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Competition between meiotic and apomictic pathways during ovule and seed development results in clonality

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Summary

• Meiotic and apomictic reproductive pathways develop simultaneously in facultative aposporous species, and compete to form a seed as a final goal. This developmental competition was evaluated in tetraploid genotypes of *Paspalum malacophyllum* in order to understand the low level of sexuality in facultative apomictic populations.

• Cyto-embryology on ovules, flow cytometry on seeds and progeny tests by DNA fingerprinting were used to measure the relative incidence of each meiotic or apomictic pathway along four different stages of the plant's life cycle, namely the beginning and end of gametogenesis, seed formation and adult offspring.

• A high variation in the frequencies of sexual and apomictic pathways occurred at the first two stages. A trend of radical decline in realized sexuality was then observed. Sexual and apomictic seeds were produced, but the efficiency of the sexual pathway dropped drastically, and exclusively clonal offspring remained.

• Both reproductive pathways are unstable at the beginning of development, and only the apomictic one remains functional. Key factors reducing sexuality are the faster growth and parthenogenetic development in the aposporous pathway, and an (epi)genetically negative background related to the extensive gene de-regulation pattern responsible for apomixis. The effects of inbreeding depression during post-fertilization development may further decrease the frequency of effective sexuality.

Introduction

Natural populations of higher eukaryotes reproduce mainly through sexuality, but sex does not always increase variation, and variation does not always increase fitness (Otto, 2009). Variation is the raw material on which natural selection acts. Sexual reproduction can bring together selectively favourable alleles present in different individuals via gene reshuffling (i.e. recombination + syngamy), although these processes may also break up favourable gene combinations built by earlier selection. Consequently, different mechanisms avoiding sex have been selectively favoured in flowering plants to exploit the advantages of clonal multiplication, covering vast areas with single and highly adapted genotypes (Johri, 1984; Stöcklin, 1992; Wesche et al., 2005). One of these mechanisms includes a pathway for asexual seed formation through parthenogenetic (fertilization-free) development of unreduced egg cells into embryos (gametophytic apomixis). Nonetheless, the production of clonal seed is not a common feature, and only a low percentage of all angiosperms reproduce by gametophytic apomixis: apospory and/or diplospory (Carman, 1997). In addition, these apomicts also have the capacity to generate variability through gene reshuffling, but the selection between both strategies is complex. Apomixis and sexuality are mutually exclusive processes

in diplosporous species, where the nonreduced embryo sac development requires meiotic failure in the megasporocytes. In contrast, apomixis occurs outside the megasporocyte in aposporous species, and hence both sexual and apomictic processes coexist. Meiotic and aposporous pathways may take place simultaneously in the same ovule, and generate seeds carrying either sexually derived or parthenogenetic embryos. Aposporous development starts by the time of, or immediately after, meiosis, when nucellar cells surrounding the germinal line (initial of apospory cells) differentiate into an unreduced gametophyte (Nogler, 1984; Asker & Jerling, 1992). Usually, the four meiotic megasporocytes degenerate, whereas the aposporous initials (AI) develop aposporous embryo sacs (AES). However, both reproductive pathways can operate independently in different ovules, or simultaneously in a single ovule, with variable frequencies among genotypes (Norrmann et al., 1989; Koltunow, 1993; Hojsgaard et al., 2008). In ovules with multiple embryo sacs, just one can be meiotic in origin, whereas the other(s) is (are) apomictic.

In summary, after meiosis, there are at least three developmental possibilities inside single ovules of aposporous apomictic plants: (1) the functional megaspore (FM) develops into a meiotic embryo sac (MES); (2) all megaspores degenerate and AI cells develop one or more AES; or (3) the FM, together with the AI cells, develops one

meiotic plus one or several aposporous sacs. Sporadically, all megaspores abort and nucellar cells fail to develop aposporous sacs. In this case, the mature ovule has no embryo sac. Along these pathways, from the end of meiosis to the mature embryo sac stage, mature seed phase, seed germination and seedling development until offspring establishment, an intensive competition can occur between apomictic and sexual pathways, towards the development of a new adult individual.

It is now widely accepted that apomixis arises as an alteration in gene expression during flower development (Polegri et al., 2010; Sharbel et al., 2010 and references therein), and that apospory is a highly plastic feature exhibiting variable penetrance in developing ovules (Norrmann et al., 1989; Burson & Hussey, 1998). Conversely, meiosis has a regular occurrence, as it is required for sex, and is well established in most angiosperms (Johri, 1984). Even though apospory and meiosis occur in tissues with different cell commitment and cell specification (Olmedo-Monfil et al., 2010), the presence of aposporous cells accelerates the onset of meiosis and sexual embryo sac formation in the diploid and sexual reproducing Sorghum bicolor (L.) Moench (Carman et al., 2011), indicating that both reproductive pathways interact in the same ovule. Even so, it is unclear whether the altered pattern of gene expression responsible for the initiation of apomixis in somatic cells of apomictic reproducing plants affects the meiotic germ line stability in the sexual pathway, and hence its efficiency. So far, the fitness amongst sexual and apomictic pathways inside the ovule has been thought to be relative to each other (Asker & Jerling, 1992; and references therein), but this relationship has not yet been investigated. The result of this competition within each individual plant (i.e. the final reproductive output) shapes directly the plant's genetic contribution to the gene pool of the population, which can have enormous consequences by altering allelic frequencies and genotypic diversity, as has been demonstrated in different apomictic species (e.g. Paun et al., 2006). Nowadays, new seed screening methods and progeny tests, aided by molecular markers, allow the extensive analysis of hundreds, or even thousands, of individuals to quantify reproductive pathways in individuals and populations (Matzk et al., 2000; Sherwood, 2001; Aliyu et al., 2010; Cosendai & Hörandl, 2010; Sartor et al., 2011). These methods, when coupled with classic embryological studies, elucidate the diverse nature of progenies derived from apomictic plants. Descendant individuals from a facultative apomictic may bear the maternal or a novel genotype, as they arise through parthenogenesis of unreduced egg cells (2n+0), or through fertilization (n+n), following a normal sexual process. When all progenies show a maternal genotype, the mother plant is considered to be an obligate apomictic genotype. Strictly speaking, obligate apomixis might occur only exceptionally in nature, as 100% apomictic progeny has been reported only rarely (Kao, 2007; Sorensen et al., 2009). It is even questionable whether obligate apomixis exists in plants, as residual sexuality has been observed even in plants previously considered to reproduce exclusively by apomixis (Asker & Jerling, 1992; Rebozzio et al., 2011).

