under a coverslip in a drop of 45% acetic acid. Squashed material was then hydrolysed in 1 N HCl at 60 °C for 10 min, stained with Feulgen (Sigma), and washed for 10 min in SO_2–water (three changes) prior to dehydration and mounting in DPX. Squashes of root tips of *Sorghum bicolour* were routinely stained for each group of *Paspalum* slides and used as an internal standard. Feulgen DNA absorptions in individual cell nuclei were measured at a wavelength of 550 nm using a Leitz MPV3 microscope photometer equipped with a mirror scanner. Forty to 50 mesophyll nuclei per plant were measured (Cáceres *et al.*, 1999).

Chromosome plates were prepared from anthers of *P. notatum* Q4188 and Q4117 genotypes. Meiocytes at late pachytene were fixed in freshly prepared ice-cold 96% ethanol:glacial acetic acid (3:1) solution for at least 3 h at 20 °C and rinsed twice in distilled water. Cell walls were digested with a mixture of pectolytic enzymes containing 0.3% (w/v) cellulase RS (Sigma-Aldrich C1184), 0.3% (w/v) pectolyase Y23 (Sigma-Aldrich P3026), and 0.3% (w/v) cytohelicase (Sigma-Aldrich C8274) in 1× phosphate buffer pH 7.5 (PBS) at 37 °C for 1 h. After two washes in sterile distilled water, each anther was carefully transferred to a grease-free slide, soaked in acetic acid (45%), sliced with a fine needle, and squashed. The chromosome preparations were frozen in liquid nitrogen and the coverslips removed. Finally, slides were air-dried at 37 ºC for 1 d and then kept at –20 ºC until use.

Cytogenetic analysis of 5mC

The immunolocalization of 5mC residues on *P. notatum* chromosomes was carried out as described by Ribeiro *et al.* (2009). Slides containing pachytene chromosomes from genotypes Q4188 and Q4117 were treated with RNase (Invitrogen, Carlsbad, CA, USA) 20 mg ml^{-1} diluted 1:200 in 2× SSC for 1 h, blocked with 1% bovine serum albumin (BSA) diluted in PBST (PBS plus 0.05% Tween-20), and incubated overnight at 4 °C with mouse anti-5-methylcytosine primary antibody (Sigma-SAB4800001, Imprint® Monoclonal

Table 1. *Origin of the RFLP probes used and polymorphisms detected*

Name	Origin	Mapped to rice chromosome	Linkage to apomixis	EST (+), genomic (–)	Polymorphisms detected between		Reference
					Restriction enzymes	Phenotypes	
C901	Rice	12	Yes	+	Yes	Yes	Nagamura *et al.* (1997)
C996	Rice	12	Yes	+	Yes	Yes	Nagamura *et al.* (1997)
C1069	Rice	12	Yes	+	Yes	Yes	Nagamura *et al.* (1997)
C454	Rice	12	Yes	+	Yes	No	Nagamura *et al.* (1997)
R1759	Rice	12	Yes	+	Yes	Yes	Nagamura *et al.* (1997)
R642	Rice	12	No	+	Yes	No	Nagamura *et al.* (1997)
R2558	Rice	5	No	+	Yes	No	Nagamura *et al.* (1997)
R1888	Rice	6	No	+	Yes	No	Nagamura *et al.* (1997)
R1506	Rice	11	No	+	Yes	No	Nagamura *et al.* (1997)
R1927	Rice	3	No	+	Yes	No	Nagamura *et al.* (1997)
PsEXS	*P. simplex*	–	Yes	–	Yes	Yes	Calderini *et al.* (2006)
PsPDK	*P. simplex*	–	Yes	–	Yes	Yes	Calderini *et al.* (2006)
B11	*P.simplex*	–	Yes	–	No	Yes	Labombarda *et al.* (2002)
Ps85	*P. simplex*	–	No	–	No	No	Pupilli *et al.* (1997)
Ps650	*P. simplex*	–	Yes	–	No	Yes	Pupilli *et al.* (2004)
pTa71	Wheat		No	–	Yes	No	Gerlach and Bedbrook (1979)

Anti-5-methylcytosine-33D3) diluted 1:100 in PBS. Then slides were washed with PBST and incubated for 60 min with the tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody [polyclonal rabbit, anti-mouse, immunoglobulins/TRITC (Code No. R 0270, DakoCytomation, Glostrup, Denmark) diluted 1:100 in PBST at 37 ºC. Finally, slides were washed in PBST and mounted with 4',6-diamidino-2-phenylindole (DAPI)/Vectashield (Vector Laboratories, Burlingame, CA, USA) solution containing 2 mg ml^{-1} DAPI.

BAC-FISH procedures

The bacterial artificial chromosome (BAC) clone 346H10 carrying a 130 kb sequence 100% linked to the *P. simplex* ACR (Calderini *et al.*, 2006) was used as a probe for BAC-FISH experiments against *P. notatum* pachytene chromosomes. The BAC clone was labelled using the Nick Translation kit, Roche (Ref. 10976776001), with dioxigenin-11-dUTP (Roche, Ref. 11573152910) as the modified base. To enhance the hybridization signal of sequences mapping at the ACR, a fragment of ~2900 bp corresponding to the *EXS* gene included in the insert of BAC clone 346H10 (Calderini *et al.*, 2006) was PCR labelled. Cycling reactions contained 1× Taq Polymerase buffer (Promega), 200 µM of dNTPs (but only 180 µM dTTP), 20 µM dig-11dUTP, 2 mM MgCl$_2$, 0.2 µM *EXS*-specific forward (5'GTTGTGGGGAGTAAATCTATGGGTCTTT3') and reverse (5'GCTATGGTGAACACTGTCAGGTAGTTGT3') primers, and 1.5 U of *Taq* polymerase (Promega). Slides previously stained with DAPI and immunodetected for 5mC were washed in 2× SSC at 42 °C to remove coverslips, washed in 2× SSC at room temperature, and then treated with ethanol:acetic acid (3:1). Slides were observed under the microscope for controlling the absence of fluorescence before performing *in situ* hybridization. FISH was carried out according to Moscone *et al.* (1996). The first antibody consisted of mouse anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC; diluted 1:30) (Sigma-Aldrich, St. Louis, MO, USA, T3523). Preparations were then rinsed and incubated in a 1:100 dilution of secondary antibody rabbit anti-mouse conjugated to TRITC (DakoCytomation). All preparations of pachytene chromosomes were photographed with a Leica DMRX epi-fluorescence microscope (Leica, Heerbrugg, Switzerland) coupled to a computer-assisted Leica DC 350 digital camera system. Red, green, and blue images were captured in black and white using IM 1000 Leica software. Images were pseudo-coloured, merged, and adjusted for brightness and contrast by using Photoshop CS6 Extended version 10.0 (Adobe, San Jose, CA, USA).

