Seed development and inheritance studies in apomictic maize-*Tripsacum* hybrids reveal barriers for the transfer of apomixis into sexual crops

OLIVIER LEBLANC*,[1], DANIEL GRIMANELLI[1], MARTHA HERNANDEZ-RODRIGUEZ[2], PABLO A. GALINDO[2], ANA M. SORIANO-MARTINEZ[2,#] and ENRICO PEROTTI[2,3]

[1]*Laboratoire Génome et Développement des Plantes (LGDP), UMR 5096 IRD-CNRS-Université de Perpignan, Montpellier, France,*
[2]*International Maize and Wheat Improvement Center, Texcoco 56130, Mexico and*
[3]*Plant Cell Biology Group, Australian National University, Canberra ACT, Australia*

ABSTRACT Apomixis in plants covers a variety of cloning systems through seeds of great potential for plant breeding. Among long-standing approaches for crop improvement is the attempt to exploit wild relatives as natural, vast reservoirs for novel genetic variation. With regard to apomixis, maize possesses an apomictic wild relative, *Tripsacum*, which we used to produce advanced maize-*Tripsacum* hybrid generations. However, introgression of apomixis in maize has failed so far. In order to understand the how's and why's, we undertook characterization of seed development and inheritance studies in these materials. We show that apomictic seeds suffer from epigenetic loads. Both seed tissues, the endosperm and the embryo, displayed developmental defects resulting from imbalanced parental genomic contributions and aberrant methylation patterns, respectively. Progeny characterization of several maize-*Tripsacum* hybrid generations allowed significant progress toward the unraveling of the genetics of apomixis. First, chromosome deletion mapping showed that expression of apomixis requires one single *Tripsacum* chromosome. However, inheritance studies revealed that female gametes inheriting this segment were unequivalent carriers depending on their origin: unreduced gametes transmit a functional segment, whereas progeny derived from reduced ones reproduced sexually. Finally, chromosomal or genomic dosage variation barely affected the apomictic phenotype suggesting no dependency for ploidy in these materials. We conclude that epigenetic information imposes constraints for apomictic seed development and seems pivotal for transgenerational propagation of apomixis. The nature of the triggering mechanisms remains unknown as-yet, but it certainly explains the modest success relative to the development of apomictic maize thus far.

KEY WORDS: *apomixis, seed development, maize, epigenetics, interspecific hybrid*

Introduction

The *Tripsacum* genus comprises wild relatives of maize (*Zea mays* L.) widely distributed across the American continent and highly variable in many aspects (Berthaud *et al.*, 1997, Randolph, 1970). Efforts towards allele mining out of this diverse, genetic reservoir have been limited so far (for instance, resistance to *Puccinia sorghi;* Bergquist, 1981). One notable exception concerns apomixis or the formation of maternal embryos within seeds (Koltunow, 1993, Nogler, 1984), a reproductive behavior of great potential for agriculture and plant breeding (Spillane *et al.*, 2004).

Apomixis in higher plants is achieved through a number of routes, most of which still lack detailed characterization. Apomictic developments have been categorized into adventitious embryony and gametophytic apomixis depending on the cell type

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Address correspondence to: Olivier Leblanc. Institut de Recherche pour le Développement, 911 Avenue Agropolis, BP 64 501, 34394 Montpellier Cedex 05, France. Fax: +33-04-6741-6181. e-mail: olivier.leblanc@ird.fr Lab web address: http://lgdp.univ-perp.fr
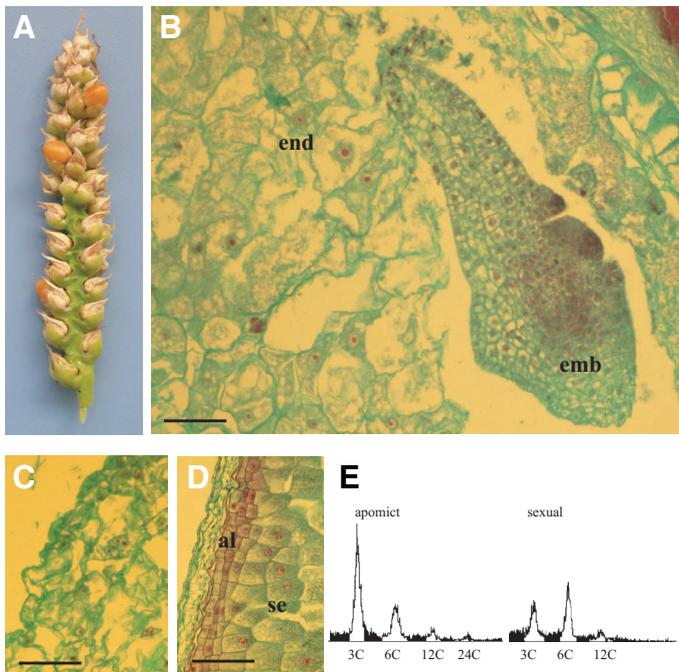
#*Present address:* Unidad de Biología Tecnología y Prototipos (UBIPRO), Universidad Nacional Autónoma de México, 54090 Tlalnepantla, Estado de México, México

that gives rise to maternal embryos, i.e. somatic cells within the ovule and parthenogenetical egg cells within unreduced megagametophytes, respectively (Gustafsson, 1946, Koltunow, 1993, Nogler, 1984). Recent works in species reproducing through distinct pathways, i.e. *Hieracium* subgenus *pilosella, Poa,* and *Tripsacum,* suggest that gametophytic apomixis (referred to as apomixis herein) relies upon either spatial or temporal misexpression of genes acting during female sexual reproduction (Albertini *et al.*, 2004, Grimanelli *et al.*, 2003, Tucker *et al.*, 2003). However, although candidate genes showing differences in spatial and temporal expression patterns between apomicts and their sexual counterparts have been identified, both their involvement in the control of apomixis and function remain largely speculative (Ozias-Akins, 2006). Inheritance studies have provided a relatively simple view for the genetics of apomixis, which was reported to depend on a few dominant genes in most species investigated (Asker and Jerling, 1992, Ozias-Akins and van Dijk, 2007). Challenging this, further molecular mapping and cytogenetic characterization of the chromosomal region(s) linked to the trait in several species have unveiled attributes frequently associated with complex loci, such as lack of recombination, trans-acting mechanism for gamete elimination, heterochromatinization and DNA rearrangements (Ozias-Akins and van Dijk, 2007). To date, the genetic control of apomixis remains confuse, as no apomictic locus has been molecularly resolved. Alike, whether the complex nature of apomictic loci or polyploidy, a usual feature of apomictic genomes, take part in the control of the trait is unknown. Fueling the debate, functional roles for polyploidy have been hypothesized, including epigenetic gene deregulation (Koltunow and Grossniklaus, 2003), ploidy-dependent gene expression (Cervigni *et al.*, 2008, Quarin *et al.*, 2001), or ectopic gene expression resulting from genome asynchrony (Carman, 1997). On the other hand, these complexities were proposed to have evolved once apomictic reproduction established, thus they may be secondary with regards to its genetic control (Ozias-Akins and van Dijk, 2007).