In the grass family, both embryological and molecular genetic studies indicate that almost all the studied aposporous species are facultative apomicts, and show some potential for sexuality which

is realized to some extent (Harlan et al., 1964; Mazzucato et al., 1995; Rebozzio et al., 2011). Paspalum malacophyllum Trin is a promising model system with which to compare the relative efficiency of sexual and apomictic pathways. Apomictic embryo sacs are structurally different from MES and, whereas three ploidy levels have been described for the species (2x, 4x, 6x), only tetraploids (2n = 4x = 40) are widely spread throughout its distribution area (Honfi et al., 1990; Morrone et al., 2000; Hojsgaard et al., 2009). The tetraploids display a remarkable level of morphological variation (Morrone et al., 2000), probably as a consequence of the plastic reproductive strategies documented for these cytotypes. Hanson & Carnahan (1956) and Brown & Emery (1958) classified tetraploid genotypes of P. malacophyllum as obligate apomicts. Bashaw et al. (1970) claimed that the species was sexual. Finally, Burson & Hussey (1998) and Hojsgaard et al. (2008) concluded that tetraploid cytotypes of P. malacophyllum reproduce by aposporous facultative apomixis. The discrepancy between these results is most probably caused by the various screening procedures employed in each study, involving the analysis of plant reproductive structures at different developmental stages of the life cycle (young and mature ovules, and offspring).

We have used tetraploids of *P. malacophyllum* to analyse the efficiency of sexual and apomictic reproductive pathways, in order to understand the functionality and main factors modulating the penetrance of sexual reproduction in facultative apomictic species. We examine the relative capability of the meiotic pathway by quantifying sexual and apomictic development at different stages of the life cycle. From the beginning of meiotic and apomictic development to the establishment of a new offspring, the interaction between both reproductive pathways is evaluated. Our results shed new light on the basis for the low incidence of sexuality in natural apomictic populations.

Materials and Methods

Plant material

Information on the genotypes of *Paspalum malacophyllum* Trin used is detailed in Table 1. There were five maternal accessions grown in a glasshouse at the germplasm bank at the Instituto de Botánica del Nordeste (IBONE), Corrientes, Argentina. Open pollinated seeds collected from these plants were sown in sterilized soil to establish new progenies. Both maternal genotypes and those of their seedlings were grown under the same environmental conditions of temperature, light regime and relative humidity. Most seedlings completed their development, although some plants died before flowering. Progeny tests were carried out with DNA extracted from those descendants that reached the adult stage.

Theoretical assumptions

We expected a variable number of young ovules carrying an FM cell as the product of meiosis, but we also expected the presence of AI cells in the nucellar tissue independently from megaspore functionality. Thus, our first measured stage, the beginning of

Table 1 Accession, identification, geographical origin, collector's name andherbarium codes of the five tetraploid¹ genotypes of Paspalummalacophyllum

Accession	Country	Collection site	Collector and vouchers ²
TK2449	Bolivia	Santa Cruz, 2 km north of Concepción, Estancia El Recreo, 16°12'S, 62°08'W	Tim Killeen; CTES, MO
V5095	Brazil	Goiás, road between Itumbiara and Bom Jesus de Goiás	José Valls; CEN
Q4286	Argentina	Tucumán, near to Graneros	Camilo Quarin; CTES, UTEP
GR564	Paraguay	Cordillera, 2 km from Altos, on the road to San Bernardino	Gabriel Rua; CTES, BAA
DH374	Argentina	Salta, Anta, 7 km northwest of El Galpón, 25°22'56''S, 64°44'54''W; 577 msm	Diego Hojsgaard; CTES

¹Hojsgaard *et al.* (2008), D. H. Hojsgaard, unpublished.

²Herbarium codes where vouchers were deposited are those according to Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum. asp).

gametogenesis, was used to set the expected frequency of sexuality and apomixis for the next stage. The competition and functionality of reproductive pathways were then evaluated, assuming that, once development starts, each reproductive pathway will have the same chance of reaching the next stage. We investigated three different cases. (1) Ovules showing only FM should develop the equivalent number (expected) of ovules with MES, which, in turn, should give rise to equal numbers of sexually derived seeds (SS). The same is expected in ovules with exclusively AI cells, and the number of AES and seeds developed by apomixis (AS). In these cases, both pathways would run independently without interaction. (2) Ovules with both FM and AI cells should develop the same (expected) number of ovules with both MES and AES, but half of this potential amount of SS and AS. (3) If proportions between the sexual and apomictic pathways shift during the developmental stages, we can postulate that they interact in a competitive way.

Reproductive pathway efficiency

Three different methods of analysis were used to measure the incidence of each reproductive path (meiotic or apomictic) at the four different assessed stages of the life cycle in five tetraploid accessions of *P. malacophyllum*: cyto-embryological analyses conducted in (1) developing and (2) mature ovules; (3) flow cytometric analysis of seeds; and (4) progeny tests using DNA fingerprinting.

The reproductive efficiency of each path on the last three stages was calculated as the ratio between the observed and expected frequencies of ovules undergoing the meiotic or apomictic pathway at each stage. To avoid an overestimation of the relative observed frequencies as a result of those ovules sharing both meiotic and apomictic reproductive pathways, the *observed* values were obtained as a ratio between $\mathbf{nm}/(\mathbf{nm} + \mathbf{na})$ for the meiotic pathway and na/(nm + na) for the apomictic pathway, where nm is the total number of ovules with a functional meiotic pathway (FM, MES, SS, SP (sexually derived progeny)) and **na** is the total number of ovules with a functional apomictic pathway (AI, AES, AS, AP (apomitic derived progeny)). However, the *expected* frequencies at any particular stage were calculated from the data of the previous stage, as a ratio between $\mathbf{nm} + 0.5 \mathbf{nma/nt}$ for the meiotic pathway and **na** + 0.5 **nma/nt** for the apomictic pathway, where **nma** is the number of observed ovules with both reproductive pathways (FM + AI, MES + AES) and **nt** is the total number of ovules analysed (Tables 2, 3).