5-Aza treatment

Paspalum simplex seeds were surface-sterilized with a mixture of 0.1% (w/v) sodium lauryl sulphate and 0.1% (w/v) mercuric chloride for 15 min, then with 0.1% (w/v) sodium lauryl sulphate for 15 min, and finally rinsed three times with sterile double-distilled water. Sterilized seeds were germinated on agar-solidified (8 gl^{-1}) MS medium (Murashige and Skoog, 1962) containing 30 mg l^{-1} sucrose together with 5, 10, 25, 50, 75, or 100 m gl^{-1} 5-Aza. Seeds were incubated at 23 ± 1 °C under a 12/12 h (day/night) photoperiod with fluorescent light at an intensity of 27 µmol m^{-2} s^{-1} and subcultured every 2 weeks. After 2 months, each plantlet was transferred to a 50 ml Erlenmeyer flask containing clay pebbles dipped in liquid MS medium with 5-Aza at the same concentration of the solid medium. The liquid medium was replaced once a week and flasks were kept in a greenhouse for 6 weeks. Plantlets were then transferred to pots with soil and irrigated according to routine practices with water containing the corresponding 5-Aza concentration.

Progeny tests

RFLP analyses were then carried out on test progeny derived from untreated controls and 5-Aza-treated apomictic MPs by employing marker loci diagnostic for deviation from apomixis. In particular, segregation of maternal bands was attributed to repression of apospory, and the presence of non-maternal bands indicated fertilization events and then repression of parthenogenesis. Confidence intervals (CIs) around observed proportions of aberrant individuals were calculated following the method described by Newcombe (1998), derived from a procedure outlined by Wilson (1927) with a correction for continuity (http://vassarstats.net/).

Results

Methylation-sensitive restriction analysis

To investigate the extent of DNA methylation of the *P. simplex* ACR, methylation-sensitive RFLP analysis was carried out by using the isoschizomers *Hpa*II and *Msp*I, which are differentially sensitive to 5mC at the CCGG cleavage site, in combination with apomixis-linked probes. *Hpa*II does not cut if the external cytosine is fully (double-strand) methylated and/or the internal cytosine is either fully or hemi- (single-strand) methylated, whereas *Msp*I cleavage is inhibited only if the outer cytosine is fully or hemi-methylated (McClelland *et al.*, 1994). Therefore, an identical migration of the hybridizing bands in both *Hpa*II and *Msp*I digests is an indication that the corresponding restriction fragment was produced from a CCGG site where the inner cytosine was unmethylated, whereas if the band is larger in the *Hpa*II digest, then the internal cytosine was methylated. It should be pointed out that since the *Hpa*II/*Msp*I analysis cannot differentiate among other methylation states of the CCGG site (such as unmethylated CCGG, fully methylated outer cytosine, or hemi-methylated inner cytosine), the percentage of total methylated DNA will probably be underestimated.

The *Hpa*II/*Msp*I digests of genomic DNA samples, originating from 25 sexual and 25 apomictic BC$_1$ plants, were hybridized with 16 probes (Table 1). Of these, one (pTa71) was a conserved sequence from a wheat rRNA gene that, being repetitive and located in highly methylated regions, was used as a positive control to test the reproducibility of the experimental procedure. The DNA of each of the 18 BC$_1$ plants (nine apomictic and nine sexual) used (Supplementary Fig. S2 at *JXB* online) showed an identical pattern of hybridization consisting of many bands whose size was <2 kb when digested with *Msp*I and of a single major band of high molecular weight probably belonging to uncut DNA when digested with *Hpa*II, indicating: (i) heavy methylation at the rRNA loci, as expected; (ii) reproducibility of the method as all the plants used showed an identical pattern; and (iii) absence of differences in the overall rDNA methylation level between apomictic and sexual genotypes in *P. simplex*. A negative control consisted of the hybridization of probe Ps85, which was isolated from a *Pst*I genomic library of *P. simplex* (Pupilli *et al.*, 1997). As *Pst*I is a methylation-sensitive endonuclease, Ps85 probably belongs to an undermethylated region of the *P. simplex* genome. Therefore, a non-polymorphic *Hpa*II/*Msp*I pattern was expected for this probe, as was observed (Supplementary Fig. S3).

The hybridization banding patterns of three rice expressed sequence tags (ESTs; (C901, C996, and C1069) together with two homologous probes, all of them linked to apomixis in *P. simplex*, are shown in Fig. 1. The three EST clones delineate the rice genomic region syntenic to the *P. simplex* ACR (Pupilli *et al.*, 2001). Clone C901, located next to the telomere of the long arm of rice chromosome 12, showed a major 3 kb band detectable in the *Hpa*II pattern, whereas the *Msp*I digest yielded a similarly intense band of lower molecular weight, indicating methylation of the CCGG site's inner cytosine near or within the sequence to which the probe hybridizes. These strong bands showed the same *Hpa*II/*Msp*I polymorphism in both apomictic and sexual genotypes. However, two *Hpa*II bands of ~7 kb and 12 kb (full arrows) together with a band