Within the Maydae tribe, apomixis occurs in *Tripsacum* only (Brown and Emery, 1958), making the genus a prime candidate to elaborate strategies for its transfer to maize either directly through backcrossing or by genetic engineering. *Tripsacum* species typically compose an agamic complex (after Babcock and Stebbins, 1938) wherein diploid individuals (x=18, 2n=2x=36) are sexual and polyploid individuals (2n=3x to 6x) reproduce apomictically. Apomixis is gametophytic of the diplosporous type (Farquharson, 1955, Leblanc *et al.*, 1995b): functional, unreduced megaspores form after omission, or early termination, of meiosis in megaspore mother cells (Grimanelli *et al.*, 2003). Further differentiation into megagametophytes resembles that of the Polygonum type found in sexual species, but cells contain nuclei of maternal genetic makeup. Activation of unreduced egg cells through unknown developmental alterations in female gametogenesis induces embryogenesis in absence of fertilization (Bantin *et al.*, 2001, Farquharson, 1955) but the developmental course of maternal embryos stops after a few rounds of mitotic divisions resulting in quiescent proembryos within unfertilized megagametophytes (Grimanelli *et al.*, 2003). Pollination, followed by the delivery of two sperm cells into the mature megagametophyte and by the fertilization of the central cell only, is required for seed development. As in many other apomicts, apomixis in *Tripsacum* plants is facultative as reproductive behaviors that allow genetic variation have been preserved through evolution. The most documented ones result from partial or complete restoration of sexual programs (Asker and Jerling, 1992, Bicknell and Koltunow, 2004), but other mechanisms such as incomplete nucleus restitution during meiosis abortion, mitotic and meiotic non-disjunction, somatic recombination, and gene

Fig. 1. Pedigree of 38-chromosome maize-*Tripsacum* hybrids (2n=20M+18Td) and derivatives. *Boxes display progeny sorting after data compilation from previous reports (Grimanelli* et al., *1998, Leblanc, 1995, Leblanc* et al., *1996) and unpublished results (O. Leblanc). Materials used in this work are from bolded boxes. Boxes with gradient color background represent undetermined progeny prior to this work. M: maize. Td:* Tripsacum dactyloides.

Fig. 2. Seed development in 2n=38 maize-*Tripsacum* (38C clone) and *Tripsacum dactyloides* apomicts. (A) *Typical inflorescence of 38C clone pollinated using diploid maize.* **(B)** *Section in mature kernel of 38C showing a normal-looking embryo (emb) but surrounded by atypical endosperm cells (end).* **(C)** *Peripheral section of 38C endosperm with altered cell differentiation.* **(D)** *Peripheral section of normal endosperm from a sexual* Tripsacum dactyloide*s strain: (al) aleurone layer cells, and (se) starchy endosperm cells.* **(E)** *Cell cycle analysis in endosperms from apomictic and sexual tetraploid* Tripsacum *strains. Bars, 300 μm.*

morphologically normal embryo (Figure 2B). This suggests that endosperm collapsing occurred after cellularization, a critical step whereby apomictic proembryos resume development (Grimanelli *et al.*, 2003). Unfavorable allelic interactions might have promoted endosperm failure, but a more plausible explanation is that development primarily suffered from maternal genomic excess. In endosperms from 2n=38 individuals pollinated with diploid maize, mother plants typically contributed six genomes (4M and 2Td) for a single paternal genome (1M). This corresponds to a strong distortion of the typical 2 maternal to 1 paternal genomic ratio (2m: 1p) required for normal seed development in many Angiosperms, including maize (Lin, 1984, Nishiyama and Inomata, 1966) and *Tripsacum*, as indicated by flow cytometrical analyses in normal and imbalanced endosperms. As shown in Figure 2E, endoreduplication in endosperms derived from 4x apomicts crossed with 4x male progenitors (2n=10x, 8m: 2p) occurred precociously compared to 6x normal endosperms (4m:2p; 4x sexuals X 4x progenitors), a difference in cell cycle progression already reported in maize as a mark for maternal excess (Leblanc *et al.*, 2002). Dosage effects incidence in the endosperm of 2n=38 apomicts was corroborated by observations made from sectioned developing kernels. Cell growth was affected similarly to that of maize endosperms suffering from maternal genomic excess (Cooper, 1951): i. e., enlarged central cells, undifferentiated basal cell layer, and poor subepidermal differentiation (compare Figure 2C and 2D). Nevertheless, deleterious effects appeared less

mutation have been reported as well (Hair, 1956, Noyes, 2005, Richards, 1996).

Since maize and *Tripsacum* were first hybridized in the early 30's (Mangelsdorf and Reeves, 1931), pathways for introgressing *Tripsacum* genetic material into the crop have been extensively scrutinized (Harlan *et al.*, 1970, Harlan and DeWet, 1977). Nevertheless, in spite of several decades of efforts (Kindiger and Sokolov, 1997, Leblanc, 1995, Leblanc *et al.*, 1996, Petrov *et al.*, 1984), no maize germplasm expressing some level of apomixis has been recovered yet. Conventional backcrossing at CIMMYT using a *T. dactyloides* apomictic donor and maize yielded facultative apomictic hybrids possessing two maize (M) genomes and one genome from *T. dactyloides* (Td) (i.e., 2n=38=20M+18Td) (Leblanc *et al.*, 1996). Here, we report on both female reproductive behavior and genomic characterization in these materials and derivative generations (Figure 1, Table 1). Our observations shed light onto critical obstacles, some of them likely of epigenetic nature, such as developmental abnormalities in apomictic seeds, methylation pattern differences following apomictic reproduction, and unexpected behavior of the chromosomal region that governs apomixis. Collectively, they provide an explanation for the modest success of transfer attempts to date.

Results

*Seed developmental abnormalities in maize-*Tripsacum *and* Tripsacum *apomicts*

Seed development in maize-*Tripsacum* individuals with 38 chromosomes (2n=20M + 18Td) hand pollinated using diploid maize individuals initiated for 36 ± 9% of the flowers (mean ± SD; >2000 flowers from 3 different cycles, 10 plants per cycle, 2 inflorescences per plant). Mature kernels were highly variable with regard to endosperm formation (Figure 2A), however with a strong trend toward defective development (65 to 80%). By contrast, 84 ± 9% kernels (mean ± SD, N=744) contained a

TABLE 1

LIST OF THE *TRIPSACUM* AND MAIZE STOCKS USED AND OBTAINED FROM CIMMYT GERMPLASM BANK

T. dactyloides ecotypes	Improved maize populations
11-36	BS13 (S) C8
61-664	BSSS (R) C13
65-1234	BS10 (FR) C11
112-1327	BS26 (S) C13
112-1328	BSCB1 (R) C13
Maize lines (CMLs)	BS11 (FR) C11
CML62 [2]	POB.21C6 x BS10 (FR) C11
CML78	POB.43C10 x BS10 (FR) C11
CML135 [1,2]	POB.24C9 x BS10 (FR) C11
CML139 [1]	POB.27C10 x BS26 (S) C3
CML204	POB.25C4 x BSCB1(R) C13
CML216	POB.27C10 x BSCB1(R) C13
CML258	POB.21C6
CML341	POB.43C10
CML346	POB.24C9
CML408,	POB.25C4
CML413	POB.32C6
CML416	POB.27C10