A standard Pearson's chi-squared test for goodness-of-fit was conducted in order to evaluate whether or not the observed and expected proportions of sexuality and apomixis between reproductive stages were significantly different. When the scored values did not fit the assumptions for Pearson's test, Yates' correction was used. Association analysis between sexual/apomictic frequencies

Table 2 Frequencies of meiotic and apomictic reproductive development in flowers of five *Paspalum malacophyllum* genotypes, as determined at two embryological stages, by the embryo : endosperm DNA ratio in seeds (Flow Cytometric Seed Screen, FCSS) and by genotype diversity within the sampled progeny (amplified fragment length polymorphism, AFLP)

	Beginning of gametogenesis ¹			End of gametogenesis ¹			Seeds			Offspring					
Genotype	п	FM	AI	FM + AI	n	MES	AES	MES + AES	п	2 : 3 (SS)	2 : 5 (AS)	3 : 5 (AS)	п	SP	AP
TK2449	16	0.125	0.375	0.500	58	0.034	0.914	0.052	73	0.110	0.863	0.027	49	0.0	1.0
V5095	11	0.0	0.818	0.182	68	0.192	0.720	0.088	78	0.117	0.870	0.013	49	0.0	1.0
Q4286	8	0.250	0.250	0.500	54	0.426	0.352	0.222	80	0.113	0.850	0.037	49	0.0	1.0
GR564	12	0.333	0.250	0.417	76	0.553	0.145	0.303	83	0.108	0.856	0.036	49	0.0	1.0
DH374	36	0.111	0.667	0.222	78	0.346	0.538	0.115	74	0.108	0.770	0.122	49	0.0	1.0
Total	83	0.145	0.530	0.325	334	0.320	0.521	0.159	388	0.111	0.842	0.047	245	0.0	1.0
(± S)		(0.098)	(0.228)	(0.138)		(0.156)	(0.231)	(0.086)		(0.003)	(0.037)	(0.038)		(0.0)	(0.0)

Gametogenesis: n, number of analysed ovules; FM, ovules with a functional megaspore cell; AI, ovules with megaspores deteriorated, but functional AI cells; FM + AI, ovules with both FM and AI cells; MES, ovules with meiotic embryo sacs; AES, ovules with aposporous embryo sacs; MES + AES, ovules with both meiotic and aposporous embryo sacs. *Seeds: n*, number of analysed seeds; SS, sexually derived seeds from a meiotic embryo sac; AS, apomictic derived seeds from an aposporous embryo sac. *Offspring: n*, number of analysed individuals; SP, sexually derived progeny; AP, apomictic derived progeny [correction added after online publication 6 November 2012: to resolve an error contained within the published footnote of Table 2, the text '*n*, number of analysed individuals' is now contained within the *Offspring* section of the footnote instead of the *Gametogenesis* section.]. \pm *S*, standard deviation of the sample.

	Embryo	sac formatio	Ц			Seed forr	nation				Offspring	formation			
	Expected	-	Realized			Expected		Realized			Expected		Realize	p	
Genotype	S	A	S	A	X^2	S	۲	S	A	X^2	S	A	S	۷	X^2
TK2449	0.375	0.625	0.082	0.918	36.63***	0.060	0.940	0.110	0.890	3.6 (NS) Y	0.110	0.890	0.0	1.0	12.36***
V5095	0.091	0.909	0.257	0.743	31.77*** Y	0.236	0.764	0.117	0.883	7.78**	0.117	0.883	0.0	1.0	13.25***
Q4286	0.500	0.500	0.530	0.470	0.36 (NS)	0.537	0.463	0.113	0.887	72.32***	0.113	0.887	0.0	1.0	12.74***
GR564	0.541	0.459	0.657	0.343	5.38*	0.704	0.296	0.108	0.892	170.41***	0.108	0.892	0.0	1.0	12.11***
DH374	0.222	0.778	0.414	0.586	20.07***	0.403	0.597	0.108	0.892	36.17***	0.108	0.892	0.0	1.0	12.11***
Total	0.307	0.693	0.388	0.612	3.07 (NS)	0.400	0.600	0.111	0.889	34.73***	0.111	0.889	0.0	1.0	12.49***
(±S)	(0.169)	(0.169)	(0,202)	(0,202)		(0,225)	(0,225)	(0,003)	(0,003)		(0,003)	(0,003)	(0.0)	(0.0)	

At the species level, goodness-of-fit values were obtained from the observed and expected genotype data. A, apomictic pathway; NS, nonsignificant; 5, sexual pathway. *, ** and *** denote significance at variables was df = 1. \pm S, standard deviation of the sample. P < 0.05, P < 0.01 and P < 0.001, respectively

reproductive pathways were made with the statistical package SPSS (SPSS Inc., Chicago, IL, USA) and with the Excel spreadsheet (2007 Microsoft Office System). Cyto-embryological analysis For the beginning and end of megagametogenesis analysis, inflorescences at the appropriate developmental stage were collected and fixed in FAA (70% ethanol, glacial acetic acid, 37% formaldehyde, 18:1:1). Spikelets belonging to these two stages of development were selected as determined by: (1) young spikelets bearing anthers with tetrads of microspores or uninucleate pollen grains, corresponding to ovaries at the end of female meiosis and early stages of embryo sac development; (2) mature spikelets collected during anthesis and before fertilization. Spikelets were dehydrated in a tertiary butyl alcohol series, embedded in paraffin, sectioned at 12-15 µm and stained with safranin-fast green. Observations were carried out with a light transmission microscope (Leica DM2500 equipped with a DFC320 camera; Leica Microsystems, Wetzlar, Germany).

and reproductive stages was quantified through Pearson's product-

moment coefficient (PPMCC), and a linear regression was applied to quantify the relationship between the proportions of sexuality at each reproductive stage. In order to illustrate the reliability of our estimations, confidence intervals for single proportions were calculated through the score method (Wilson, 1927; Newcombe, 1998), for a confidence level of 95%. All analyses for efficiency of

Flow cytometric seed analysis

Seeds from open-pollinated flowers for all five genotypes were collected in a time period of 30 d during the flowering season, and a Flow Cytometric Seed Screen (FCSS) protocol described by Matzk *et al.* (2000) was followed to reconstruct the reproductive pathways of mature seeds. The fluorescence intensity of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei was determined using a Partec PA II Flow Cytometer (Partec GmbH, Münster, Germany) with the detector operating at 355 nm. Ploidy levels of the endosperm and embryo tissues were estimated by comparing the different peak configurations. Approximately 3000 nuclei were measured per sample. Data analysis was performed using PA II's Partec FloMax software. The mean values of DNA content (C-values) for embryo and endosperm of single seeds were established to infer the sexual or apomictic origin of each seed. The coefficient of variation for each sampled peak was 5% or less.

