Sexual reproduction is the default mode in apomictic *Hieracium* subgenus *Pilosella*, in which two dominant loci function to enable apomixis

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SUMMARY

Asexual seed formation, or apomixis, in the *Hieracium* subgenus *Pilosella* is controlled by two dominant independent genetic loci, *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*). We examined apomixis mutants that had lost function in one or both loci to establish their developmental roles during seed formation. In apomicts, sexual reproduction is initiated first. Somatic aposporous initial (AI) cells differentiate near meiotic cells, and the sexual pathway is terminated as AI cells undergo mitotic embryo sac formation. Seed initiation is fertilization-independent. Using a partially penetrant cytotoxic reporter to inhibit meioisis, we showed that developmental events leading to the completion of meiotic tetrad formation are required for AI cell formation. Sexual initiation may therefore stimulate activity of the *LOA* locus, which was found to be required for AI cell formation and subsequent suppression of the sexual pathway. AI cells undergo nuclear division to form embryo sacs, in which *LOP* functions gametophytically to stimulate fertilization-independent embryo and endosperm formation. Loss of function in either locus results in partial reversion to sexual reproduction, and loss of function in both loci results in total reversion to sexual reproduction. Therefore, in these apomicts, sexual reproduction is the default reproductive mode upon which apomixis is superimposed. These loci are unlikely to encode genes essential for sexual reproduction, but may function to recruit the sexual machinery at specific time points to enable apomixis.

Keywords: apomixis, seed, apospory, fertilization, embryo, endosperm.

INTRODUCTION

Seed development in angiosperms usually occurs by sexual reproduction. This involves the formation of male and female floral organs, in which meiotic reduction, nuclear mitosis and differentiation occur to give rise to male sperm cells and the female gametophyte or embryo sac. Double fertilization occurs within the embryo sac, whereby the female egg and central cell nuclei each fuse with a sperm cell to initiate development of the embryo and endosperm compartments of the seed.

By contrast, some angiosperms form seeds asexually by apomixis, a process that gives rise to seedlings that are genetically identical clones of the mother. Apomixis is generally controlled by a few dominant loci with unknown identity and function (Koltunow and Grossniklaus, 2003; Ozias-Akins and van Dijk, 2007). We use *Hieracium* species (Asteraceae; Figure 1a) to understand how apomixis is manifested and controlled (Bicknell and Koltunow, 2004). Apomixis in *Hieracium* is characterized by mitotic

Figure 1. Cytological analysis of reproduction in the apomict *H. praealtum* (R35) and derived irradiation mutants.

(a) Stages of R35 capitulum development. Scale bars = 1 cm (top left) and 100 μm (individual floret).

(b–e) Cleared ovules from the apomict parent R35 at capitulum stages indicted at bottom right. Scale bars = 20 μm (b) and 100 μm (c–e).

(f–j) Mutant 179 (*LOAlop*). (j) Fertilized embryo sac. Scale bars = 20 μm (f, h, i) and 100 μm (g, j).

(k–n) Mutant 134 (*loaLOP*). Scale bars = 20 μm (k), 100 μm (l) and 50 μm (m, n).

(o–r) Mutant 115 (*loalop*). (q) Fertilized embryo sac. Scale bars = 20 μm (o) and 100 μm (p–r).