(1) and (2): lines used to derive H1and H3 maize hybrids, respectively.
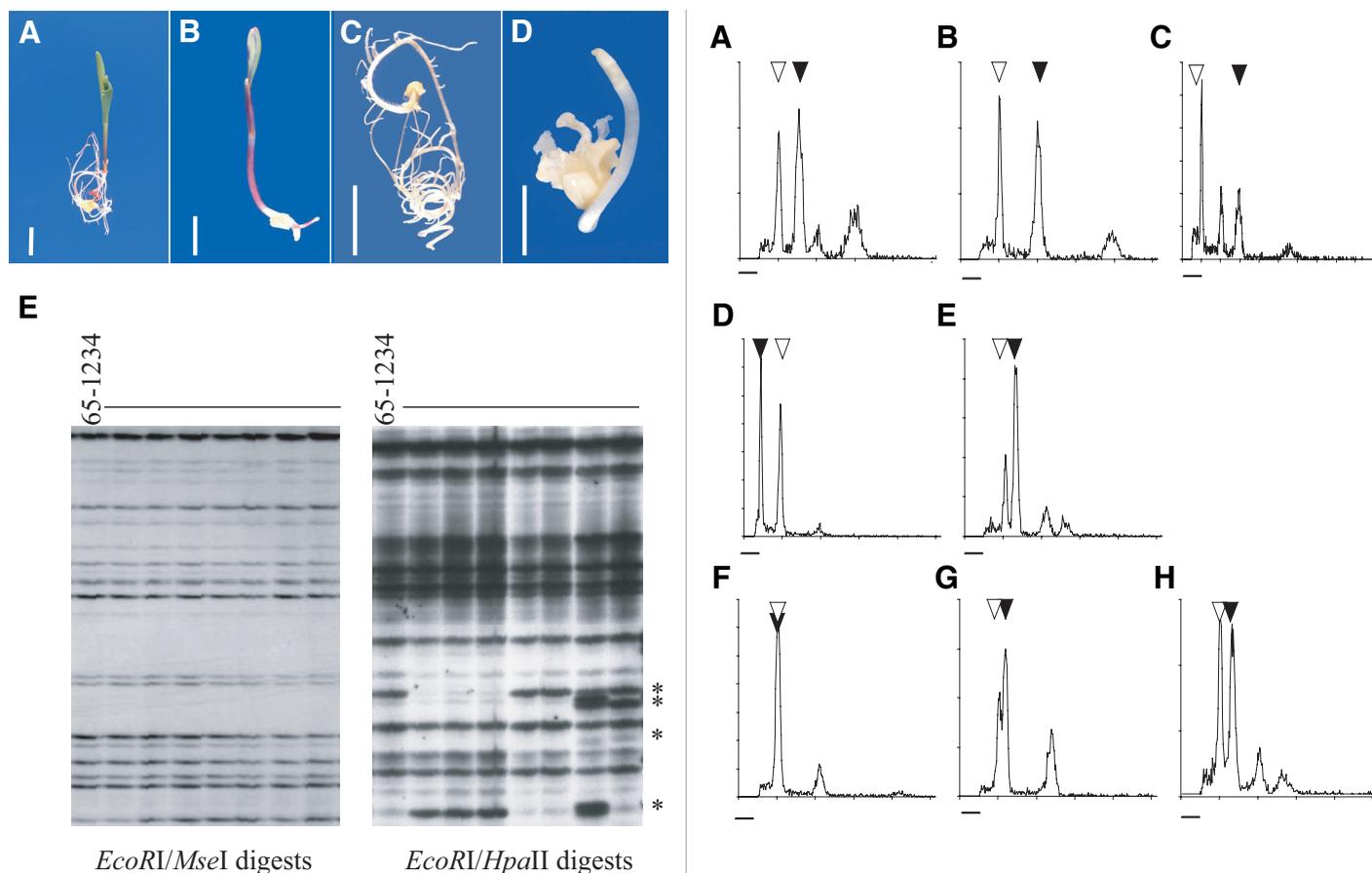
Fig. 3 (Left). Maternal embryo defects in maize-*Tripsacum* and *Tripsacum* apomicts. (A-D) *Germination of maternal seedlings from seeds from maize-Tripsacum apomict 38C showing normal* **(A)** *and aberrant* **(B-D)** *developments. Bars, 1 cm.* **(E)** *Genomic cloning (*EcoRI*/*MseI *digests) and detection of methylation-associated polymorphisms (*EcoRI*/*HpaII *digests) through AFLP fingerprinting in maternal progenies of 65-1234, a tetraploid, apomictic* T. dactyloides *ecotype. Asterisks indicate differentially methylated restriction sites.*

Fig. 4 (Right). Progeny sorting of apomictic clone 38C after DNA content analyses in young seedlings. (A) *Maternal clone with 2n=38.* **(B,C)** *Off type progeny with higher DNA contents than that of maternal clones: 2n=48* **(B)** *and 2n=76* **(C)**. **(D-H)** *Off type progeny with lower DNA contents than that of maternal clones: 2n=10* **(D)**, *2n=28* **(E)**, *2n=20* **(F)**, *2n=22* **(G)**, *2n=27* **(H)**. *Open and dark triangles indicate 2C peaks produced by nuclei isolated from CML62, the diploid maize reference, and from the sample, respectively. Chromosome numbers were determined by root tip counts. Bar, 50-channel intensity.*

critical in our materials than those reported for maize in which <1% of the 4x X 2x kernels produce an endosperm (Cooper, 1951, Leblanc *et al.*, 2002). Developmental adaptations that ultimately restore the endosperm balance requirement are frequent in apomicts (Grimanelli *et al.*, 1997, Grossniklaus *et al.*, 2001, Nogler, 1984). Among these, the union of polar nuclei with two sperm nuclei could have contributed to dosage effect alleviation in 38C X 2x endosperms. To further investigate the importance of such fertilization events in seed development of apomicts, we directly estimated DNA contents in growing endosperms from normally developed kernels obtained 15 days after pollination with diploid maize (H3). Flow cytometry analyses indicated that 1x paternal contribution (fertilization by a single sperm cell) was predominant (80%, n=86). Therefore, fertilization of the central cell by two sperm cells unlikely served as a critical factor to mitigate dosage effects in endosperms of 2n=38 apomicts.

Defects were also common during embryo germination. Despite the use of an *in vitro* procedure, germination of kernels with developed endosperm usually expanded over several weeks and produced less than 60% viable seedlings (Table 2). Low seed fertility resulted from lack of germination but also from developmental alterations, including early developmental arrest, undifferentiated growth, and differentiation failure of shoot or roots (Figures 3A to 3D). Polyembryonic kernels were frequent (Table 2), a trend that is usually associated to apomictic developments (Hanna and Bashaw, 1987) and for which we have provided recently a detailed characterization in *Tripsacum* and maize-*Tripsacum* hybrids (Grimanelli *et al.*, 2003). Finally, although development initiated to a lesser extent than in embryos accompanied by an endosperm, embryos dissected from defective kernels and cultured on a hormone-free medium exhibited similar defective developmental behaviors (data not shown).

In cloning of both animals and plants, analogous developmental defects have been correlated to inaccurate DNA methylation patterns for key developmental gene (Kaeppler *et al.*, 2000, Reik and Dean, 2001, Rideout *et al.*, 2001). To investigate whether this