The rationale for using FCSS is based on the different embryoto-endosperm relative DNA content among seeds as a consequence of fertilization on embryo sacs having diverse ploidies (Supporting Information Fig. S1).

Progeny test

A progeny test using DNA fingerprints generated with amplified fragment length polymorphism (AFLP) markers was carried out for each of the five tetraploid accessions of *P. malacophyllum* to establish whether or not meiotic and gamete segregation take place

in the formation of the progenies. The rationale for using AFLPs is based on the consequences of meiosis and syngamy in terms of the genetic profiles of the progeny. Contrary to parthenogenetically derived descendants, the genetic pattern of sexually derived descendants shows different patterns of polymorphisms relative to the parents.

Each progeny was composed of first-generation plants obtained from open-pollinated seeds. Between 150 and 200 seeds per maternal genotype were germinated in steam-sterilized soil, and seedlings were then separately transplanted into pots. A total of 245 adult plants (49 for each accession) was randomly selected, and their DNAs were extracted following the procedure described in Martínez et al. (2003). AFLP profiles were generated according to the method described by Vos et al. (1995), with modifications: genomic DNA (1 µg) was digested at 37°C overnight with 5 U EcoRI (Promega) and 2.5 U Msel (New England BioLabs Inc., Ipswich, MA, USA) in 2 × Restriction-Ligation (R/L) Buffer (50 mM Tris-HCl, pH 7.5; 50 mM magnesium acetate (C₄H₆MgO₄); 250 mM potassium acetate (C₂H₃KO₂); 25 mM dithiothreitol (DTT)) and 10 mg ml⁻¹ bovine serum albumin (BSA) to a final volume of 25 µl. Reactions were stopped on ice and a ligation mixture (5 pM EcoRI (UBC) and 30 pM Msel adapters (KeyGene, Wageningen, the Netherlands), 1 Weiss U T4 DNA ligase (Promega), 10 mM ATP and $1 \times R/L$ buffer) was added to each tube, which was then incubated for 6 h at 37°C. A preselective amplification was performed in a final volume of 25 µl, and preselective amplicons were diluted five-fold in ultrapure water, and $5\,\mu$ l of the dilution was then added to the selective amplification mixture under the standard PCR programme. Aliquots of the selective amplification were loaded in 5% (w/v) acrylamide/1 × TBE (Tris/Borate/EDTA) denaturing gel. Electrophoresis was carried out at 60 W for 2 h. Gels were fixed in 10% (v/v) acetic acid for 20 min and silver stained by the DNA Silver Staining System (Promega). Two (or three) replicates were carried out per genotype to corroborate the amplification pattern of the selected primer combinations. The proportion of progenies originating by apomixis was determined according to the pattern of banding, as apomixis avoids meiosis and syngamy, and new offspring have the same genotype as the mother plant.

Results

Female gametogenesis during meiotic and apomictic pathways

Almost 500 ovules from five accessions of *P. malacophyllum* were evaluated to analyse the frequencies of meiotic and apomictic pathways in young and mature ovules. At the beginning of gametogenesis, in 47% of 83 ovules, the chalazal megaspore remained healthy and functional (Table 2). At this stage, in most of the ovules, regardless of whether or not all megaspores degenerated, one to several active nucellar cells (AI) were observed (Table 2, Fig. 1a,b). AES developed from these cells. At the end of megagametogenesis, each gametophyte was distinguishable by its structural anatomy (see scheme in Fig. S1). An FM undergoes three mitotic divisions (eight nuclei as product), whereas, in AI cells, only



Fig. 1 Reproductive developmental stages evaluated in ovules of tetraploid *Paspalum malacophyllum*. (a, b) Young ovule at the end of meiosis/ beginning of gametogenesis showing two aborted megaspores (red) and a chalazal functional megaspore (FM) (yellow) embedded in nucellar tissue (grey), and accompanied by an initial of apospory cell (white blue). (c, d) Mature ovule at the end of gametogenesis showing a reduced gametophyte (yellow) accompanied by two unreduced gametophytes. Note that one AES is dislocated in the chalazal pole, whereas the other grows towards the micropile (asterisk), where the MES is well inserted and the male gametes will be delivered and discharged during fertilization. Egg cells are white. Two sinergid cells in the MES and one in the AES, placed close to the micropile, are not shown. ii, internal integuments; black dot, chalazal pole. Bar, 30 µm.

two divisions (plus one more in only one of the nuclei) are necessary to form a mature female gametophyte (four to five nuclei as product). At anthesis, MES had an egg cell, two synergids, a central cell with two polar nuclei and three to several antipodals towards the chalaza (originated from extra divisions on antipodal cells; Fig. 1c,d). AES had an egg cell, one or two synergids (occasionally synergids were not observed) and a central cell with two (eventually with one or three) polar nuclei (Fig. 1c,d). A total of 334 mature ovules was observed for the five genotypes. One MES was observed in 160 ovules (47.9%), independent of the presence or absence of AES in these ovules (Table 2). The number of AES observed per mature ovule was variable, and usually one or two were fully developed at anthesis (Table 2, Fig. 1c,d). In addition, some ovules lacking embryo sacs were also observed.

The proportions of different types of megagametophytes, and the category of mature ovules, varied greatly among the five accessions (Tables S1, S2). All genotypes were constitutively facultative apomicts, as they developed both MES and AES. Proembryos were eventually observed by the time of anthesis in AES of accessions TK2449 (4.5%) and V5095 (5.3%).

More than 70 ovules were at intermediate stages of development, between the beginning and end of gametogenesis (data not shown).

In these ovules, the first stages of megagametogenesis were identical in both MES and AES. However, when more than one embryo sac developed in the same ovule, it was possible to differentiate the unreduced gametophyte from the reduced one by its position in the ovule (see Fig. 1d), and its relatively more rapid development according to the timing of mitotic cell divisions.