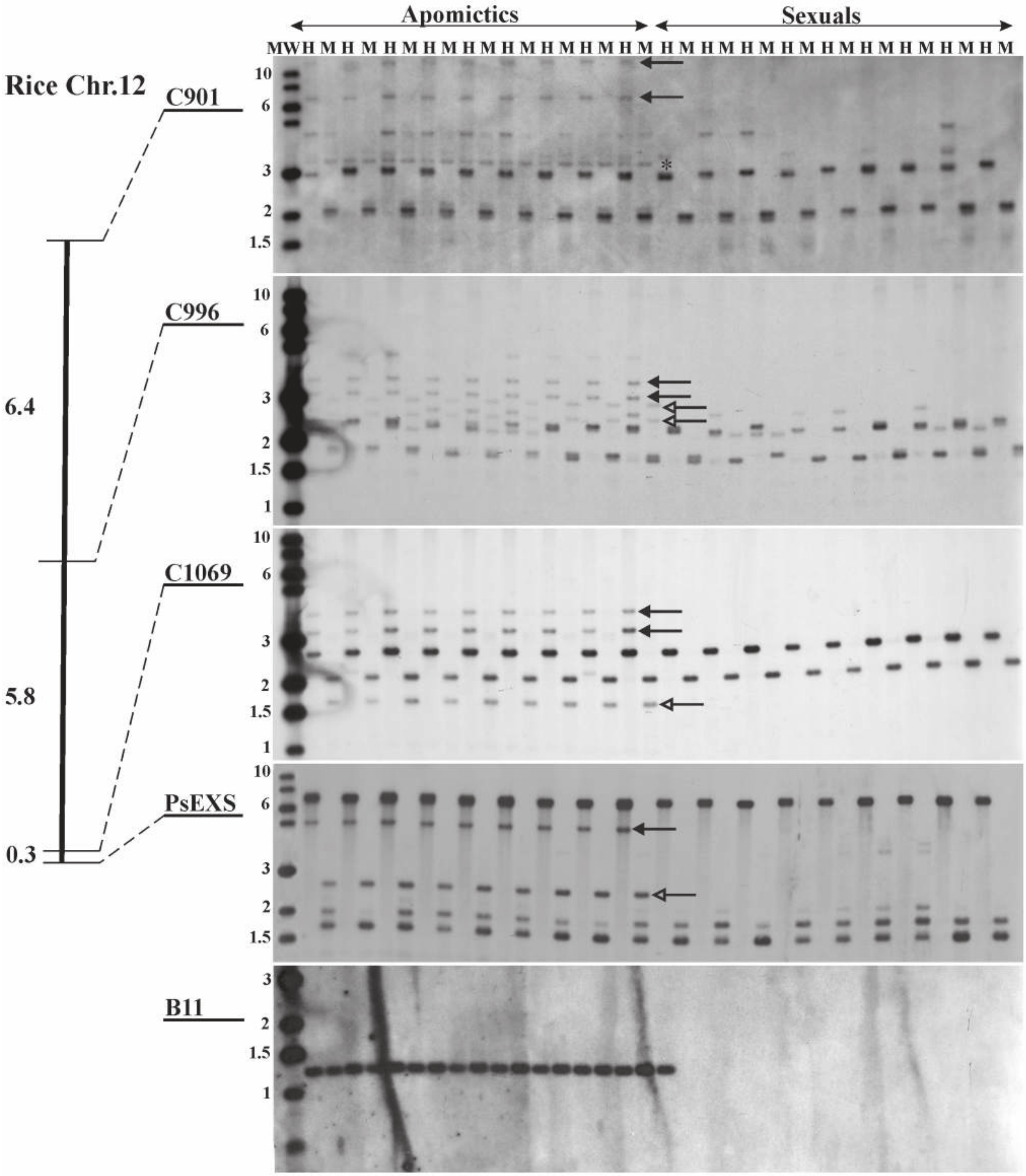

Fig. 1. Hybridization banding pattern of apomixis-linked probes with *Hpa*II (H)/*Msp*I (M) DNA digests of apomictic and sexual plants of *P. simplex*. Only apomixis-specific bands are indicated by arrows and an asterisk. Filled and open arrows point to *Hpa*II and *Msp*I apomixis-specific bands, respectively, whereas the asterisk indicates a non-polymorphic apomixis-specific band. Map distances on the rice chromosome are expressed in centiMorgans, and molecular weights (M) in kilobases.

of 3.5 kb common to both *Hpa*II/*Msp*I patterns were clearly detectable in apomictic plants only. This observation indicates the existence of an additional allele in apomictic plants, that had both methylated and non-methylated cytosines at the tested CCGG sites. The probe C996 produced a conserved *Hpa*II/*Msp*I polymorphism for major bands in both apomictic and sexual plants, together with an apomixis-specific polymorphism revealed by two *Hpa*II-specific (filled arrows) and two *Msp*I-specific (open arrows) faint bands. In this case, no common bands between the *Hpa*II/*Msp*I patterns of the apomictic genotypes were detected, indicating an absence of non-methylated areas in the vicinity of the hybridization site of the apomictic allele. Again, probe C1069 showed a methylated conserved pattern for major bands together with an apomixis-specific *Hpa*II/*Msp*I polymorphic pattern for less intense bands, in which two *Hpa*II bands of 4.2 kb and 3.2 kb were replaced by a single band of 1.7 kb in the *Msp*I pattern, indicating methylation of the internal cytosine at the related restriction site. A similar hybridization pattern (i.e. conserved *Hpa*II/*Msp*I polymorphisms for major bands together with apomixis-specific polymorphisms for weaker bands) was detected for C454 and R1759 (not shown).

To verify whether this particular pattern of hybridization revealed by rice EST probes could be due to their partial homology with *P. simplex* DNA, homologous probes were developed on the sequence of the protein-coding genes *PsEXS* and *PsPDK*, whose rice homologues were located in the vicinity of the apomixis-linked ESTs (Calderini *et al.*, 2006). The common pattern of hybridization of the apomixis-linked ESTs was confirmed with both probes, but, as expected, the apomixis-specific bands were much more intense than in the former cases (Fig. 1, PsEXS).

Furthermore, to investigate whether this hybridization pattern was also prevalent in hemizygous non-coding DNA regions of the ACR, a probe was developed from an apomixis-specific amplified fragment length polymorphism (AFLP), and hybridized to the *Hpa*II/*Msp*I blots. As expected, no hybridizing signals were present in sexual plants, whereas a single band of ~1.3 kb was detected in the *Hpa*II/*Msp*I pattern of apomictic genotypes (Fig. 1, B11). No *Hpa*II/*Msp*I polymorphisms were detected for this probe, indicating no methylation, at least in this particular hemizygous non-coding region. Another apomixis-linked genomic sequence belonging to the non-hemizygous non-coding region of the ACR (Ps650) showed an absence of methylation of the related CCGG sites in both sexual and apomictic plants (not shown). In summary, only probes originating from expressed sequences (ACR-mapping ESTs) located in non-hemizygous regions detected differential methylation in apomictic and sexual plants. Non-expressed regions were unmethylated, regardless of their hemizygosity. Altogether, these results indicate that 5mCs were prevalently located within the body of protein-coding genes and were differentially represented in sexual and apomictic genotypes.

