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Apospory and parthenogenesis may be uncoupled in *Poa pratensis*: a cytological investigation

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Abstract Despite the potential that apomixis has for agriculture, there is little information regarding the genetic control of its functional components. We carried out a cytohistological investigation on an F_1 segregating population of *Poa pratensis* obtained from a cross between a sexual and an apomictic parent. About half of the F_1 progeny plants were parthenogenic, as adjudicated by an auxin test. The degree of parthenogenesis ranged from 1.44% to 92.9%. Apospory was detected in parthenogenetic plants as well as in two non-parthenogenetic individuals. These results indicate that two distinct genetic factors control apospory and parthenogenesis in *P. pratensis* and that apospory and parthenogenesis may be developmentally uncoupled.

Keywords *Poa pratensis* L. · Apomixis · Apospory · Parthenogenesis · Cytohistology

Introduction

Kentucky bluegrass (*Poa pratensis* L.), an attractive forage and turf grass, reproduces mainly through pseudogamous facultative aposporous apomixis (Müntzing 1933). Aposporous apomixis is functionally composed of two processes: apospory and parthenogenesis. Apospory is the development of embryo sacs starting from cells that differentiate from the nucellus. In *P. pratensis*, these aposporous embryo sacs develop through parthenogenesis to viable apomictic seeds if the unreduced polar nuclei fuse with a sperm cell from the male gametophyte (pseudogamy). This mode of reproduction is highly prized by breeders, as propagating the maternal genotype allows the development of varieties that breed true regardless of heterozygosity (Hanna and Bashaw 1987; Savidan and Dujardin 1992). Proper manipulation of apomixis requires knowledge of at least three different aspects of the trait: (1) unreduced megagametophyte formation; (2) parthenogenesis; (3) endosperm development. Despite the potential this trait has for agriculture, we know very little about the genetic control of these aspects.

Data so far accumulated on *P. pratensis* (Matzk 1991a; Barcaccia et al. 1997, 1998; Matzk et al. 1997) suggest that apomixis is controlled by a single genetic locus of as yet undetermined size and structure. This implies that the unreduced egg has a built-in tendency to autonomous parthenogenesis, and that apospory and parthenogenesis are pleiotropic (Mazzucato et al. 1996; Barcaccia et al. 1997; Matzk et al. 1997). Similar pictures have been described in *Ranunculus* (Nogler 1984) and in *Panicum* (Savidan 1980). However, data to support the alternative hypothesis may have been overlooked. The reason could be in part historical, as most classical studies have failed to consider parthenogenesis and apospory as distinct tractable traits (Noyes and Riesberg 2000). The finding that haploid parthenogenesis occurs at low frequencies in *P. pratensis* (Brittingham 1943; den Nijs and Winkelhorst 1992; Mazzucato et al. 1996; Barcaccia et al. 1998), together with the inherent difficulties encountered in performing extensive cytological investigations on apospory, have led to breeders focusing their studies on apomixis as a whole rather than on its functional components. Apospory and parthenogenesis now need to be analyzed separately to uncover the mechanisms that control each process.

Cytological investigations conducted through light microscopy have been used to identify features diagnostic of aposporous initial cells (Grazi et al. 1961; Gröber

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1986; Naumova et al. 1993; Mazzucato et al. 1995; Naumova 1997). The functional megaspores and aposporous initial cells, for instance, appear to differ in their overall thickness, the presence of multiple membrane structures, and the number of vacuoles, mitochondria and vesicles (Naumova and Willemse 1995). However, the cytological organization of sexual and unreduced embryo sacs is extremely similar and it is very difficult to distinguish between them (Koltunow 1993). To date, few studies have attempted to determine the genetic control of parthenogenesis in *P. pratensis* (Matzk 1991a; Barcaccia et al. 1998). Barcaccia et al. (1998) reported the identification of AFLP markers that map to both sides of the parthenogenesis locus. However, as this study was based on bulk segregant analysis, it treated parthenogenesis as a qualitative rather than a quantitative trait. We now need map-based studies to gain detailed information on the genetic basis that controls the major variability of parthenogenesis.