Flow cytometric seed screen

A total of 388 seeds was analysed by flow cytometry for the five accessions, and an average of 77 seeds per accession. The FCSS, performed through seed-by-seed analysis, showed three types of histogram peaks in all accessions, corresponding to three different embryo : endosperm ratios of relative DNA contents: 2C: 3C, 2C: 5C and 3C: 5C (Table 2, Fig. S1). In seeds, a fixed variation in sex proportion among genotypes was observed (Table S1). Only *c*. 11% of seeds produced 2C: 3C histogram peaks, which indicated the occurrence of a reductional division (meiosis) and double fertilization. Most seeds, ranging from 77% to 87% depending on accession, displayed 2C: 5C embryo : endosperm peaks (apospory + pseudogamy), and a small proportion of seeds exhibited 3C: 5C peaks (apospory + double fertilization), indicating that parthenogenesis failed in some aposporous sacs, and hence seeds bearing 2n + n embryos were formed.

Reproductive origin of the progeny

DNA fingerprints generated with five AFLP primer combinations (*Mse*I-AAC/*Eco*RI-AAT; *Mse*I-AAC/*Eco*RI-AGA; *Mse*I-AAC/ *Eco*RI-AGC; *Mse*I-ACA/*Eco*RI-AAC; *Mse*I-AGC/-*Eco*RI-AAC) were used to analyse each tetraploid accession and their progeny. These primers were selected because they produced a large number of clear and informative bands among a total of 36 random pairs of primers assayed in one maternal accession and two of its descendants.

A total of 1272 DNA fragments with different lengths (identifiable bands) was generated for the species, with an average of 254 bands (ranging between 218 and 285) for each genotype. Between 40 and 60 bands were amplified per primer combination, and c. 28% (range, 8-40%) of these bands represented genotype-specific fragments (Fig. S2). Replicates validate the reproducibility of DNA fragment lengths under the PCR conditions used (not shown). AFLP molecular analysis showed that the five accessions were different genotypes with specific genetic profiles, which belonged to morphologically and geographically differentiated populations (Hojsgaard et al., 2008; Table 1). The same allelic profile, that is 100% maternal type, was shared between every single individual in the whole progeny and their mother plant (Fig. S2). No polymorphic fragments were recovered, and genotypic diversity within each sample was G=1. Although self-pollinated progeny can hardly be discriminated from apomixis-derived individuals when using dominant markers in highly homozygous plants, proportions of genotypespecific fragments and inter simple sequence repeat (ISSR) data on the five analysed genotypes showed relatively high levels of polymorphism among P. malacophyllum accessions (present work; E. J. Martínez, unpublished). It is widely known that polyploid apomicts show high levels of heterozygosity (Paun et al., 2006). Hence, a self-pollination event in polyploid polymorphic and/or heterozygous P. malacophyllum should be easily revealed with AFLP markers by segregating or shifting a number of alleles to a recessive homozygous condition (see details in the Discussion section). However, progeny derived from a cross-pollination event between genetically divergent genotypes could be simply identified by characteristic parental fragments. The only mechanism able to maintain unmodified the genetic profile of any plant when propagating through a seed is apomixis. Accordingly, it was concluded that all of the plants derived from seed originated solely through the apomictic reproductive pathway (Table 2). The absence of polymorphisms in the progenies also suggests that no 2n + n individuals occurred within each adult progeny.

Rates of reproductive pathway efficiency

The >1150 ovules, seeds and offspring evaluated provide an appreciable view of the reproductive efficiency of each meiotic and apomictic pathway from early ovule development throughout the reproductive process and up to the establishment of a new F_1 generation, and outline a clear reproductive trend in *P. malacophyllum*.

In Paspalum species, as in many other apomictic complexes, sexual diploids express a stable pathway, whereas polyploids exhibit a widely variable incidence of sexuality and seed production (Fig. 2). The five tetraploid accessions of P. malacophyllum displayed a highly variable capacity for sexual and apomictic reproduction in embryological stages, depicted by wide confidence intervals, but a tighter variation in either seeds or progeny stages (Fig. S3a,b; Tables S1-S3). Values of realized sexuality at the blooming stage ranged from near 8% (TK2449) to almost 66% (GR564) (Table 3) and, with the exception of genotype Q4286, all observed values from accessions were significantly different from those expected at the previous reproductive stage (Tables 2, 3). This absence of statistical correspondence in frequencies for each reproductive path is caused by genotype-specific variation (Fig. 3a), and depicts different tendencies to increase or decrease in their relative capacities for sexual and apomictic reproduction. Nevertheless, both meiotic and apomictic frequencies at the two embryological stages showed a medium (r=0.5) and strong (r=0.69) correlation, respectively, between genotypes, suggesting that the values follow a general trend (see later in this section). At the species level, a slight tendency to increase the percentage of sexuality between the beginning and end of gametogenesis was observed (Table 3), although the differences in the frequency of meiotic and apomictic pathways were not significant for $\alpha = 0.05$ (P = 0.08, $X^2 = 3.07$; Table 3). The observed proportion of ovules in which the meiotic pathway alone was active showed a shift from 14.5% to 32% between these two embryological stages, and represents a 2.2 times increase, whereas the proportion of ovules in which only the apomictic pathway was active remained at c. 53% (Fig. 3b, Table 2). The increase in the relative number of ovules with a functional meiotic pathway occurred at the expense of those ovules sharing both reproductive pathways, as its frequency decreased



Fig. 2 Proportion of functional reproductive pathways at different stages collected from 35 genotypes belonging to 12 Paspalum species. Only species from subgenus Anachyris were considered as for polyploid data. A grey boxplot over a white bottom represents data from diploid material. A striped boxplot over a shaded bottom represents data from tetraploid materials. The steady reproductive programme of diploids is altered by ploidy and apomixis, most probably as a consequence of genetic and epigenetic variation in polyploid counterparts (Cervigni et al., 2008; Ochogavía et al., 2009; Verhoeven et al., 2010), which depict a highly variable and lower relative productivity. Ab, ovules showing no functional pathways, i.e. ovules without FM or AI cells, or ovules without functional embryo sacs; AES, aposporous embryo sacs; AI, ovules with a functional apomictic pathway; FM, ovules with a functional meiotic pathway; MES, meiotic embryo sacs; OP, seeds yielded after open pollination; SP, seeds yielded after self-pollination. Outlier values are represented by circles, and stars represent extreme values. Whiskers correspond to confidence intervals of 95%.

two-fold, from 32.5% to 15.9% during the same reproductive stages (Figs 3b, S4). By the end of female gametogenesis, when blooming starts, this situation was also reflected in a higher efficiency of the meiotic path compared with the apomictic one, relative to the beginning of gametogenesis (Table 4).