Abbreviations: ai, aposporous initial cell; cc, central cell; dm, degenerating megaspores (with arrowheads); e, egg; em, embryo; en, endosperm; end, endothelium; es, embryo sac; fm, functional megaspore; int, integument; ne, nucellar epidermis.
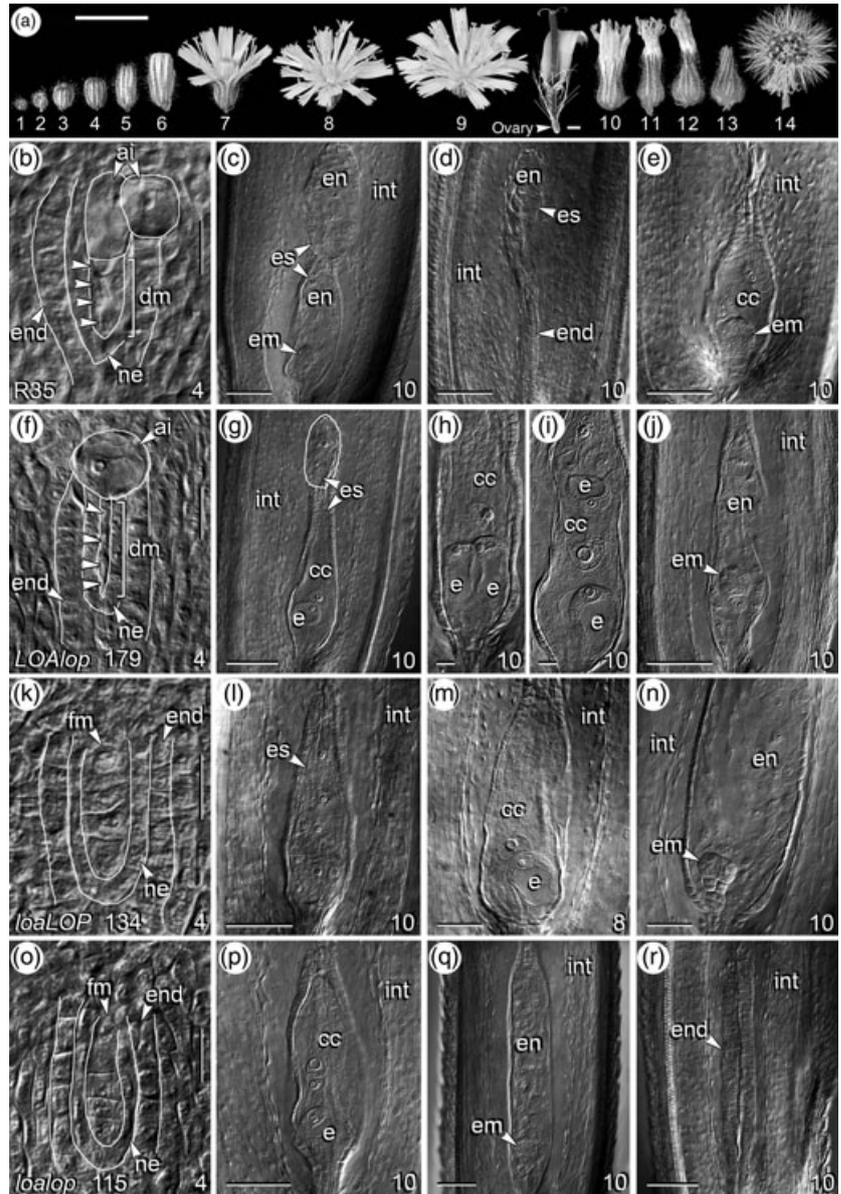
development of embryo sacs without prior meiosis, followed by fertilization-independent (autonomous) embryo and endosperm formation. Two mechanisms of mitotic embryo sac formation, termed diplospory and apospory, have evolved in two subgenera, *Hieracium* and *Pilosella*, respectively, which also include sexual species (Figure S1; Fehrer *et al.*, 2007a). We have focused on the self-incompatible *Hieracium* subgenus *Pilosella*, in which apomictic and sexual species co-exist in two divergent chloroplast haplotype network groups (Figure S1; Fehrer *et al.*, 2007b).

Sexual reproduction initiates first in the ovules of aposporous subgenus *Pilosella* species with formation of a megaspore mother cell (MMC) and meiosis. It is unknown if these events are a prerequisite for initiation of apospory, but somatic AI cells differentiate close to sexually determined cells. Nuclear mitosis of AI cells during aposporous embryo sac formation and subsequent embryo sac expansion towards meiotic cells correlate with the cessation of sexual gametophyte development. The apomictic embryo sacs assume the position of the degraded sexual products (Koltunow *et al.*, 1998a, 2000). The autonomous seed development observed in apomictic Asteraceae is rare in apomictic plants (Bicknell and Koltunow, 2004; Ozias-Akins and van Dijk, 2007).