phenomenon perturbs embryo development in apomicts, we studied progeny from five apomictic *Tripsacum dactyloides* individuals. First, similarly to that observed in maize-*Tripsacum* hybrids, germination rates of normal seeds were low (33 to 70%) and a significant proportion (4 to 55%) of the germinating seeds were affected in development (Table 3). Altogether, viable adult plants were recovered for only 25% of the seeds. Late-occurring defects were also observed in adult plants, including sterility and delayed flowering time (respectively, 8% and 4%, n=70). We further performed a genome-wide characterization of DNA methylation for each progeny. As control experiments, we used two populations representing non-apomictically derived, but genetic replica, of the mother plant; the first one (n=10) consisted of cuttings of a single apomictic genotype, 65-1234 while the second one (n=12) was generated by selfing CML216, a maize homozygous line. We first verified that the three procedures we used reproduced faithfully mother plant genotypes by generating AFLP fingerprints from DNAs cleaved using *EcoR*I and–*Mse*I, two methyl-insensitive restriction enzymes. All loci scored (n > 250) were strictly monomorphic in all cases studied (mother plant vs. progeny/replica, amongst progeny; Figure 3E). Then, we assessed methylation status of CCGG sites using a modified AFLP procedure using *EcoR*I/*Msp*I or *EcoR*I/*Hpa*II digests as preamplification DNA templates (Cervera *et al.*, 2002). For both *EcoR*I/*Hpa*II and *EcoR*I/*Msp*I digests, we observed identical fingerprints among replica used for control experiments, indicating conservation of genome methylation patterns (data not shown). By contrast, patterns of digestion among clonal individuals derived apomictically showed methylation-sensitive polymorphisms: firstly, pairwise comparisons of methylation patterns revealed that a significant fraction of the fragments detected using *EcoR*1/
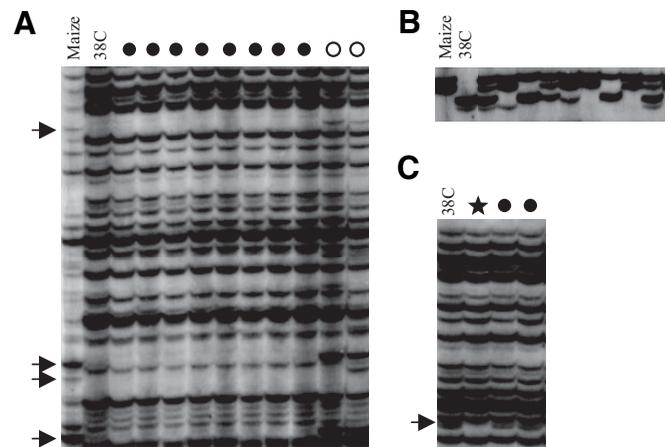
Fig. 5. AFLP genotyping of progeny from maize-*Tripsacum* apomictic clone 38C. (A) *Derivatives through unreduced female gametes. Dark and open circles respectively indicate maternal progeny or off type progeny after fertilization (genomic accumulation, open circles). Arrows show maize fragments specific to the male progenitor.* **(B)** *Sexual derivatives.* **(C)** *Sporadic chromosome loss in one individual derived from unreduced, parthenogenetical female gametes (star). The arrow indicates one missing fragment.*

*Hpa*II digests (5.8%; n > 250) were polymorphic (Figure 3E) whilst they appeared monomorphic using *EcoR*1/*Msp*1 digests. Furthermore, we also detected polymorphisms for 2.4% of the fragments amplified from *EcoR*1/*Msp*1 digests. We thus conclude that apomictic clones differed for methylation patterns within CCGG regions despite genomic identity. These results suggest that inaccurate erasing, maintenance or establishment of DNA methylation might occur during apomictic reproduction and that, as observed in animals (Dean *et al.*, 2001), this could explain the defects noted in maternal embryos.

Reproduction in 38-chromosome apomictic clones can occur through a variety of mechanisms

Variation in the reproductive behavior within a single individual is common in both natural and artificial apomicts (Koltunow *et al.*, 2000, Leblanc, 1995, Nogler, 1984, Noyes, 2005, Richards, 1996, Van Dijk *et al.*, 1999). In order to assess the nature and the extent of variation in reproductive behavior in 38C, an apomictic clone, we collected flow cytometrical, cytogenetical and molecular data within progenies obtained after pollination using 2x maize progenitors. Although the majority of viable seedlings consisted of maternal individuals (similar DNA content and fingerprinting patterns, Figures 4A and 5A respectively), progeny also contained a mixture of non-maternal genotypes (9%, Table 2; Figures 4 and 5). The prevailing triggering mechanism consisted of unreduced egg cell fertilization as shown by DNA content and fingerprinting analyses, both indicative of the addition of one maize haploid genome to that of 38C (Figures 4B and 5A). The remaining non-maternal progeny were infrequent (<0.2% overall, Table 2) and split into two categories regarding DNA content: (1) values twice that of the mother clone (Figure 4C) resulting from parthenogenesis in egg cells derived from unreduced megaspores that underwent spontaneous chromosome doubling (n=218; 2n=76, data not shown), and (2) a wide range of values significantly lower than that of the mother clone (n=249; Figures 4D to 4H).

TABLE 2

PROGENY CLASSES FROM 38C APOMICTS POLLINATED USING DIPLOID MAIZE AS DETERMINED BY DNA CONTENT ANALYSES

| Male	Nt	Ge (%)	Me (%)	Npr	Mp (%)	Inc (%)	Dec (%)
H1[(1)]	7,393	54	17.3	4,691	93.7	6.1	0.2
H3[(2)]	10,206	49.5	13.0	5,740	88.2	11.5	0.3
All	-	-	-	>100,000	91.0	8.8	0.2

Nt: total number of kernels; Ge: kernels that produced viable seedlings; Me: multiple embryos; Npr: number of viable progeny recovered; Mp: maternal progeny (same DNA content as mother plants); Inc: off type progeny showing increase in DNA content; Dec: off type progeny showing decrease in DNA content. (1) 2001 winter cycle; (2) 2002 summer cycle

TABLE 3

DEVELOPMENTAL DEFECTS IN APOMICTIC *TRIPSACUM DACTYLOIDES* PROGENIES

Accession number	Germinating seeds (%)	Early arrest (%)[a]	Undifferentiated growth (%)[a]	Viable adult plant (%)
65-1234 (n=145)	35	33	22	16
11-36 (n=34)	68	0	4	65
61-664 (n=22)	73	19	31	36
122-1327 (n=42)	33	7	14	26
112-1338 (n=38)	32	17	33	16
Total (n=281)	41	20	20	25

[a] values shown were determined for germinated seeds
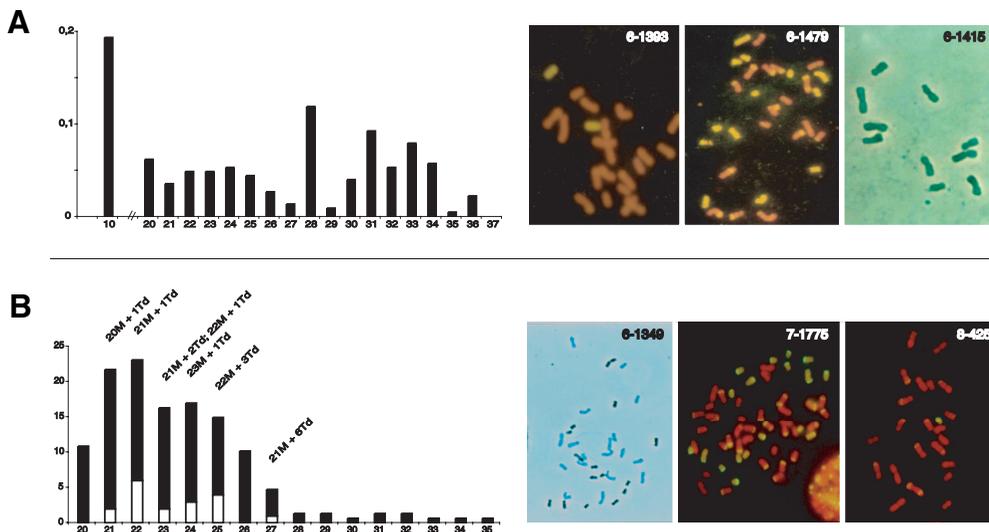
Fig. 6. Cytogenetical characterization of off type progeny obtained from apomictic 38C clones (A) and from highly facultative 2n=43 hybrids (B). (A) *Chromosome number frequencies (n=228) and chromosome painting in three archetypal derivatives with regard to their origin: 6-1393, sexually derived with 2n=20M+2Td; 6-1479, asexually derived, but chromosomally imbalanced, with 2n=31=17M+14Td, and; 6-1415, a maize haploid.* **(B)** *Chromosome number distribution (n=127) and chromosome painting in 6-1349 (2n=33=18M+15Td); in 7-1775, one derivative of 6-1349 through genomic accumulation (2n=43=28M+15Td), and; in 8-425, a sexual offspring of 7-1775 (2n=23=22M+1Td). Sexual individuals that carried the DNA segment associated with diplospory distributed according to white bars and chromosome complements are indicated on top of corresponding bars. Chromosome painting was obtained using digoxygenin labeled* Tripsacum *DNA as a probe detected with either FITC or horseradish peoxidase as anti-digoxygenin conjugate (* Tripsacum *chromosomes are yellow and dark blue respectively).*