Finally, to investigate the methylation-sensitive restriction pattern of genes not related to apomixis, the hybridizing banding patterns of five rice ESTs spread over five different chromosomes (R642, R2558, R1888, R1506, and R1927; Table 1) were assayed. Polymorphic *Hpa*II/*Msp*I patterns were revealed for all of them, including R642, which was located in a region of chromosome 12 of rice unrelated to apomixis, indicating a methylated status of the corresponding genes, but no methylation differences between sexual and apomictic genotypes were detected (not shown).

To sum up, methylation of CCGG inner cytosines is common at *P. simplex* coding regions, whether located or not on the ACR, whereas non-coding low-copy intergenic regions seem to be less methylated. Within the ACR, apomictic genotypes showed additional alleles whose methylation level depends on the specific clone taken into account. Since the *Hpa*II/*Msp*I polymorphisms corresponding to major bands looked identical in apomictic and sexual plants, the presence of the apomixis-specific alleles did not alter the methylation status of their 'sexual' allelic counterparts.

Immunodetection of 5mC and in situ *hybridization with apomixis-linked BAC clone 364H10*

To obtain an overall view of the methylation genomic landscape of the ACR and to establish parallelisms between *P. simplex* and the related species *P. notatum* regarding the heterochromatin/euchromatin structural context in which the apomixis locus is embedded, BAC-FISH analysis of pachytene chromosomes was undertaken in the latter species using the apomixis-linked 346H10 *P. simplex* BAC as a probe. To verify whether the clone 346H10 is located in the *P. notatum* ACR, a blot containing the DNA digests of apomictic and sexual plants of the same species was probed with a sequence belonging to the gene *PsEXS* included in the same BAC. The resultant hybridization pattern showed a band of high molecular weight present only in apomictic plants and absent in sexual plants, confirming the linkage between this sequence and apomixis in the species (Supplementary Fig. S4 at *JXB* online). Once this association was proven, clone 346H10 was used for FISH analysis in combination with 5mC immunodetection. First, DAPI was used to counterstain chromosome preparations and obtain a C-banding-like pattern under fluorescence microscopy. Low DAPI fluorescence intensity revealed a loose euchromatic organization, while high-intensity fluorescence revealed major heterochromatic regions. Overall, chromosomes from both Q4117 (apomict) and Q4188 (sexual) genotypes showed a prevalence of euchromatin, interspersed with several heterochromatin knobs. No major differences in the heterochromatin distribution were evidenced between genotypes (Fig. 2A, D). 5mC immunodetection revealed dispersed signals along all chromosomes together with some heavily methylated regions in both genotypes. Several stronger signals of 5mC immunolocalization overlapped with the highly condensed chromatin regions previously revealed by DAPI staining (Fig. 2B, E).

BAC-FISH hybridization showed a single region with high hybridization intensity in Q4117 (arrow, Fig. 2C), while a similar signal was not detected in the sexual strain Q4188 (Fig. 2F). Moreover some faint hybridization signals randomly distributed along chromosomes of both apomictic and sexual genotypes were also detected. This could be due

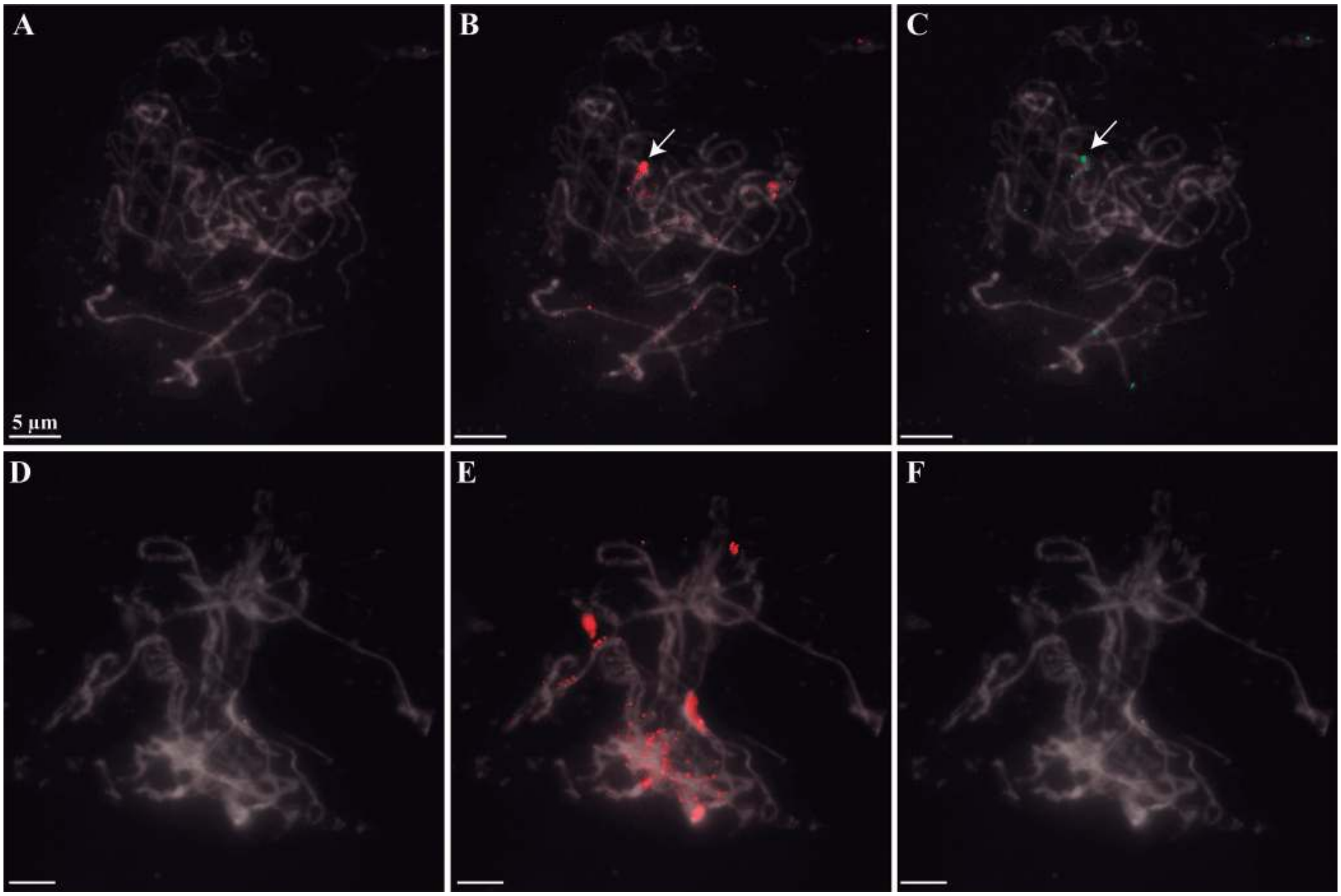

Fig. 2. Immunodetection of 5-methylcytosine and BAC-FISH analysis on pachytene chromosomes of apomictic (Q4117, A–C) and sexual (Q4188, D–F) *P. notatum*. (A, D) DAPI staining, (B, E) immunodetection of 5-methylcytosine, (C, F) BAC-FISH hybridization. The arrows indicate the position of the BAC clone on pachytene chromosomes of Q4117 (C) and in relation to an immunodetected heterochromatin knob (B). (This figure is available in colour at *JXB* online.)