Apomixis in *Hieracium* subgenus *Pilosella* is genetically controlled by two dominant independent genetic loci termed *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*; Catanach *et al.*, 2006). The developmental roles of these loci during seed formation and the genes they encode are unknown. Sexual reproduction is not completely

eliminated in apomictic subgenus *Pilosella* species. Rare, sexually derived progeny called 'off-types' are produced, including sexual hybrids and progeny exhibiting higher and lower ploidy states relative to the parental apomict (Bicknell *et al.*, 2003; Bicknell and Koltunow, 2004; Ozias-Akins and van Dijk, 2007; Fehrer *et al.*, 2007b). This suggests interplay between sexual and apomictic pathways in these apomicts. Developmental marker analyses have shown that gene expression programs are indeed shared during reproduction in sexual and apomictic *Hieracium* species once the AI cell has undergone mitotic nuclear division to form aposporous embryo sacs and also during subsequent mitotic events of autonomous embryo and endosperm formation (Tucker *et al.*, 2003). This suggests that apomixis may recruit the sexual machinery, and various models have been proposed for hypothetical apomixis gene function (Koltunow and Grossniklaus, 2003; Bicknell and Koltunow, 2004). It is not known whether the loci controlling apomixis in *Hieracium* subgenus *Pilosella* species are also essential for the events of sexual reproduction.

Developmental analysis of a unique set of *Hieracium* apomixis mutants that have lost either or both of the dominant *LOA* and *LOP* loci provides the opportunity to establish the roles that these loci play in seed development and to evaluate interactions between apomixis and sexual reproduction. Catanach *et al.* (2006) generated 79 *Hieracium* apomixis mutants by gamma irradiation and used them to identify markers associated with *LOA* and *LOP*. On the basis of phenotypic analyses at the whole-plant level, examining seed set and seedling ploidy by flow cytometry, mechanistic assumptions concerning derivation of the observed progeny classes were made, sub-dividing them into three phenotypic mutant classes. One class appeared to have lost the ability to form unreduced gametophytes but retained fertilization-independent seed initiation (*loaLOP*). A second class required pollination to set seed (*LOAlop*). The third phenotypic class appeared to have lost both components of apomixis (*loalop*). Comparative use of amplified fragment length polymorphisms (AFLPs) as a genomic fingerprinting tool between the apomict and the mutants identified clusters of markers linked to *LOA* and *LOP* loci that were present in the apomict but lost in the mutants (Catanach *et al.*, 2006). However, the mutants and their progeny were not developmentally analyzed in that study to determine the cell types and changes in reproductive development involved in expression of the observed phenotypes. Therefore, the type and number of developmental parameters that these loci influence are unknown.

Here, we examined the developmental differences between the apomict used for irradiation and a subset of mutant plants containing the smallest regions of linked marker loss at *LOA* and *LOP* loci. The developmental roles of the *LOA* and *LOP* loci were identified in terms of the timing of their function during ovule development and the cell types that they influence to enable apomixis. We also established that initiation of sexual reproduction is a prerequisite for initiation of apomixis. Furthermore, we observed that sexual reproduction remains intact in mutants when *LOA* and *LOP* loci are deleted, indicating it is the default reproductive mode in apomictic aposporous *Hieracium*. *LOA* and *LOP* are therefore unlikely to encode factors that are essential for sexual reproduction. These findings have enabled us to propose mechanistic pathways incorporating the roles of *LOA* and *LOP* and their interaction with the sexual pathway that explain the generation of both apomictic progeny and rare sexually derived 'off-type' progeny in *Hieracium* subgenus *Pilosella* species.

RESULTS

Apomixis in *H. praealtum* (R35), the plant used for seed irradiation

Taxonomic examination revealed that the apomict used for seed irradiation containing single copies of the *LOA* and *LOP* loci was *H. praealtum* rather than *H. caespitosum* as previously described by Catanach *et al.* (2006). Karyotype analysis (Mukai *et al.*, 1990) revealed that this plant contains 35 chromosomes rather than the 36 chromosomes expected for a tetraploid with a base chromosome number of 9 (Fehrer *et al.*, 2007a,b; Krahulcová *et al.*, 2009). The plant was renamed R35, and its karyotype is distinguished by a long chromosome (Figure S1). R35 and other karyotyped experimental plant material used in this study were mapped to the subgenus *Pilosella* chloroplast haplotype network using *trnT–trnL* sequences (Figure S1; Fehrer *et al.*, 2007a,b).