We have explored the basis of apomixis in *P. pratensis* by studying apospory and parthenogenesis independently in a progeny segregating for the mode of reproduction.

Materials and methods

Plant material

A segregating F_1 population of 38 plants was produced by crossing a completely sexual clone (S1/1-7, non-aposporic and non-parthenogenetic) with a highly apomictic genotype (RS7-3, aposporic and parthenogenetic). The sexual clone was derived from a cross between two completely sexual genotypes selected from German cultivars (Matzk 1991a; Barcaccia et al. 1998), while the apomictic clone was recovered within a natural Italian population (Mazzucato 1995). Cytological investigations showed that the chromosome number of parental genoypes was $2n=36$ for S1/1-7 and $2n=64$ for RS7-3. All progeny plants investigated had a complement of 50 chromosomes (Porceddu et al. 2001).

Auxin test and degree of parthenogenesis

The genetic capacity for parthenogenesis was estimated in a total of 38 F_1 plants by the auxin test (Matzk 1991b) performed for 2 years. The auxin treatment of panicles was done according to Mazzucato et al. (1996).

Cytohistological investigations of apospory

Spikelets were harvested for cytohistological analysis at different developing stages: from glumes opening to full anthesis. Ovaries and young developing caryopses were dissected from spikelets, fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7, for 6 h at room temperature and rinsed four times with the same buffer. Samples were then post-fixed for 2 h with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in an ethanol series and embedded in epon-araldite. Semi-thin sections were cut with a glass knife on a Reichert OmU2 ultramicrotome and then stained with toluidine blue. Investigations of a minimum of 30 ovaries per plant were conducted using a Leica DMR light microscope equipped with a Leica DC 200 digital photo camera.

Results

Analysis of molecular markers indicated that all the 38 plants analyzed were sired by the agamospermous pollen parent (Porceddu et al. 2001). These plants were cloned and subjected to both auxin test and cytohistological analysis.

Auxin test and degree of parthenogenesis

The evaluation of samples after auxin treatments allowed sterile ovaries, non-induced and auxin-induced grains to be discriminated. Of the 38 F_1 plants, 22 entirely lacked any parthenogenic activity and were classified as non-parthenogenetic (Table 1) whereas 16 exhibited a highly variable parthenogenic capability, ranging from 1.44% to 92.9% (mean of 25.6) and were classified as parthenogenetic (Table 1). The overall segregation ratio between plants scoring a null degree of parthenogenesis and plants showing on average a degree of parthenogenesis of 25.6% did not differ significantly from the 1:1 ($\chi^2=0.974$, 1 *df*) expected in the case of a monogenic control (Matzk 1991a).

Cytohistological investigations of apospory

Spikelets of *P. pratensis* had 3–4 flowers acropetally arrayed, the most basal of which developed earlier. At the beginning of megagametogenesis, the ovary had, on average, a size of 180×245 µm and contained an ovule of 75×62 µm. All the examined genotypes (parentals and 38 F_1 plants) showed a regular meiosis of the megaspore mother cell (Fig. 1A). The calazal megaspore became the functional megaspore (Fig. 1B) whereas the others – in the micropylar region – degenerated, leaving behind intense colored spots (Fig. 1B).

All the individuals classified as parthenogenetic showed aposporous initial cell(s) arising from a somatic cell usually located in the middle of the nucellus (Fig. 1C). Aposporous initials were easily distinguishable among other cells of the nucellus because of their increased size, the occurrence of numerous small vacuoles and a large nucleolus (Fig. 1C). The time of appearance of aposporous initials and the fate of the meiotic embryo sac varied among individuals. In this respect, two different behaviors were observed. In most of the aposporic genotypes, the first symptom of collapse and the beginning of degeneration of the functional megaspore was evident soon after the end of meiosis while some nucellar cells enlarged to become aposporous initials and prepared to divide (Fig. 1C, D). In the remaining few cases the functional megaspore inside the ovule began megagametogenesis but the developing embryo sac never accomplished its full development. The young megagametophyte degenerated and was surrounded by several nucellar cells, which resembled aposporous initials. While every genotype showed several aposporous