to partial homology of the BAC clone with TEs distributed throughout the genome as observed in *P. simplex* (Calderini *et al.*, 2006). Interestingly, the region where the BAC clone revealed a major hybridization signal in Q4117 was coincident with a heterochromatic region detected by DAPI staining and a heavily methylated region identified by 5mC immunodetection (arrow, Fig. 2B).

These results indicated that the *P. notatum* ACR is located on a heterochromatic knob characterized by a high content of 5mCs. These results are in agreement with those reported in *P. simplex* by Calderini *et al.* (2006), and in *P. notatum* by Podio *et al.* (2012).

5-Aza treatment

To investigate whether DNA demethylation can affect apospory and/or parthenogenesis, the effect of 5-Aza on apomictic *P. simplex* reproductive development was studied. A small-scale pilot study was carried out to establish a threshold value of 5-Aza concentration to obtain the maximum effect on DNA demethylation without dramatic effects on plant survival. Five apomictic families (47, 48, 50, 65, and 71) were selected and 10–60 open pollinated seeds from each family were cultured aseptically in M medium containing 5, 10, and 25 mg l^{-1} 5-Aza for all families and 50 mg l^{-1} and 100 mg l^{-1} 5-Aza for family 71 only. After 3 months of culture, 22–27% of seedlings were still viable in media containing 5 mg l^{-1} and 10 mg l^{-1} 5-Aza, respectively, whereas seedling survival dropped dramatically to 14.4% in media with 25 mg l^{-1} 5-Aza. Treatment with 5-Aza at 50 mg l^{-1} and 100 mg l^{-1} was lethal for almost all seeds of family 71 (Supplementary Table S1 at *JXB* online).

To test the effect of 5-Aza on cytosine demethylation, the *Hpa*II/*Msp*I restriction patterns of the plants recovered from 5-Aza treatment were compared with those of their related untreated families. Since the *Msp*I pattern did not vary substantially as a consequence of 5-Aza treatment, the increase in the percentage of monomorphic bands (i.e. the appearance of novel *Hpa*II bands at the same position as the pre-existing *Msp*I bands, indicating demethylation of the related CCGG site) in the *Hpa*II/*Msp*I pattern of treated plants compared with untreated controls was taken as an indicator of the effectiveness of the demethylation. The *Hpa*II/*Msp*I banding patterns produced with the probes C996 and C1069 were analysed on the DNA digests of 5–10 treated plants for each family (Table 2). Treatments with 5 mg l^{-1} and 10 mg l^{-1} 5-Aza did not affect the methylation status of the apomixis locus in any of the treated plants, with the exception of those belonging to family 71, whereas the 25 mg l^{-1} dose induced a variable increase (from 2.3- to 3.7-fold) in the percentage of monomorphic bands in the treated plants of families 48, 50, and 71. Treated plants from families 65 and 47 were less

Table 2. *Effect of 5′-azacytidine treatment on average number of monomorphic bands in the HpaII/MspI pattern*

5-Aza (mg l^{-1})	Families				
	47	48	50	65	71
0	14.28	7.14	7.69	6.67	7.69
5	18.43 ± 4.4	9.88 ± 4.76	9.29 ± 2.99	6.82 ± 0.24	17.80 ± 7.46
10	22.17 ± 5.8	16.04 ± 8.15	13.82 ± 5.30	7.07 ± 0.18	17.77 ± 10.49
25	20.32 ± 6.1	26.47 ± 7.74	17.79 ± 10.65	11.96 ± 5.78	19.50 ± 9.06

affected by the 5-Aza treatment. Thus, the concentration of 25 mg l^{-1} 5-Aza was considered a good compromise between plant survival (14.4%) and effective cytosine demethylation. Differences in DNA demethylation rates among plants might be related to differential drug uptake rather than to genotype-specific responses.

The variation on apospory/parthenogenesis rates was evaluated on a total of 374 open-pollinated progeny (test progeny) from 29 MPs treated with 25 mg l^{-1} 5-Aza. For each family, the open-pollinated progeny of a single untreated MP was taken as a control (Table 3). Segregation of maternal bands was observed in only two individuals belonging to two different MPs, rendering proportions with 95% CIs overlapping with those corresponding to the controls (Newcombe, 1998). These results indicated that the 5-Aza treatment had no significant effect on apospory (with a confidence of 95%). Conversely, seven MPs showed a percentage of progeny, ranging from 4% to 100%, displaying novel non-maternal bands. The proportion of aberrant progeny was 0.0802, with a 95% CI not overlapping with that of the controls. This indicated a significant increment in the occurrence of fertilization events which was assumed to be derived from a detrimental effect of 5-Aza on parthenogenesis. Two examples of these non-maternal bands are shown in Fig. 3 where six out of the 13 test progeny derived from a treated MP showed two non-maternal bands evidenced by two independent probes additional to the whole maternal banding pattern. These non-maternal bands were the same as recorded in the original BC_1 population and they probably came from potential pollinating parents present in the same population (see Supplementary Fig. S1 at *JXB* online for probe Ps71). Therefore, their presence strongly suggests the occurrence of fertilization events.