Developmental analysis of apomixis in R35 was performed across the stages of capitulum development shown in Figure 1(a). Sexual reproduction initiated first, with differentiation of a single MMC in ovules of stage 2 capitula, and meiosis occurred so that tetrads were evident in stage 3 and 4 capitula. One to four large aposporous initial (AI) cells differentiated close to cells completing meiosis (Figure 1b). Nuclear mitosis occurred in some AI cells, and these aposporous embryo sacs expanded towards the degenerating megaspores. This coincided with the degeneration of all four megaspores and the cessation of sexual female gametophyte development (Figure 1b). Multiple aposporous embryo sac formation was frequent in R35, as was AI cell persistence and/or differentiation in later stages (Figure 1c and Table 1). Embryo sac collapse was also observed (Figure 1d). These features were also evident in C36 and D36, two other apomictic species grown at the same time (Table 1).

Autonomous seed formation in the apomict R35 was precocious, commencing in 37% of the embryo sacs as soon as egg and central cells developed in unopened florets (stage 6; Figure 1a). By the time of petal senescence, 2% of ovules had quiescent egg and central cells (Table 1), with the remainder undergoing embryogenesis (Figure 1e). Apomictic

Table 1 Analysis of embryo sac and early seed development in cleared ovaries of wild-type and mutant plants (%)[a]

Plant identity	Phenotype[b]	AI Stage 6	AI	Multiple ES	Collapsed ES	Immature ES (2–8 nuclei)	EC + CC	EM + EN	Endosperm no embryo[c]	Abnormal embryos	% seed germination per capitulum
						Stage 10					
P36	Sexual	0	0	0	6	8	86	0	0	0	62 (×D36)
A35	Apomict	1[d]	4	1	15	7	5	55	17	0	nd
D36	Apomict	21(st8)	22	12	23	2	6	57	5	0	64
C36	Apomict	26	23	20	6	3	0	85	6	0	79
R35	*LOALOP*	45	36	19	39	5	2	50	4	5	53
R35 × A35	*LOALOP*	ND	35	19	35	1	1	60	4	0	ND
133[e]	*LOALOP* (?)	8	9	0	0	0	0	1	1	100	ND
125[e]	*LOALOP*	32	6	2	49	26	9	10	6	69	ND
179	*LOAlop*	50	43	14	58	3	38[f]	0	0	0	0
179 × A35	*LOAlop*	ND	35	18	55	5	25	12	2	4	31
179 × P36	*LOAlop*	ND	ND	ND	ND	ND	ND	ND	ND	ND	8
136[e]	*LOAlop* (?)	67	60	27	8	0	7	0	0	0	0
116[e]	*LOAlop* (?)	ND	9	3	0	0	0	0	0	0	0
138	*LOAlop*	83(st8)	64	48	21	7	72[g]	0	2[h]	0	0
156	*LOAlop*	28	14	4	58	12	15	0	0	0	0
143[e]	*LOAlop*	71	44	19	40	22	1	0	0	0	0
118	*LOAlop*	28	9	5	41	3	56	0	0	0	0
144	*LOAlop*	25	14	5	30	12	59	0	0	0	0
119[e]	*LOAlop*	31	38	12	58	9	30	0	3	0	0
134	*loaLOP*	0	0	0	12	8	38	39	4	20	10
134 × P36	*loaLOP*	0	0	0	16	5	3	74	2	17	31
134 × A35	*loaLOP*	ND	ND	ND	ND	ND	ND	ND	ND	ND	18
146	*loaLOP*	0	0	0	28	13	35	13[i]	10	43	ND
165	*loaLOP*	0	0	0	43	23	21	6[i]	6	80	ND
152	*loaLOP*	0	0	0	71	15	3	11	0	86	ND
135	*loaLOP*	0	0	0	36	7	23	33	0	32	ND
124	*loaLOP*	0	0	0	48	30	13	8	1	71	ND
115	*loalop*	0	0	0	48	20	31	0	1	0	0
115 × P36	*loalop*	0	0	0	56	11	2	28	4	0	24
168[e]	*loalop*	0	0	0	61	17	17	0	1	0	0

AI, aposporous initial cells with one nucleus; ES, embryo sac with more than one nucleus; EC + CC, egg cell and central cell present in embryo sac; EM + EN, embryo and endosperm present in embryo sac; ND, not determined.