Table 1 Results of the auxin test and estimated degree of parthenogenesis of plants classified as non-parthenogenetic and as parthenogenetic (*bold*)

Progeny plant no.	Total cariopses	Sterile cariopses	% of sterile	Full cariopses with endosperm	Empty cariopses (without endosperm)		Degree of parthenogenesis
					With embryo	Without embryo	
1	234	19	8.12	55	0	160	0.00
3	381	64	16.80	187	0	130	0.00
4	128	25	19.53	0	0	103	0.00
5[a]	643	64	9.95	35	0	544	0.00
8	324	124	38.27	72	0	128	0.00
10[a]	329	32	9.73	20	0	277	0.00
12	220	5	2.27	22	0	193	0.00
13	620	184	29.68	109	0	327	0.00
16	242	34	14.05	29	0	179	0.00
18	184	55	29.89	16	0	113	0.00
19	204	31	15.20	19	0	154	0.00
21	204	82	40.20	11	0	111	0.00
22	407	223	54.79	37	0	147	0.00
23	154	18	11.69	10	0	126	0.00
24	190	35	18.42	15	0	140	0.00
25	216	47	21.76	65	0	104	0.00
27	235	47	20.00	38	0	150	0.00
29	235	39	16.60	24	0	172	0.00
30	154	25	16.23	21	0	108	0.00
41	183	38	20.77	3	0	142	0.00
42	199	34	17.09	8	0	157	0.00
43	229	53	23.14	24	0	152	0.00
2	**756**	**85**	**13.24**	**151**	**13**	**507**	**2.50**
6	**333**	**44**	**13.21**	**96**	**9**	**184**	**4.66**
7	**237**	**9**	**3.80**	**41**	**99**	**88**	**52.94**
9	**520**	**253**	**48.65**	**120**	**6**	**141**	**4.08**
14	**134**	**42**	**31.34**	**49**	**18**	**25**	**41.86**
15	**335**	**29**	**8.66**	**137**	**128**	**41**	**75.74**
17	**398**	**27**	**6.78**	**54**	**8**	**309**	**2.52**
26	**403**	**90**	**22.33**	**88**	**191**	**34**	**84.89**
29	**466**	**163**	**34.98**	**78**	**37**	**188**	**16.44**
31	**376**	**46**	**12.23**	**88**	**7**	**235**	**2.89**
32	**153**	**25**	**16.34**	**17**	**6**	**105**	**5.41**
33	**215**	**5**	**2.33**	**49**	**23**	**138**	**14.29**
34	**229**	**72**	**31.44**	**20**	**5**	**132**	**3.65**
36	**261**	**47**	**18.01**	**5**	**3**	**206**	**1.44**
37	**445**	**19**	**4.27**	**74**	**12**	**340**	**3.41**
46	**205**	**12**	**5.85**	**38**	**144**	**11**	**92.90**

[a] Recombinant plants (no parthenogenesis but presence of aposporous initials and embryo sacs)

initials within an ovule, variation was noticed in the number of developed aposporous embryo sacs per ovule. In some genotypes, only one aposporous initial developed in a mature embryo sac with egg apparatus and proliferating antipodals. In ovules of the majority of genotypes, however, more than one embryo sac reached full development (Fig. 1E) but only one was normally oriented with the egg apparatus toward the micropyle (Fig. 1F).

Of the 22 non-parthenogenetic plants, 20 showed a normal meiotic pathway and none showed aposporous initials. In these genotypes, it was possible to observe the functional megaspore that started the development of a *Polygonum*-type embryo sac with an egg apparatus formed by a pear-shaped large egg cell and two synergids. The central cell usually enlarged and the antipodals (Fig. 1G) proliferated, pushing polar nuclei toward the egg apparatus. The remaining two non-parthenogenetic plants, namely plants 5 and 10, showed several aposporous initials and embryo sacs (Fig. 1H).