Going through the next stages of analysis, FCSS showed that the realized sexuality in seeds was uniform among the five accessions (11.1% on average; ranging from 10.8% to 11.7%), and significantly lower than the overall rate of expected sexuality (40%) observed at flowering (P < 0.001, $X^2 = 34.73$, Table 3). Less than one-quarter of the total number of MES present at flowering, and only about one-third of the ovules carrying exclusively MES, were able to produce a seed. This means a large reduction in the expected level of sexuality, which renders a very low reproductive efficiency of the meiotic pathway at this stage (Table 4). Only accession TK2449 showed nonsignificant differences in the level of sexuality and apomixis observed by both cyto-embryological and FCSS procedures, the remaining genotypes showing a significant drop in



Fig. 3 Proportions of three different categories of ovules bearing functional reproductive pathways at the two analysed developmental stages in *Paspalum malacophyllum*: at the beginning and the end of gametogenesis. (a) Genotype-specific tendencies on ovules with only one functional pathway (either meiotic or apomictic) and ovules with both paths functional. (b) General tendency for all accessions. The resolution of competition between meiotic and apomictic paths is illustrated by the reduction in the proportion of ovules sharing both reproductive modes. AES, aposporous embryo sacs; AI, initials of apospory cells; FM, functional megaspores; FM + AI, functional megaspores + initials of apospory cells; MES, meiotic embryo sacs; MES + AES, meiotic + aposporous embryo sacs.

Table 4 Ratio between the observed and expected frequencies of meiotic (M) and apomictic (A) reproductive pathways in *Paspalum malacophyllum*, as evaluated at three different developmental stages during a new individual's formation and establishment

	Anthesis	S	Seeds		Proger	ıy
	Μ	А	Μ	А	M	А
TK2449	0.17	1.50	1.82	0.95	0.0	1.12
V5095	2.59	0.84	0.50	1.15	0.0	1.13
Q4286	1.07	0.93	0.21	1.91	0.0	1.13
GR564	1.30	0.65	0.15	3.01	0.0	1.12
DH374	1.82	0.77	0.27	1.50	0.0	1.12
Total	1.30	0.92	0.28	1.48	0.0	1.12

Values > 1 indicate a proportional increase in the number of ovules observed.

the observed rates of sexuality, together with a prevalence, and high reproductive efficiency, of apomixis (Tables 3, 4). At the seed stage, the large reduction in the frequency of sexuality derives mainly from those ovules with solitary MES, as almost two-thirds were not able to develop a seed (Table 3, Fig. S4).

At the last stage, the progeny test analysis showed that each one of the five sampled F_1 progenies displayed a single consistent genetic profile, identical to the maternal profile. The 245 fully developed

descendants originated through the apomictic pathway. Again, the levels of sexuality and apomixis observed were significantly different from those expected by FCSS analysis for all accessions (P < 0.001, $X^2 = 12.49$), and for each genotype (Table 3). Reproductive efficiency was > 1 for the apomictic path, but was null for the meiotic path, as no sexual offspring were produced (Table 4).

Linear regression analysis on the complete dataset (Fig. 4) summarizes the main trend. In addition to the relative increase in the frequency of ovules with a functional meiotic pathway between the first and second reproductive stages, the realized sexuality clearly tended to decrease throughout the different stages of the reproductive process. The strongest decline was observed between MES and SS, but sexuality dropped to zero during the establishment of the F_1 generation.

Discussion

The levels of sexuality at four stages of the life cycle in five *P. malacophyllum* genotypes were shown to vary greatly and to exhibit a marked decline towards the final steps of development and offspring formation. The reasons for the decline in sexuality observed differ according to the developmental stage examined.

Developmental instabilities and reproductive efficiency during gametogenesis

Flowers of *P. malacophyllum* display no significant difference in the proportion of ovules with meiotic or apomictic development (e.g. 47% of ovules have a viable FM at the beginning of gametogenesis, whereas, at the end of gametogenesis, a MES was present in 48% of ovules). Nevertheless, as is usually observed, the penetrance of sexuality and apomixis at both developmental stages is highly variable among accessions (Savidan, 1982; Mazzucato *et al.*, 1995; Burson & Hussey, 1998; Naumova *et al.*, 1999; Hojsgaard *et al.*, 2008). This variation is probably a result of dissimilar genetic backgrounds among genotypes, as they are adapted to divergent geographical and environmental conditions. Despite the fact that sexual–asexual proportions may be modified by geographical and seasonal effects (Quarin, 1986; Rebozzio *et al.*, 2011), the course of the interaction between reproductive pathways during flower



Fig. 4 Trend obtained after a multiple linear regression on the frequencies of realized sexuality in five *Paspalum malacophyllum* genotypes. The third-order polynomial regression shows the fittest coefficient of determination (R^2) , which explains most of the observed variability on the dataset. FM, functional megaspores; MES, meiotic embryo sacs; SP, sexually derived individuals; SS, sexual seeds. Whiskers represent the minimum and maximum values in the dataset.

development should not change greatly. Within P. malacophyllum genotypes, the highly significant differences between the observed and expected frequencies of either sexuality or apomixis suggest developmental instabilities in both reproductive pathways. For example, the decrease in the expected sexuality observed in TK2449 is based on a drastic reduction in ovules carrying MES or MES + AES, when compared with ovules carrying FM or FM + AI. This reduction in the sexual capacity of TK2449 shows that the meiotic pathway is highly unstable in this genotype, as a very low proportion of FM undergoes successful gametogenesis. Conversely, in accession Q4286, meiotic and apomictic pathways are stable, as a higher proportion than expected from ovules with FM or AI alone gives rise to ovules with only MES or AES. In genotypes V5095, GR564 and DH374, the sexual capacity increases notably at flowering, mainly because of those ovules with FM + AI, wherein the aposporous path failed to develop an unreduced embryo sac. As meiosis is the default mode in apomicts (Koltunow et al., 2011), the success of the meiotic pathway at this stage in these accessions depends on the instabilities of aposporous pathways rather than a buttressed sexuality.