Figure 6A displays chromosome numbers in 228 out of the 249 individuals of the latter class. Further cytogenetical and molecular characterization provided more comprehensive views for their nature and origin. A first set (n=83) consisted of individuals derived sexually as molecular analyses revealed both segregating maternal markers and paternally inherited markers (Figure 5B). In addition, all plants determined for chromosomal complements (n=62) combined a diploid maize genome with a few extra *Tripsacum* chromosomes (i.e., 6-1393, Figure 6A). The remaining individuals were categorized as follows: hybrids carrying aneuploid chromosome sets of maize, *Tripsacum* or both (n=80; 2n=29 to 2n=36, i.e., 6-1479; Figure 6A) with fingerprint patterns indicating no paternal DNA (absence of karyogamy) and sporadic loss of maternal markers (Figure 5C); maize haploids (n=40; 2n=10, i.e., 6-1415; Figure 6A) of maternal and paternal origin (data not shown), and; allodihaploid hybrids with 2n=10M+18Td (n=25). All allodihaploids genotyped (n=9) lacked paternal fragments indicating that they arose parthenogenetically from allodihaploid egg cells. However, we could not resolve unambiguously whether these eggs derived meiotically or ameiotically because of the lack of heterozygous maize markers detectable in 38C and of the small number of plants analyzed.

Finally, to test whether the viable seedlings we analyzed consisted in a biased sample for inferring reproductive behavior of 38C clones, we also estimated DNA contents in embryos dissected from defective kernels and in embryos that exhibited growth defects (≥ 250 for each type; data not shown). In both cases, they arranged in a similar manner to that of viable seedlings.

Here, as in many other apomictic species, 38C clones reproduced through facultative apomixis. Although genomic accumulation was predominant, genetic variation within progeny arose through a number of mechanisms. While incomplete nucleus restitution at meiosis I is a known side effect of diplosporous pathways (Nogler, 1984, Noyes, 2005, Richards, 1996) likely promoted by meiotic asynchrony between the maize and *Tripsacum* chromosomes in our materials (Grimanelli *et al.*, 2003, Harlan *et al.*, 1970), parthenogenesis in reduced gametes and spontaneous chromosome doubling did not occur at greater incidences than that observed in many sexual plant species (de Wet, 1979, Kimber and Riley, 1963) or in interspecific hybrids (Nasrallah *et al.*, 2000, Soltis and Soltis, 1999).

Transgenerational propagation of apomixis

Understandably, the genetics of apomixis has been investigated mostly after transmission through reduced male gametes. However, recent progress toward the molecular deciphering of plant reproduction has revealed pivotal roles for female gametophytic factors during early seed development. Therefore, conceivably, maternal and paternal transmission might impact differently critical regulatory factors governing the apomictic phenotype. With regards to this, the collection of non-maternal off-types we produced from clone 38C allowed us to examine maternal transmission of apomixis for a variety of modalities. In particular, we determined reproductive behaviors in non maternal derivatives with lower DNA content values than that of 38C based on progeny tests and Southern hybridizations using two diagnosis probes for diplospory, umc28 and cdo202 (Leblanc *et al.*, 1995a). Data were in agreement for most hybrids analyzed (n=106), except for three sexually derived individuals carrying the chromosome harboring the diplospory locus (Table 4). Considering the small number of incongruent individuals, we further wanted to
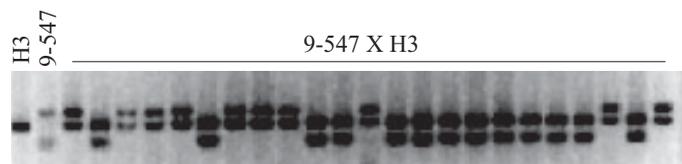
Fig. 7. Sexual behavior in 9-547, a maize-*Tripsacum* hybrid with 2n=22=19M+3Td and carrying the chromosome associated with apomixis. *Progeny were derived after crossing with a maize hybrid (H3) and amplicons were obatined using a SSR primer combination (bnlg339 locus).*

TABLE 4

REPRODUCTIVE MODE IN FERTILE, NON-MATERNAL DERIVATIVES OF 38C AS DIAGNOSED BY RFLP ANALYSES AND PROGENY TESTING USING AT LEAST 50 INDIVIDUALS

Off type nature	N	RFLP	Pr. test
Allodihaploids	9	+	A
Maize haploids	25	-	S
Asexually-derived aneuploids	39	+	A
Asexually-derived aneuploids	2	-	S
Sexually-derived addition lines	28	-	S
Sexually-derived addition lines	3	+	S

A: Apomictic. S: Sexual.

validate these observations using a larger population. To achieve this, the unusual reproductive behavior of derivatives of 6-1349, which reproduced through highly facultative apomixis (see next section for a detailed analysis), allowed the recovery of 166 individuals through sexuality. Southern hybridizations using umc28 identified 29 individuals that carried the DNA segment linked to diplospory, out of which 18 set seeds after crosses with diploid maize (see Figure 6B and next section for cytogenetical characterization). Lack of progeny in the remaining inviduals resulted from early developmental arrest, flowering failure, or seed defects. With regards to this, we observed a similar fertile-to-sterile ratio in the 137 inviduals diagnosed as sexuals (18:29 and 58:79, respectively), thus discarding strong deleterious effects induced by the apomixis-associated chromosome. Flow cytometrical and molecular data collected from a total of 1190 progenies indicated that all 18 fertile individuals selected as putative apomicts reproduced sexually (Figure 7). Although we cannot discard potential for functional apomixis in the sterile individuals, these results show that the segment defined by umc28 and cdo202 is not sufficient to confer apomixis by it-self. This suggests that apomixis might depend on critical *Tripsacum* genetic factors, unidentified in previous studies, and segregated away during meiosis. Alternatively, loss of apomixis in these materials might denote trans-acting chromosomal dosage effects modulating gene expression within the umc28/cdo202 segment (i.e., aneuploid syndrome).