[a]Approximately 80–200 ovules from unpollinated florets (unless stated otherwise) collected from a minimum of three individual plants were examined per stage. Multiple embryo sacs were found in an ovule at stage 10 and all were scored.

[b]*LOALOP* indicates both loci are functional; *LOAlop* indicates *LOP* is dysfunctional; *loaLOP* indicates *LOA* is dysfunctional; *loalop*, indicates both loci are dysfunctional.

[c]Cellular endosperm-like cells present in the embryo sac but an embryo not observed.

[d]A35 initiates AIs early. Most coalesce into a single embryo sac at stage 6.

[e]Significant ovule deformity.

[f]3.5% of embryo sacs contained two egg cells.

[g]5% of embryo sacs contained two or three egg cells.

[h]Found only in a secondary chalazal embryo sac.

[i]Seed initiation frequency increased at later stages.

development in R35 is very similar to that in D36 and C36. Four centrally located *LOA*-linked sequence characterized amplified region (SCAR) markers and one of the three *LOP*-linked SCAR markers examined are also conserved in these three apomictic species (Figure 2 and Table S1).

Apomixis mutants differ from apomict R35 at two distinct time points in ovule development

For developmental analyses in this study, we used a subset of 19 of the original 79 irradiation mutants generated by Catanach *et al.* (2006). Each is named using a three-number code (Table 1). The selected mutants exhibit the smallest regions of marker loss at *LOA* or *LOP* loci (Figure 2). These mutants, with a differing extent of marker loss at each locus, should represent an allelic mutant series. However, gamma irradiation causes point mutations, deletions, translocations and inversions throughout the genome. In addition to alterations at *LOA* and *LOP*, the mutants are therefore likely to carry changes in other parts of the genome that may also influence reproductive events. We
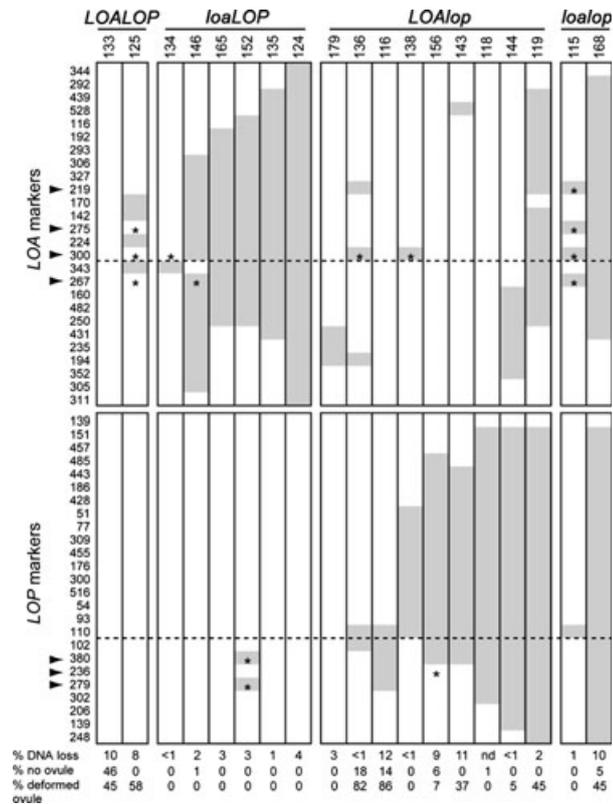
Figure 2. Sub-division of the 19 mutant plants into phenotypes based on analyses performed in this study.
Upper-case *LOA* and *LOP* indicate functional loci. Mutant plants are listed in vertical columns; horizontal rows indicate AFLP markers. Shaded regions show marker loss. Dotted lines are the central regions of each locus (Catanach *et al.*, 2006). Mutants 115, 125 and 133 had revised phenotypes; phenotypes of mutants 136 and 116 were inconclusive. Arrowheads indicate SCAR markers examined in the mutant set, and asterisks indicate differences in SCAR marker presence relative to original AFLP determinations by Catanach *et al.* (2006).

reasoned that, if common reproductive features were observed in irradiation mutants and these correlated with common regions of marker loss at each locus, it would be possible to establish consensus functions for the *LOA* and *LOP* loci. The 19 mutants were initially examined cytologically at informative stages of capitulum development, and grouped according to similarities in developmental phenotypes (Figure 2 and Table 1).