The test progeny showing non-maternal bands could be B_{III} hybrids (i.e. individuals originating from fertilization of unreduced egg cells). If this was the case, these individuals should have a DNA content 50% higher than that of the other progeny; that is, they should be hexaploid ($2n=4x+2x$), while the other sister progeny should be tetraploid ($2n=4x$). To assess the ploidy level of the putative B_{III} hybrids, Feulgen analysis of somatic DNA was performed on an individual's subset of test progeny, and the results are reported in Table 4. The Feulgen reaction allows DNA *in situ* to be specifically stained based on the reaction of Schiff reagents with aldehyde groups generated in the DNA molecules by HCl hydrolysis (Feulgen and Rossenbeck, 1924). As the staining intensity is proportional to the DNA concentration, Feulgen analysis of DNA content has been used to estimate the ploidy level in plants (Bennett and Smith, 1976; Bennett *et al.*, 1982). Moreover, correlation between Feulgen analysis and parameters linked to the ploidy level [i.e. pollen diameter (Cáceres *et al.* (1999)] as well as more refined systems of nuclear DNA content analysis [i.e. flow cytometry (Michaelson *et al.*, 1991; Cáceres *et al.*, 2001)] were reported. Significant differences in the Feulgen absorption between putative B_{III} and non-B_{III} hybrids used as a negative control corresponded to the expected values of the DNA content for a hexaploid compared with a tetraploid genotype of *P. simplex* (Table 4).

To sum up, artificial demethylation had little or no effect on apospory, whereas it induced a highly significant depression of parthenogenesis, involving eight out of 33 treated MPs. This phenomenon was particularly evident in family 48, for which three of the treated MPs showed parthenogenesis depression at a highly significant level. Moreover, parthenogenesis depression could have been underestimated because occasional self-pollination could have masked some egg fertilization events, as was probably the case for plant no. 8 from family 48 (Table 4) for which a DNA content similar to that of hexaploids was measured, but in no case were non-maternal bands detected.

Discussion

DNA methylation at the cytosine residues in a symmetrical CG context is an evolutionarily conserved modification of DNA commonly detectable in several living forms including animals, plants, and fungi (Chan *et al.*, 2005; Freitag and Selker, 2005; Goll and Bestor, 2005; Klose and Bird, 2006). Plants have evolved unique additional mechanisms of cytosine methylation in symmetrical (CNG) and asymmetrical (CNN) contexts, where N could be either A, T, or G (Finnegan and Kovac, 2000). In all cases, plant DNA methylation is related to transcription repression, either by preventing the binding of transcription factors to promoters (Bird, 2002) or by blocking the binding of RNA polymerase to promoters (Baylin and Herman, 2000) through the mediation of methyl-CpG-binding domain (MBD) proteins, which recognize methylation sites on DNA (Ballestar and Wolffe, 2001; Straussmann *et al.*, 2009). The specific function of cytosine methylation depends on its genomic context: within repetitive non-coding genomic regions, DNA methylation (either in CG or non-CG contexts) acts as a defence against TE proliferation (Lisch, 2009), whereas when methylation is located within protein-coding regions its function is less clear. Several hypotheses have been formulated about the possible function of gene body methylation, from transcription regulation to no function at all (reviewed in Takuno and Gaut, 2013). In *Arabidopsis* genes, body methylation tends to be associated with constitutive expression (Zhang *et al.*, 2006; Zilberman *et al.*, 2007).

A high level of gene body methylation was detected in CG contexts for (pseudo)genes linked to the ACR of *P. simplex*, suggesting that their expression might be deregulated, as already noticed for apomixis-linked alleles by Polegri *et al.* (2010). Although heavy DNA methylation was detected in an apomixis-linked allele related to the retrotransposon sequence

Table 3. *Analysis of the mode of reproduction of open-pollinated MPs treated with 25 m gl^{-1} 5′-azacytidine*

Family	Names of MPs	No. of test progeny	Off-type offspring showing		Proportion (95% confidence interval)	
			Absence of maternal bands	Presence of non-maternal bands	Absence of maternal bands	Presence of non-maternal bands
71	Control	31	–	–	0 (0–0.1373)	0 (0–0.1373)
	#19	12	–	–	0 (0–0.3013)	0 (0–0.3013)
48	Control	25	–	–	0 (0–0.1658)	0 (0–0.1658)
	#6	5	–	–	0 (0–0.5371)	0 (0–0.5371)
	#9	23	–	1	0 (0–0.1781)	0.0435 (0.0023–0.2397)
	#10	6	–	6	0 (0–0.4832)	1 (0.5168–1)[a]
	#11	40	–	4	0 (0–0.1091)	0.1 (0.0325–0.246)
	#14	13	–	–	0 (0–0.2834)	0 (0–0.2834)
	#15	5	–	1	0 (0–0.5371)	0.2 (0.0105–0.7012)
	#205	6	–	–	0 (0–0.4832)	0 (0–0.4832)
	#206	4	–	–	0 (0–0.6042)	0 (0–0.6042)
	#211	13	–	6	0 (0–0.2834)	0.4615 (0.204–0.7388)[a]
	#219	12	–	12	0 (0–0.3013)	1 (0.6987–1)[a]
	#222	23	–	–	0 (0–0.1781)	0 (0–0.1781)
	#223	10	–	–	0 (0–0.3445)	0 (0–0.3445)
47	Control	15	–	–	0 (0–0.2535)	0 (0–0.2535)
	#6	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#93	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#96	9	–	–	0 (0–0.3712)	0 (0–0.3712)
	#103	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#110	6	–	–	0 (0–0.4832)	0 (0–0.4832)
	#111	7	1	3	0.1429 (0.0075–0.58)	0.4286 (0.1181–0.7976)
	#113	8	–	–	0 (0–0.4023)	0 (0–0.4023)
65	Control	19	–	–	0 (0–0.1682)	0 (0–0.1682)
	#6	23	1	–	0.0435 (0.0023–0.2397)	0 (0–0.1781)
	#26	9	–	–	0 (0–0.3712)	0 (0–0.3712)
	#27	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#29	9	–	–	0 (0–0.3712)	0 (0–0.3712)
	#30	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#32	10	–	–	0 (0–0.3445)	0 (0–0.3445)
	#34	34	–	–	0 (0–0.1264)	0 (0–0.1264)
50	Control	12	–	–	0 (0–0.3013)	0 (0–0.3013)
	#2	12	–	–	0 (0–0.3013)	0 (0–0.3013)
	#4	25	–	–	0 (0–0.1658)	0 (0–0.1658)
Total						
Control	5	102	0	0	0 (0–0.0452)	0 (0–0.0452)
Treated	29	374	2	33	0.0053 (0.0009–0.0213)	0.0802 (0.0556–0.1137)*

[a] Significant at 95% confidence (including continuity correction).