Assessment of the impact of genomic and chromosomal dosage variation on apomixis

In natural populations, apomixis and polyploidy are usually associated. However, artificial ploidy variation in apomicts and related sexuals has produced ambiguous data, i.e., isolation of diploid or dihaploid apomicts (Bicknell, 1997, Dujardin and Hanna, 1986, Leblanc *et al.*, 1996, Sharbel and Mitchell-Olds, 2001) vs. apomixis induction after ploidy rise in sexual *Paspalum notatum* (Quarin *et al.*, 2001). Thus, whether polyploidy has a role in reshaping the sexual pathway towards apomixis remains to be elucidated. In higher organisms, many regulatory complexes for gene expression are dosage-dependent, mainly because of stoechiometric requirements among components (Birchler *et al.*, 2001). As a result, changes in genome structure (aneuploidy, ploidy variation) often impact gene expression levels and, ultimately, phenotypes. Genomic accumulation and chromosome losses allowed the recovery of maize-*Tripsacum* lineages convenient for addressing the influence of dosage variation in tuning the balance between sexual and apomictic reproduction. First, we addressed the effects of aneuploidy through a chromosome deletion mapping approach by taking advantage of the chromosomally imbalanced apomicts previously determined for the mode of reproduction (Table 4). They were genotyped using a set of seventy-seven AFLP and twenty-seven SSR markers specifically amplified from the *Tripsacum* complement of 38C genome (Figure 8A). As shown in Figure 8B, most markers (92%) failed to amplify the expected fragment in at least one individual. Despite small scale discrepancies in grass genome synteny (Devos, 2005, Goff *et al.*, 2002), we assume that the marker set we used likely covered the entire *Tripsacum* genome. Therefore, chromosomal variation, except for that harboring the umc28/cdo202 region, had no significant effect on diplospory; although their developmental fate could vary across genotypes, most female gametes were unreduced. Secondly, we examined the impact of ploidy variation throughout genomic accumulation series derived from clones of 38C and chromosomally imbalanced derivatives that showed contrasted *Tripsacum* genome landscapes (Table 5). In order to limit the impact of allelic variation introduced through the male, plants were pollinated with H3, the male progenitor used in 38C pedigree. All progenies, but that obtained from hybrid 6-1349 (2n=33=18M+15Td, Figure 6B), showed a propensity for unreduced female gamete formation similar to that of their respective mother plant (Table 5). Therefore, although variations for parthenogenesis vs. fertilization in unreduced egg cells could be observed in some instances (a phenomenon noticed in most hybrid generations we generated), apomictic reproduction was prevalent in these materials. In derivatives of 6-1349 with 2n=43=28M+15Td (Figure 6B), DNA content estimates

TABLE 5

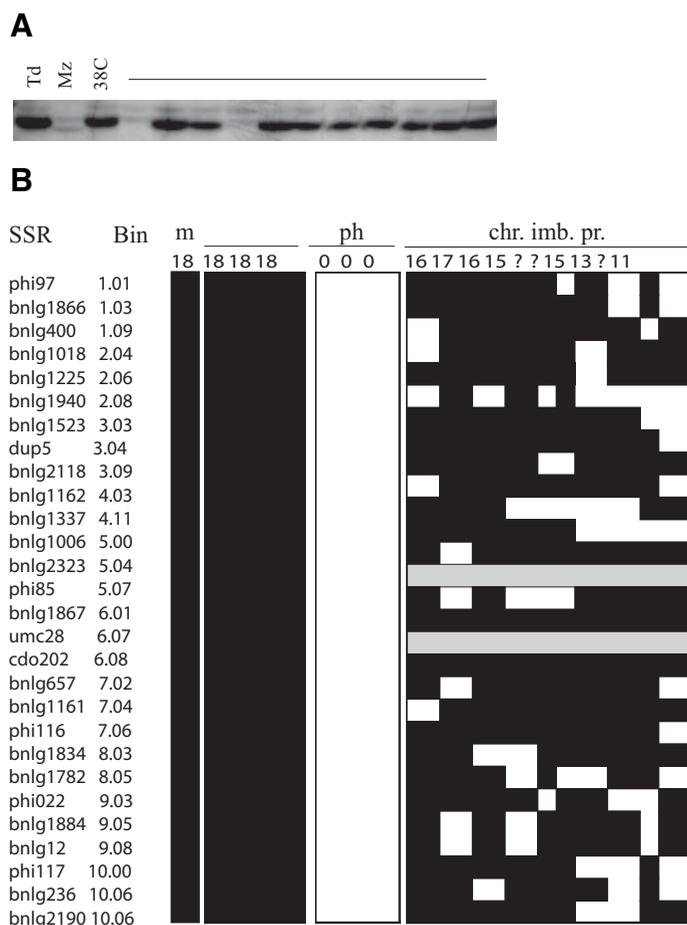**DNA-CONTENT-BASED PROGENY TESTS IN APOMICTIC CLONES WITH DIFFERENT CHROMOSOMAL COMPLEMENTS**

Clone	A – Apomictic clones				B – 2n+n derivatives		
	2n	N	Npr	UR	N	N_{pr}	UR
38C	38	nd	-	0.99	27	726	0.93*
6-1313	36	4	310	1.00	5	41	0.96*
6-1349	33	>50	5150	0.99	66	630	0.18
6-1394	34	12	1660	1.00	19	137	0.97*
6-1439	31	3	51	0.95	1	7	1.00[nd]
6-1544	35	9	1096	1.00	45	493	0.95*
6-1580	36	10	1112	1.00	25	278	0.97*
6-1583	36	15	1100	1.00	18	199	0.95*

* indicates no significant difference to that observed in mother clone (α = 0.05). nd: not determined

revealed that only a small fraction originated from unreduced megagametophytes through either apomixis or genomic accumulation (18%, Table 5B). The remainders arose sexually; they displayed a wide range of chromosome numbers and cytogenetical analyses indicated aneuploid maize chromosome complements (Figure 6B). Interestingly, 26 out of the 66 individuals with 43 chromosomes produced 100% sexual progeny. However, progeny size was generally low and it remained unclear whether reversion to sexuality was complete or partial. We further investigated possible male effects by pollinating clones of the seven hybrids originally selected (Table 5) using a set of 8 diverse maize CIMMYT lines (CML78, CML204, CML258, CML341, CML346, CML408, CML413 and CML416). Derivatives through genomic accumulation, including those derived from 6-1349, showed similar behavior to that of their original counterparts produced using H3 (data not shown), thus discarding that the phenomenon

A

B

Fig. 8. *Tripsacum* genome landscapes in chromosomally imbalanced, asexual derivatives of maize-*Tripsacum* apomictic hybrid 38C. (A) *Segregating pattern of one AFLP fragment specific to 65-1234, the* Tripsacum dactyloides *donnor. Mz: maize male progenitor. Td: 65-1234. 38C: 2n=38 maize-*Tripsacum *mother plant.* **(B)** Tripsacum *genomic contributions as determined by maize SSR markers detecting a fragment specific of the* Tripsacum *genome in 38C clones. Each column shows one individual, which* Tripsacum *chromosome number is indicated on top. m: maternal clone. chr. imb. pr.: chromosomally imbalanced progeny. mdh: maternal dihaploid (2n=28). ph: paternal haploid (2n=10). Gray areas: not determined.*

resulted from specific allelic interactions.

These findings indicate that neither chromosome depletion (except for that causing the removal of the segment homologous to maize 6L) nor maize genome addition can promote reversion towards sexuality, when endured by maize-*Tripsacum* chromosome complements proper for functional apomixis. Collectively, they suggest that apomixis responds little to genome plasticity, a behavior also documented recently in alloploid apomicts of the *Boechera holboellii* complex (Kantama *et al.*, 2007). However, although the general mechanisms governing apomictic reproduction in maize-*Tripsacum* apomicts unlikely operate in a dosage dependent manner, the recurring reproductive shift between 6-1349 and its derivatives after genomic accumulation suggests that dosage dependency occurs to some extent and may account, at least partially, for variations in facultativeness commonly observed in apomictic species.

Discussion

Although the proposition for an epigenetic role have been put forward for the emergence of apomixis from the sexual pathway (regulation through epialleles, relaxation of genomic imprinting), its relevance remains largely unexplored (Grossniklaus *et al.*, 2001, Koltunow and Grossniklaus, 2003, Ranganath, 2004). As discussed below, our observations lend support to epigenetics as a critical component in the biology of apomicts along two different lines: seed development and transgenerational propagation of apomixis. However, apomixis was expressed in our materials with no dependency for polyploidy nor for chromosomal variation, two mechanisms usually epigenetically regulated (Chen, 2007). This suggests that epigenetic effects associated with polyploidy unlikely participate in re-directing the sexual development towards apomixis, supporting the proposition that the usual association of polyploidy and apomixis can be regarded as an evolutionary adaptation, i.e. for balancing natural selection against apomictic genomes that accumulate deleterious mutations generation-wise (Mogie, 1992) and/or for preventing deleterious effects induced by apomictic DNA segments when transmitted through haploid gametes (Nogler, 1984).