Discussion

The results of our cytohistological investigation into parthenogenesis and apospory in *P. pratensis* show that the F_1 population resulting from a cross between a sexual (non-aposporic and non-parthenogenetic) and an apomictic (aposporic and parthenogenetic) parent segregated for both apospory and parthenogenesis, and so indicated that the traits are both genetically dominant and heterozygous in the apomictic parent. In addition, while apospory was scored in all parthenogenetic individuals, two non-parthenogenetic genotypes showed aposporous ini-

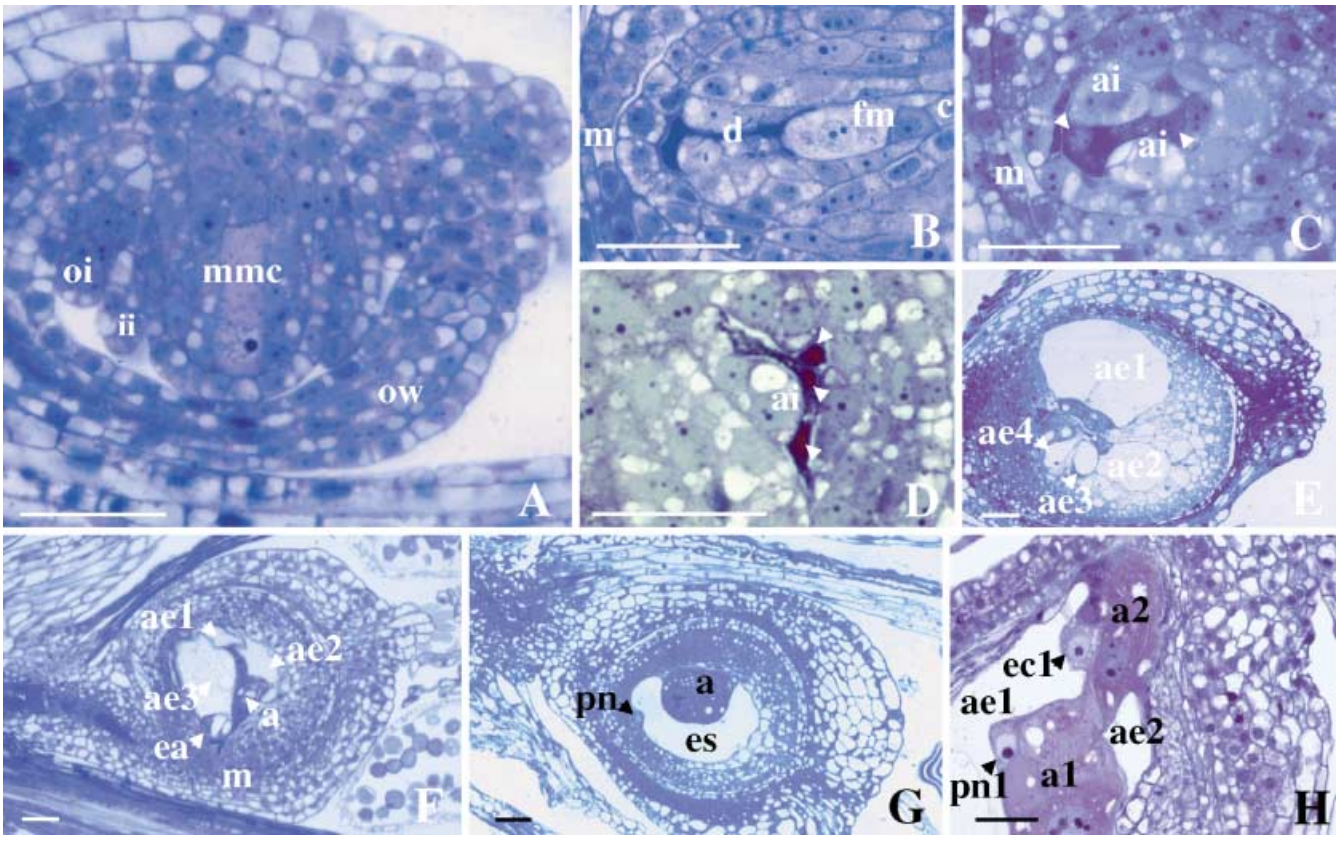

Fig. 1A–H Semi-thin sections of *Poa pratensis* ovaries (*bars* 40 µm). **A, C–F** Apomictic genotypes; **B, G, H** sexual genotypes. **A** Megaspore mother cell (*mmc*) immediately before meiosis, genotype 33. **B** Functional (*fm*) and degenerated (*d*) megaspores, genotype 19. **C** Aposporous initials (*ai*) surrounding megaspores (*white arrows*) at the end of meiosis, genotype RS7-3. **D** Aposporous initial (*ai*) after the first mitotic division to form an aposporous embryo sac; the degenerate megaspores residue is dark blue (*white arrows*), genotype 29. **E** More aposporous embryo sacs (*ae1*, *ae2*, *ae3*, *ae4*) randomly arranged, genotype 7. **F** Three aposporous embryo sacs (*ae1*, *ae2*, *ae3*), only one (*ae3*) is correctly oriented, genotype 15. **G** Antipodal cells (*a*) and polar nuclei (*pn*) of a sexual embryo sac (*es*). **H** Micropylar portion of ovule with two aposporous embryo sacs (*ae1*, *ae2*) differentially oriented, genotype 10. Abbreviations: *oi* outer integument, *ow* ovary wall, *a* antipodal cells, *ae* aposporous embryo sac, *ai* initial cell of aposporous embryo sac, *c* calaza, *d* degenerated megaspore, *ea* egg apparatus, *ec* egg cell, *es* sexual embryo sac, *fm* functional megaspore, *m* micropyle, *pn* polar nuclei

tials. These findings suggest that: (1) parthenogenesis is contingent on apospory but not vice-versa; (2) the traits are controlled by distinct genetic factors.

The main objection to the first point could be that the present data set was of limited size. However, numerous reports that haploid parthenogenesis is usually low in *P. pratensis* (Brittingham 1943; den Nijs and Winkelhorst 1992; Mazzucato et al. 1996; Barcaccia et al. 1998) lend weight to this hypothesis.