The idea explaining asynchronous ovule development in apomicts on the basis of the temporal or spatial de-regulation of genes controlling the reproductive programme (Carman, 1997; Grimanelli et al., 2003; Koltunow & Grossniklaus, 2003; Curtis & Grossniklaus, 2007) is now supported by transcriptome analysis, showing large numbers of differentially expressed genes between sexual and apomictic types (Sharbel et al., 2009, 2010; Polegri et al., 2010). Most of these genes appear during sporogenesis and early gametogenesis, in premeiotic and meiotic/apomictic stages, which correlate with AI or diplosporous cell emergence in developing ovules of apomictic systems. Interestingly, the somatic vs germ line cell fate specification is controlled by RNA-dependent DNA methylation mechanisms, and is possibly involved in the expression of apomictic phenotypes (Grimanelli, 2012). In this context, if apomixis arises as a consequence of epigenetic alterations affecting cell specifications at early developmental stages, which trigger a downstream cascade of gene de-regulations, and the differences observed in the frequencies of FM and AI cells on P. malacophyllum ovules are a result of the dissimilar evolutionary histories among apomictic genotypes, these dissimilarities may have set up different genetic and epigenetic frameworks responsible for apomixis and for the observed plant-specific varying instabilities in developmental programmes.

Furthermore, our data show that the occurrence of polyploid apomixis destabilizes the sexual pathway, but the apomictic pathway also starts by being developmentally unstable. As a consequence, ovules of *P. malacophyllum* sharing both reproductive pathways at early gametogenesis depict a relatively more 'steady' and competitively efficient meiotic programme, which contributes to increase the fraction of MES ovules. The relative (in)stability and developmental capacity of the aposporous pathway seem to be affected only when a meiotic cell remains functional in the same ovule. Indeed, evidence in other model systems declared apomixis as a variable-in-penetrance feature (e.g. *Poa pratensis*; see Matzk *et al.*, 2005). Assuming that those ovules of *P. malacophyllum* showing meiotic and apomictic functional cells result from a weak penetrance of apomixis (e.g. minor level of de-regulated genes allowing the simultaneous presence of both pathways), this would explain its relative instability and lower efficiency in such ovules. Next, a strong penetrance of the character (e.g. superior level of deregulated genes) should bring in the emergence of the apomictic pathway as the sole option (keeping it stable, as observed in ovules displaying exclusively AI and/or AES), and might be responsible for meiotic cell abortion in these ovules as a result of the same extensive gene de-regulations.

AES are competitively advantageous for seed formation

Although, in P. malacophyllum, close to 50% of mature ovules bear one sexual embryo sac, only c. 22% of these ovules were able to generate seeds (c. 11% of the total number of ovules). This entails some competitive advantage favouring seed development from AES against MES. Differential timings of development between reproductive pathways can constitute an advantage for AS formation, whereby it benefits aposporous sacs when competing for space and resources inside the ovule. Nygren (1948) was the first to recognize variation in developmental timings of sexual and apomictic embryos in Calamagrostis. More recently, Savidan (1982, 2000) and Leblanc & Savidan (1994) observed analogous differences between meiotic and aposporous pathways in Megathyrsus maximus (Jacq.) B. K. Simon & S. W. L. Jacobs (syn. Panicum maximum Jacq.) and Tripsacum dactyloides L., respectively. Finally, as mentioned previously, Sharbel et al. (2009, 2010) provided evidence for a general up-regulation of genes in apomictic ovules of Boechera retrofracta (Graham) Á. Löve and D. Löve, and hypothesized that it was mediated by either ploidy or parthenogenetic development. A scenario such as this can be a feasible cause for developmental heterochrony between meiotic (reduced) and apomictic (unreduced) cells. Although, in P. malacophyllum, the 'ploidy hypothesis' by itself does not constitute a clear advantage during gametogenesis, as the meiotic pathway is more competent than the apomictic one (see the previous section), the general pattern of de-regulated genes may be better tolerated by unreduced than reduced megagametophytes, which preserve heterozygosity and have twice the number of gene copies. In addition, parthenogenetic development (i.e. fertilization-independent embryogenesis activation) must also play a crucial role, increasing the efficiency of apospory for seed formation. Early pro-embryos have been observed in different Paspalum species (present work; Burson & Bennett, 1971; Quarin et al., 1996; Espinoza et al., 2001; Hojsgaard et al., 2008), as well as in other apomicts, for example Allium tuberosum (Kojima & Nagato, 1992) and Taraxacum officinale (Cooper & Brink, 1949). In these apomicts, the fusion of the unreduced egg cell with one sperm cell is not necessary to restore a 2n zygote ploidy and initiate embryogenesis, as occurs in the meiotic pathway. Furthermore, if pollination is anticipated, the refractory nature of unreduced egg cells to fertilization is unlocked (Martínez et al., 1994), indicating that parthenogenesis on unreduced sacs probably speeds up those normal events that avoid polyspermy (multiple fertilization) in the sexual pathway (Scott et al., 2008). Our results in P. malacophyllum point towards a substantial competitive advantage of apospory after gametogenesis

and during seed formation, mainly mediated by an accelerated developmental timing. Moreover, as parthenogenesis allows precocious embryo initiation, when pollination triggers endosperm development in those ovules of pseudogamous species, the growth of the asexual embryo is much more advanced than that of the sexual one.

Even without competition, MES of apomicts show a low performance

Competition of sexual/apomictic reproductive pathways reduces the fraction of SS, but ovules with no cytological signs of aposporous growth in the surrounding nucellar tissue are not subordinated to this interaction. Hence, we should expect as many SS as ovules bearing a single meiotic sac. Even so, the difference between the observed and expected numbers of SS is significant at the species level, and highly significant in four of five maternal genotypes of *P. malacophyllum*. These data suggest that, in addition to competitive limitations of the meiotic pathway, some genetic restraint not derived from a competitive environment in the ovule, and constitutively present, is responsible for the poor performance of MES when developing in the absence of any other embryo sac.