Two epigenetic mechanisms controlling sexual plant reproduction act as barriers for apomictic seed development

Development of both the embryo and the endosperm was affected in 38-chromosome apomictic clones. Deleterious dosage effects in endosperm development likely resulted from differential parental imprinting, a well described, but molecularly undetermined as yet, epigenetic phenomenon in seed plants. On the other hand, the epigenetic nature of embryo defects is less clear. The developmental defects we observed may denote genome wide effects resulting from the genetic makeup of this material as reported in newly formed allopolyploids (reviewed in Chen, 2007, Rapp and Wendel, 2005). In such hybrids, certain gene expression patterns vary suddenly and often reflect nonequivalent parental contributions to the transcriptome. Heritable changes, some of them of epigenetic nature (DNA methylation, chromatin structure), have been documented to explain the novel transcriptome observed in hybrids as well as its potential to persist over time (Adams *et al.*, 2003, Kellogg, 2003). Since the genome of the maize-*Tripsacum* generations we described here likely has

been subjected to allopolyploidization-induced effects, this material seems by nature unsuitable to investigate the impact of apomictic reproduction on DNA methylation patterns. On the other hand, although the polyploid origin of apomictic genomes in *Tripsacum* still needs clarification (Grimanelli *et al.*, 1998) and in the light of recent data in resynthesized *Brassica napus* lines (Gaeta *et al.*, 2007), we assume that possible epigenetic effects of polyploidization have been long stabilized. We show that *Tripsacum* apomictic progeny suffer from developmental defects and that methylation patterns within a single genomic clone (same or different generations) vary significantly. Aberrant genome methylation patterns have been shown to induce large pleiotropic effects on development in plants, including after cloning procedures (Cao and Jacobsen, 2002, Finnegan *et al.*, 1996). All together, this suggests that apomictic progeny in *Tripsacum* carry an epigenetic load that arises from improper genome reprogramming of methylation states during apomixis. Therefore, in the light of the impact of the phenomenon in *Tripsacum* apomicts (Table 3), we believe that it explained most embryo developmental defects we recorded in maize-*Tripsacum* progeny.

Furthermore, the difference in viability between sexually and asexually derived offspring in *Tripsacum* likely results from the maintenance of critical epigenetic mechanisms acting during sexual plant reproduction. Whether the phenomenon is exclusive to our materials or expands to other apomictic systems remains to be investigated. Notably, short-circuiting of sexual programs in some apomicts allows to override epigenetic constraints for endosperm development (Grimanelli *et al.*, 1997, Grossniklaus *et al.*, 2001, Nogler, 1984), but data in aposporous *Hieracium* also support a role for epigenetics in apomictic embryo developmental defects (Koltunow *et al.*, 2000). Clearly, further research will be required to determine the nature of the mechanisms responsible for both endosperm and embryo alterations in apomictic seeds.

Restoration of sexuality upon transmission through female sexual gametes

Data obtained from asexual, chromosomally imbalanced, derivatives of 2n=38 apomictic hybrids strongly suggest that expression of apomixis in maize-*Tripsacum* hybrids relies upon one single *Tripsacum* chromosomal region: its removal caused reversion to sexuality and expression of the trait was independent of its chromosomal complement recipient as no response after genome or chromosomal dosage variation was observed. In view of these results, sexual reproduction in hybrids that derived sexually from apomictic mother plants and that carried the chromosomal segment associated with diplospory unlikely resulted from a missing chromosomal region critical for apomixis to function, but segregated away during meiosis. Likewise, chromosomal rearrangements were also discarded as we found only one rearranged chromosome among all the complements we determined cytogenetically (n=37; data not shown). Apart from its chromosomal environment (that appeared neutral with regards to completion of the apomictic phenotype), another distinctive feature for the chromosome harboring the diplospory locus between sexually derived hybrids and chromosomally imbalanced hybrids stands in its transgenerational propagation, i.e. reduced, fertilized vs. unreduced, parthenogenetical female gametes, respectively. With regard to unreduced gametes, fertilization had little impact on the

reproductive mode in the resulting derivatives as shown from data collected in genomic accumulation series. Finally, since our materials were fully male sterile, we could not perform reciprocal (sexual X apomict) crosses to examine transmission through male gametes. However, it is worth mentioning that transmission through male *Tripsacum* gametes did not result in segregation distortion against apomixis (Leblanc *et al.*, 1995a). Therefore, our data strongly suggest non-equivalency of the DNA region controlling apomixis when transmitted through reduced female gametes on one side and through unreduced female gametes and reduced male gametes on the other side, i. e. non functional vs. functional, respectively. These findings support the proposition for an epigenetic mechanism by which sexuality is restored when apomixis regulatory components are inherited through female reduced gametes. Such female germ line specific epigenetic repression has been reported in *Drosophila* as responsible for hybrid dysgenesis (Blumenstiel and Hartl, 2005) and telomeric *Trans*-Silencing Effect or TSE (Josse *et al.*, 2007). Both phenomena result from maternal repression of transposable elements (TE) through a RNA silencing pathway while the paternal germ line transmits active TEs. In addition, TSE repressing mechanism is induced for TEs, or trans-genes, inserted into subtelomeric heterochromatin and shows variegation when silencing is not complete (Josse *et al.*, 2007). Whether maternal repression of apomixis mechanistically relies on similar bases is unknown, but note that, in most cases investigated including *Tripsacum*, "apomixis DNA segments" consist of non-recombining chromosomal blocks. Finally, reports in *Pennisetum* indicate similar strong segregation distortion for the apospory-specific genomic region (AGSR) when inherited through sexual female gametes (Roche *et al.*, 2001). Interestingly, the two DNA segments associated with apomixis, and cytogenetically best characterized to date, contain rearrangements of high repetitive sequences derived from transposable elements (Akiyama *et al.*, 2004, Calderini *et al.*, 2006). It is thus conceivable that the phenomenon has evolved in species reproducing through different apomictic pathways. A role for epigenetic repression of apomixis may be the maintenance of sexual reproduction within agamic complexes, a critical evolutionary force for population dynamics and adaptation.

Efforts towards the characterization of apomictic developments by molecular and cellular means have proven by far more complicated than thought in the 80-90s. Particularly, complexity comes from the diversity of phenotypes but also from the divergence between genetic analyses mostly pointing out a single dominant factor and either molecular mapping in apomicts or reproductive mutants analyses in model plants. In this report, we have provided evidence that apomictic seed likely suffer from an epigenetic load and that epigenetics influences, at least partially, inheritance of apomixis in maize-*Tripsacum* hybrids. The limited knowledge for the molecular basis underlying apomictic phenotypes strongly hampers the assessment of the role of epigenetics in the trait. Nevertheless, although a small number of key events of the sexual pathway is believed to be sufficient for inducing apomixis from the sexual pathway (Bicknell and Koltunow, 2004, Grimanelli *et al.*, 2001), the nature of the mechanisms required to sustain this switch might entail, and trigger, profound changes in the reproductive

biology of sexual plants, therefore strongly limiting the efficiency of the conversion of sexual crop into apomicts.

Materials and Methods

Plant material

All *Tripsacum* and maize materials were obtained from CIMMYT Plant Genetic Resources Center (Table 1). The five apomictic *Tripsacum dactyloides* accessions used in this report (65-1234, 11-36, 61-664, 112-1327, 112-1328) have been previously characterized for the mode of reproduction and classified as diplosporous apomicts of the Antennaria type (Leblanc, 1995, Leblanc *et al.*, 1995b).