The segregation data indicate that about half the progeny plants had parthenogenic capabilities, a finding that can be explained by a genetic model for the control of parthenogenesis based on a dominant allele present in a single copy in one parent. The high variability of the measured degree of parthenogenesis, however, argued against the assumption that parthenogenesis is a discrete genetic trait. The apparent contradiction can be solved by considering parthenogenesis as a trait of low hereditability or hypothesizing a genetic control with the involvement of unlinked modifier genes (Matzk 1991a). Several lines of evidence support the latter hypothesis. In *Arabidopsis*, studies indicated that fertilization-independent seed development may be initiated by mutations in single genes (Luo et al. 1999; Vielle-Calzada et al. 1999). The wild type of these genes is thought to repress autonomous embryo and seed development. The high variation of autonomous endosperm development scored in individuals bearing mutant alleles of these genes indicates that the expression of parthenogenesis may be influenced by unlinked modifier genes. Albertini et al. (2001) have recently estimated the number of genes controlling the degree of parthenogenesis by multiple regression analysis. Molecular marker segregation data showed that a minimum of four genes from the sexual parent and one from the apomictic parent influence the expression of parthenogenesis.

Apospory was detected in 18 of the 38 individuals. However, detection of the trait was not simple, as individuals varied in the number of embryo sacs and the timing of the appearance of aposporous initials. Studies on large populations will be of value in determining whether these aspects represent measurable features of the de-

gree of apospory. If they do, studies on the relationship between the number of aposporous initials, number of unreduced embryo sacs, and parthenogenesis, should shed light on the genetic control of apomixis and suggest strategies for its manipulation.

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