As mentioned in the Introduction, apomicts show a general pattern of altered gene expression in the ovule (Polegri et al., 2010; Sharbel et al., 2010; Baroux et al., 2011; Pupilli & Barcaccia, 2012) and, whether initiated by one master gene or several genes, still remains unclear. New studies on megaspore mother cell determination during ovule development provide a clue to the role of genes involved in de novo DNA methylation and post-transcriptional gene silencing mediated by small RNAs. Loss of function of these genes causes unrestricted specification of female gametophytic precursors and AI-like cells in nucellar tissues of Arabidopsis and maize (Zea mays) (García-Aguilar et al., 2010; Olmedo-Monfil et al., 2010), a phenotype reminiscent of aposporous apomixes, or functional gamete formation without meiosis in maize (Singh et al., 2011), a phenotype reminiscent of diplosporous apomixis. If transient populations of small RNAs present in reproductive organs are important regulators of extensive epigenetic changes taking place during gametogenesis and early seed development (Bourc'his & Voinnet, 2010), once apomixis is triggered, the gene(s) involved must amplify the signal impelling an apomixis-like phenotype and gradually disturbing the progression of the meiotic programme. This idea fits with our observations of developmental instabilities in ovules of *P. malacophyllum*, and suggests that, in natural apomicts, once the (epi)genetic state responsible for apomixis is activated, it turns into a stable phenotype by the end of gametogenesis, affecting increasingly and negatively the sexual pathway of seed formation.

Unbalanced fractions between sexual and apomictic progeny: foundations for clonal populations

No offspring of tetraploid *P. malacophyllum* genotypes originated from sexual reproduction, that is by the fusion of two meiotically reduced gametes. Even though AFLPs are useful for the screening of genetic changes throughout all the genome, they do not

discriminate heterozygous from dominant homozygous genotypes (hence this variation remains hidden). In an autotetraploid individual heterozygous for one locus AAaa, 17% or 21% of its gametes are recessive homozygous *aa* (depending on whether they are linked or not to the centromere, respectively) (Lacadena, 1988; Comai, 2005). When self-pollinated, the chances of getting one individual *aaaa* would be as low as $0.17 \times 0.17 = 0.17^2 = 0.0289$, too low to discriminate between polymorphic and nonpolymorphic individuals for that locus. However, if we consider not one, but 30 loci, the chances of getting one individual without showing any of the expected recessive homozygous changes within its genetic profile is $0.83^{30} = 0.0037$, that is one of 250 F₁ individuals. According to the classic view on population genetic analysis, each fragment length is considered as one allele (representing one locus); therefore, after analysing > 200 loci in tetraploid *P. malacophyllum*, the expectations of having one individual generated through sexuality and selfing without showing any genetic variation in its genetic profile, as found in our case, would be extremely low. Thus, the observed lack of sexually derived individuals again implies a significant difference from that expected from SS proportions. Although most apomictic species have some potential for sexual reproduction, usually few (or no) adult plants originate through sexuality. Examples can be found in monocots, such as the Bothriochloa-Dichantium complex (Harlan et al., 1964) or Paspalum notatum (Rebozzio et al., 2011), and dicots, such as the alpine plant Ranunculus kuepferi (Cosendai & Hörandl, 2010) or Boechera holboelli (Aliyu et al., 2010). Even after successful seed formation, P. malacophyllum shows a strong restriction to establish fully developed sexually produced individuals. As discussed before, the low efficiency of the sexual pathway during seed formation is probably caused by the altered (epi)genetic state of de-regulated genes that characterize apomictic flowers and engender developmental instabilities. The same pattern of de-regulated gene networks must be interfering and modifying the subsequent seedling development, with the probable additive effects of an increased homozygosity. The progenies of heterozygous P. malacophyllum were grown from open-pollinated seeds. However, although diploid sexual relatives of apomicts are self-incompatible, apomicts usually show self-fertility (Hörandl, 2010). Tetraploid cytotypes of P. malacophyllum are all selfcompatible (Hojsgaard et al., 2008; D. H. Hojsgaard, unpublished); florets are hermaphrodite and organized in racemes. At blooming, anther dehiscence takes place within the first minute after flower opening; consequently, the more likely scenario at flowering is a self-fertilized spikelet. Self-pollination in plants increases homozygosity, resulting in inbreeding depression (reviewed in Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996; Keller & Waller, 2002; Comai, 2005). In apomictic plants, inbreeding depression would become effective only in progenies originating from sexuality by the expression of recessive deleterious/lethal genes unmasked after zygote formation in a self-fertilized meiotic-derived egg cell. In the apomictic pathway, parthenogenesis avoids meiotic and gametic recombination phases of sexual reproduction, and thus eludes homozygous allele combinations and inbreeding consequences. Although reaching homozygosity at any locus would be slower in

autotetraploids than in diploids, self-pollination fosters this process and represents a disadvantage when compared with cross-pollination. If the growth of SS of *P. malacophyllum* is affected by selfing and increased homozygosity, this result is another cause explaining the failure of MES after fertilization and the poorer seed set in selfpollinated flowers of *Paspalum* species. Low vigour and slow growth rates of seedlings are consequences of inbreeding in both allogamous and autogamous plants (Husband & Schemske, 1996). These effects were observed in some of the *P. malacophyllum* seedlings in the glasshouse. Certainly, SS of tetraploid *P. malacophyllum* loses its germinative capacity (as no sexual individuals were recovered) or, if germinated, they were unable to survive.

Conclusion

The sexual reproductive pathway in facultative apomictic plants of *P. malacophyllum* is functional, but dramatically less effective than apospory followed by parthenogenesis. Apomixis starts as a developmentally unstable alternative to meiosis that becomes progressively stabilized, and has a detrimental effect on the meiotic developmental programme. The strong competition, the proper (epi)genetic de-regulation that causes apomixis and modifies the normal progression of the meiotic pathway, and the consequences of inbreeding depression are major factors supporting the establishment of clonality.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Ratio of relative embryo : endosperm DNA content in facultative apomictic *Paspalum* species according to the reproductive pathway followed during seed formation.

Fig. S2 Progeny test on two *Paspalum malacophyllum* genotypes and 30 F_1 plants.

Fig. S3 Variation in the proportion of sexuality and apomixis observed in five *Paspalum malacophyllum* genotypes throughout four different stages of the plant cycle.

Fig. S4 Column chart showing the meiotic and apomictic frequencies observed at different stages of the life cycle in *Paspalum malacophyllum*.

Table S1 Range of confidence intervals for reproductive pathwaysat four developmental stages of five Paspalum malacophyllumgenotypes

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