C1069 in *P. simplex*, non-consistent methylation was detected for the same probe in *P. notatum* (Podio *et al.*, 2012). On the other hand, differences in the global methylation patterns between sexual and apomictic genotypes of the latter species were highlighted by clustering the two groups using methylation-sensitive molecular markers (Rodriguez *et al.*, 2012). Finally, FISH analysis coupled with immunodetection of 5mC revealed heavy DNA methylation at the ACR of *P. notatum*.

Taken together, these results indicated that although differences may exist between *P. simplex* and *P. notatum* for the level of cytosine methylation at single specific genes, reflecting slight differences in time and modality by which these species diverged from a common ancestor, heavy cytosine methylation and location on heterocromatin knobs are common features of the ACR in the two species.

The overall epigenomic landscape of a plant is not static, but rather the result of the dynamic action of evolutionary forces that act during development under the influence of the environment (Zhong *et al.*, 2013). An induced modification of the epigenetic status by artificially decreasing the frequency of cytosine methylation causes transcriptional reactivation of silenced genes and leads to the alteration of plant growth and development (Zhang *et al.*, 2012). One of the several methods used to modify the frequency of methylcytosine in the genome of living forms is to treat the organism with 5-Aza (Jones and Taylor, 1980). This nucleoside analogue specifically inhibits DNA methyltransferases, thus preventing DNA methylation. Furthermore, treatment with 5-Aza causes chromatin decondensation and mediates an increase in H3 acetylation and a decrease on H3K9 methylation during interphase and

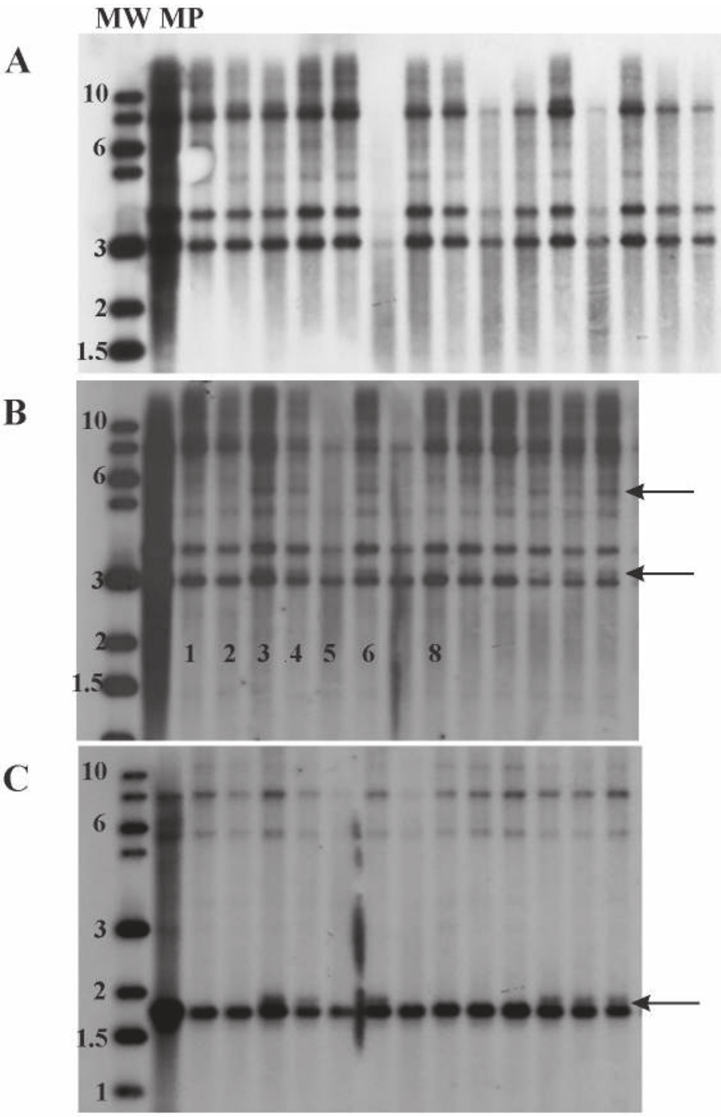

Fig. 3. Hybridization banding pattern of diagnostic molecular probes with the *Eco*RI DNA digests of MPs and test progeny of *P. simplex*. (A) Untreated MP and 26 test progeny DNA digests and (B) treated MP and 13 test progeny DNA digests probed with Ps71; (C) as B, probed with Ps96. Arrows points to non-maternal bands. Numbers mark the plants chosen for Feulgen analysis reported in Table 4. Molecular weights (M) are expressed in kilobases.

Table 4. *Feulgen analysis of off-type individuals detected in test progeny*

Family 48, MP #211		Family 47, MP #111	
Plant number[a]	**Feulgen adsorption**[c]	**Plant number**	**Feulgen adsorption**
Control MP (4*x*)	13.64 ± 0.85	Control MP (4*x*)	12.22 ± 0.35
#1 (–)	12.37 ± 0.94	#1 (–)	12.92 ± 0.41
#2 (–)	13.20 ± 0.46	#2 (+)	19.07 ± 0.64
#3 (+)	19.70 ± 0.94	#3 (–)	12.25 ± 0.44
#4 (+)	18.42 ± 0.62	#4 (+)	17.36 ± 0.58
#5 (–)	14.64 ± 0.54	#7 (+)	18.73 ± 0.77
#6 (+)	18.62 ± 0.67		
#8 (–)	18.01 ± 0.89		

[a] Putative ploidy level: (–), 4*x*; (+), 6*x*.
[b] Arbitrary units ±SE.

mitosis (Yang *et al.*, 2010). Among several effects produced by 5-Aza in plants, those related to embryogenesis are of particular interest for the present research. It had been reported that 5-Aza treatment induced repression of somatic embryogenesis in coffee (Nic-Can *et al.*, 2013), carrot (Yamamoto *et al.*, 2005), and *Medicago truncatula* (Santos and Fevereiro, 2002), whereas in *Acca sellowiana* 5-Aza-mediated cytosine demethylation enhanced somatic embryogenesis, although the embryo-to-plant conversion rate was negatively affected (Fraga *et al.*, 2012). The dynamics of cytosine methylation during zygotic embryogenesis seem to resemble those of somatic embryogenesis, as demethylation represses embryogenesis (cell proliferation) and, conversely, methylation marks the quiescent state of the fully differentiated embryos in *Brassica* (Solís *et al.*, 2012), *Arabidopsis* (Xiao *et al.*, 2006), rice (Abiko *et al.*, 2013), and *Castanea* (Viejo *et al.*, 2010). In the present results, 5-Aza treatment had little or no effect on the formation of non-reduced embryo sacs from nucellar cells (apospory), whereas it seemed to affect negatively the autonomous embryo development from unreduced egg cells (parthenogenesis) thus allowing their fertilization to form B_{III} hybrids. These results suggest that key factors affecting parthenogenesis are under epigenetic control and in particular cytosine methylation may represent at least one of the mechanisms by which this control is exercised. Experiments carried out on mice oocytes provide definitive evidence that mechanisms preventing parthenogenetic development of the embryos are under epigenetic control and in particular, on the DNA imprinting marks originating from the paternal parent (Kono *et al.*, 2004; Kawahara *et al.*, 2007).