The hybridization scheme we followed for the production of sexual and apomictic 38-chromosome maize-*Tripsacum* hybrids (2n=20M+18Td, M and Td indicate a maize and a *Tripsacum* origin, respectively) is detailed elsewhere comprehensively (Leblanc *et al.*, 1996, Savidan, 2000) and summarized in Figure 1. The *T. dactyloides* donor used in the initial cross was 65-1234, a tetraploid apomict with 2n=72. Note also that all maize-*Tripsacum* hybrids used in this work derived from individuals of a single apomictic allodihaploid clone (2n=28=10M+18Td) and that 28- and 38-chromosomes individuals are fully male sterile and were used as pistillate progenitors exclusively.

Occasional fertilization of unreduced female gametes in all dihaploid clones by a maize sperm cell (15%; Leblanc *et al.*, 1996) yielded 38-chromosome hybrids. Male progenitors were as follows: H1 and H3, two maize F1 hybrids respectively derived from CML135 and CML139, and from CML135 and CML62, and individuals from 18 CIMMYT improved maize populations listed in Table 1. Subsequent backcrossing generations were derived using H1, H3 and CIMMYT Maize lines (CMLs, Table 1).

In most cases, *Tripsacum* and maize-*Tripsacum* progeny were grown from endosperm-filled kernels, but embryos dissected out of defective kernels were also rescued. Kernels were germinated in a dark growth chamber at 27°C on agar-solidified N6 medium (Chu *et al.*, 1975) while dissected embryos were grown under similar conditions on N6 medium supplemented with 4% sucrose. Once selected, seedlings were acclimatized into Jiffy pots prior to transplanting into 20 inches pots.

Determination of reproductive behavior in maize-*Tripsacum* hybrids

Female-fertile hybrids were systematically progeny-tested. Evaluation of genetic hetero / homogeneity within progeny was primarily assessed by comparing DNA content estimates (collected through flow cytometry, see section below) in mother plants to that of offspring. In the backcrossing scheme we followed, similar DNA contents to that of the mother plant denoted apomixis whereas a significant difference indicated its failure. Typically, individuals resulting from the fertilization of an unreduced gamete showed an increase in DNA content while sexuality or haploidization caused a reduction (Figure 1). Further characterization was carried out for selected individuals by gathering cytogenetic and molecular data (see corresponding sections for experimental procedures). Molecular analyses consisted in detecting the chromosomal segment responsible for diplospory through RFLP analyses using umc28 and cdo202 clones (Leblanc *et al.*, 1995a), and in genotyping progeny using maize Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (Vos *et al.*, 1995).

Flow cytometric analyses

DNA content values were estimated based on flow cytometric analyses (FCM) performed using a Partec CA-II device (PARTEC, Munster, Germany) and following a procedure adapted from Galbraith *et al.* (1983). Nuclei preparations consisted of filtrates (90μm mesh) of small pieces of tissues (e.g. leaf from young seedlings, dissected embryos, dissected endosperms) chopped using a razor blade into Galbraith's buffer containing 1mg/l of 33342 Hoechst DNA dye (Sigma Aldricht, France).

Typically, fluorescence signals were collected from at least 2000 intact excited nuclei and they were plotted into intensity (x axis, 512 channels) X nuclei counts (y axis) histograms. The flow cytometer was calibrated regularly by adjusting on channel 50 the fluorescence intensity of 2C peaks produced by leaf nuclei extracted from a diploid maize inbreed (CML62, CIMMYT line). Finally, the ratio of the mean intensity of the 2C peak (CV<5%) produced by the mother plant to that of each individual within progeny were used to assay the clonal nature of offspring, i.e. maternal clones produce a ratio of 1 while for off type individuals it deviates from 1.

Chromosomal analyses

Chromosome numbers were obtained from root tip preparations collected from young seedlings following Jewel and Islam-Faridi (1994). Chromosomal complements were determined using genomic *in-situ* hybridization as described by Islam-Faridi and Mujeeb-Kazi (1995).

Molecular procedures

Total genomic DNA extraction, Southern hybridizations and SSR detection were performed following standard procedures for maize.

Twenty-seven maize SSRs out of 235 were selected based on DNA amplification of *T. dactyloides* 65-1234, of maize (H1, H3), and of several individuals of a 2n=38 clone (data not shown) using the following criteria: detection of loci spread out in the maize genome and production of amplicons specific to the *T. dactyloides* genome in 2n=38 maize-*Tripsacum* hybrids. Loci (name and bin) in the final selection were as follows: phi97 (1.01); bnlg1866 (1.03); bnlg400 (1.09); bnlg1018 (2.04); bnlg1225 (2.06); bnlg1940 (2.08); bnlg1523 (3.03); dup5 (3.04); bnlg2118 (3.09); bnlg1162 (4.03); bnlg1337 (4.11); bnlg1006 (5.00); bnlg2323 (5.04); phi85 (5.07); bnlg1867 (6.01); bngl657 (7.02); bnlg1161 (7.04); phi116 (7.06); bnlg1834 (8.03); bnlg1863 (8.04); bnlg1782 (8.05); phi022 (9.03); bnlg1884 (9.05); bnlgl128 (9.08); phi117 (10.00); bnlg236 (10.06); bnlg2190 (10.06) (for details see http://www.maizegdb.org/probes).

Fingerprinting was performed using an AFLP procedure modified from Vos *et al.* (1995) that allowed chemiluminescent visualization of the amplified fragments. Ligation reactions were performed using DNA fragments produced by the *EcoR*I and *Mse*I restriction enzymes. For pre-selective amplifications, non-selective primers complementary to *EcoR*1 and *Mse*1 adapters were used (5'-GACTGCGTACCAATTC-3'. 5'-GATGAGTCCTGAGTAA-3', respectively). Selective amplifications were performed using identical primers with three additional nucleotides at the 3' ends (ACA for *EcoR*1 and CAA, CAT, CAC, CAG, CTC, CGG, and CTT for *Mse*1).

For assessing genome-wide DNA methylation, we followed the modified AFLP procedure of Cervera *et al.* (2002) to compare DNA fingerprints obtained from *EcoR*I/*Mse*I digests (methylation-insensitive polymorphisms) and from *EcoR*I/*Hpa*II and *EcoR*1/*Msp*1 digests (methylation-sensitive polymorphisms). In order to avoid polymorphisms arising from differences in developmental stages (Finnegan *et al.*, 1998), all DNA samples were obtained from the terminal leaf beneath the inflorescence.

Histology

Freshly collected kernels were fixed in FAA for 24 hours. Dehydratation, paraffin infiltration, sectioning (10 μm), and staining (safranin / fast green) were made following standard procedures (Freeling and Walbot, 1994). Preparations were examined under a Zeiss Axiophot or Leitz Aristoplan light microscope.

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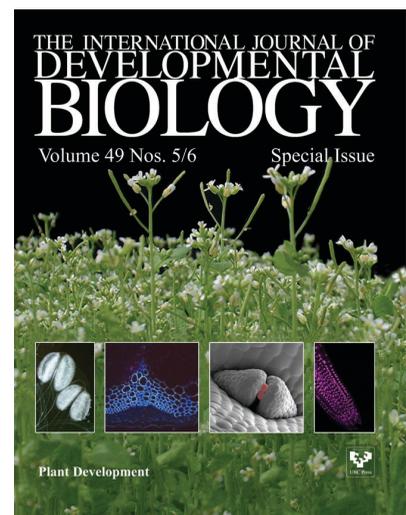**Characterization of seed storage proteins and their synthesis during seed development in Olea europaea**
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