Imprinting in plants is traditionally considered to be a phenomenon restricted to the endosperm, although recent research reveals a parent-of-origin gene expression in the embryos (Jiang and Kholer, 2012). Although mechanisms of imprinting in early embryo development are similar in mammals and plants, gene regulation control in plants seems more flexible, as parthenogenesis is commonly detectable either as an individual feature or associated with diplospory or apospory. The genetic determinant(s) of parthenogenesis should enable autonomous embryo development and, at the same time, prevent the egg cells (either of mitotic or meiotic origins) from being fertilized. The possibility of enhancing the penetrance of parthenogenesis by anticipating the timing of pollination has been reported in *Cenchrus ciliaris* (Burson *et al.*, 2002), *Pennisetum* (Bashaw *et al.*, 1992), and *Tripsacum* (Kindiger and Dewald, 1994). All these species are characterized by protogynous flowering behaviour according to which stigmas mature several days before anthers, and, since unreduced egg cells committed to parthenogenesis are accelerated compared with reduced egg cells, fertilization of the latter cells is favoured by early pollination. However, the success of unreduced egg cell fertilization and therefore the repression of parthenogenesis is species dependent: early pollination enhances fertilization of accelerated unreduced egg cells in non-protogynous *P. notatum* (Martínez *et al.*, 1994) whereas the opposite is true when development of unreduced egg cells is delayed compared with that of the meiotic cells as reported in wild apple (Liu *et al.*, 2014). Using electron microscopy, Vielle *et al.* (1995) found that a cell wall covered the plasma membrane of the aposporous egg cell of *C. ciliaris* several hours before a pollen tube entered the female gametophyte, thereby providing a physical barrier to fertilization. Such a barrier was not present in reduced egg cells of the same species. Conversely, no barriers to fertilization were found in unreduced egg cells of *Panicum maximum* (Naumova and Willemse, 1995) or in reduced egg cells of lines of barley committed to haploid parthenogenesis (Mogensen, 1982).

Accelerated development of unreduced and parthenogenetically committed egg cells is probably related to loss of their receptivity for sperm fusion. As an example, Felitti *et al.* (2011) reported the differential expression of the *lorelei*-family-like ACR-linked *n20gap-1* gene, in flowers of sexual and apomictic *P. notatum*. *LORELEI* was identified as a controller of the sperm discharge onto the egg cell in *Arabidopsis*. Moreover, escape from fertilization is guaranteed by acceleration of autonomous embryo and endosperm development compared with sexual embryogenesis in hybrids resulting from sexual×apomictic crosses in *Hieracium* (Koltunow *et al.*, 2011; Rosenbaumová *et al.*, 2012). A precocious fertilization-independent metabolic activation was noticed by Naumova and Matzk (1998) in parthenogenetic lines compared with their sexual counterparts of the Salmon system in wheat. In this case, no structural barriers hindering fertilization were detected between the two isogenic lines but, rather, the precocious initiation of the parthenogenesis pathway is under strict genetic control and depends on the presence of a parthenogenesis-inducing gene (*Ptg*) and the absence of a parthenogenesis-suppressing gene (*Spg*). This genetic set up depends on the substitution of the short arm of wheat chromosome 1B with the short arm of chromosome 1 of rye (Tsunewaki and Mukai, 1990). Such a model of a parthenogenetic inducer and repressor has been adapted by Matzk *et al.* (2005) to explain the genetic control of parthenogenesis in apomictic *Poa*, a species for which independent segregation of apospory and parthenogenesis is well documented (Albertini *et al.*, 2001). According to these antecedents, it can be hypothesized that both parthenogenesis activator and suppressor genes are present in the recombinationally blocked ACR and that the suppressor might be inactivated by cytosine methylation in apomictic *P. simplex*. Artificial demethylation of the suppressor by 5-Aza treatment could then allow fertilization of unreduced egg cells.

To the authors' knowledge, this is the first report of artificial phenotype reversion in a natural apomictic plant that is probably related to an epigenetically induced variation of gene expression patterns. This achievement has important implications from the perspective of parthenogenesis candidate gene(s) isolation and characterization in natural apomictic systems. Demethylated genes, whose differential expression is linked to phenotype reversion, might represent interesting candidates. Further work should be focused on the following aspects: (i) the presence of potential suppressors of parthenogenesis as well as fertilization promoters within/near the ACR needs to be confirmed; (ii) the influence of the differential methylation detected on the ACR on the activity of these particular genes should be investigated; and (iii) the effect of the differential activity of these genes on reproductive development should be examined by reverse genetics. In any case, the possibility of inducing a phenotype reversion experimentally is an additional tool to others already available (Ortiz *et al.*, 2013) that makes *Paspalum* an excellent biological system to study apomictic reproduction.

Supplementary data

Supplementary data are avialable at *JXB* online.

Figure S1. Hybridization banding pattern of the probe Ps71 with *Eco*RI DNA digests of part of the BC_1 population of *P. simplex* from which families were selected.

Figure S2. Hybridization banding pattern of probe pTa71 with *Hpa*II (H)/*Msp*I (M) DNA digests of *P. simplex* apomictic and sexual plants.

Figure S3. Hybridization banding pattern of the probe Ps85 with *Hpa*II (H)/*Msp*I (M) DNA digests of *P. simplex* apomictic and sexual plants.

Figure S4. Hybridizing banding pattern of the probe PsEXS with *Eco*RI DNA digests of apomictic and sexual *P. notatum* hybrids together with their parental lines.

Table S1. Plant survival after 5′-azacytidine treatment.

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