Nine mutants had defects in ovule development of varying severity and penetrance that ranged from an inability to form an ovule to various structural aberrations in ovule formation, including altered funiculi, deformed integuments, absence of micropyle and endothelium, and an inability to fully expand in the ovary cavity (Figure 2 and Figure S2). These phenotypes did not appear to correlate with specific regions of marker loss, and thus are likely to result from the influence of unlinked mutations. Four plants were excluded from further analyses. Mutants 125 and 133

were re-scored as apomicts because AI cell formation and autonomous embryos were observed despite ovule defects. In mutant 136, aposporous embryo sac formation also occurred in ectopic locations (Figure S2). In both the 116 and 136 mutants, severe ovule deformity appeared to compromise completion of aposporous embryo sac formation, and thus the potential for seed development could not be evaluated (Figure S2).

The remaining 15 mutants were found to be defective at two stages of apomictic development (Figure 2 and Table 1). They had either lost the ability to form AI cells or initiate autonomous seed development, or both. This led to partial or complete reversion to sexual reproduction. The analysis of these 15 mutants, and their progeny, summarized below, provided information concerning the roles of *LOA* and *LOP* in enabling apomixis, and their interactions with the events of sexual reproduction.

LOP stimulates autonomous seed formation but is not essential for fertilization-induced seed development

Seven mutants (179, 138, 156, 118, 143, 144 and 119; Table 1) formed large rounded AI cells at stages 3 and 4 of capitulum development during the later events of sexual meiosis and megaspore selection. The events of aposporous embryo sac formation were similar to the apomicts R35 (Figure 1f,g). However, in unfertilized embryo sacs, the egg and central cells remained arrested in development and autonomous seed initiation was not observed (Figure 1h,i and Table 1). Mutants 179 and 138 developed two or three eggs in individual embryo sacs at low frequency (Table 1) in both the micropylar (Figure 1h) and chalazal regions (Figure 1i). Loss of autonomous embryo formation was associated with loss of the capacity to autonomously form endosperm in all of the mutants (Table 1). All mutants except 179 lost *LOP*-linked markers (Figure 2). These mutants were assigned the phenotype *LOAlop* (where uppercase letters indicate the locus is functional).

Seeds did not form following emasculation in cut capitula or in the absence of pollination in uncut capitula of the *LOAlop* mutants. They only initiated seed formation after fertilization (Figure 1j and Table 2). The hybrid nature of the progeny was evident following pollination of the yellow-flowered R35 mutants with the red-flowered plant A35 because they formed dual-colored flowers (Figure S3). As both parents contain 35 chromosomes (Figure S1), we used flow cytometry to compare the ploidy of hybrid seedlings to that of the parent R35 plant to which we assigned a relative ploidy value of 1×. The majority of the progeny (61/63; Table 2) had a higher ploidy equating to 1.5× that of the apomict R35 parent, indicating that fertilization of unreduced eggs had occurred, to give rise to higher-ploidy progeny. The events of aposporous embryo sac formation are therefore functional in these mutants, but autonomous seed development is no longer functional.

Table 2 Analysis of the ploidy level of seedlings arising with and without cross-pollination using flow cytometry, relative to R35 with a ploidy value of 1×

Plant	Phenotype	Cut capitula[a] Seed germination (*n* from *n* capitula)	Ploidy 0.5×	Uncut capitula Seed germination (*n* from *n* capitula)	Ploidy 0.5×	1×	1.5×	Crossed with A35[b] Seed germination (number per capitulum)	Ploidy 0.5×	1×	1.5×
R35	*LOALOP*			168/7		20/20		17–25		25/25	
179	*LOAlop*	0/3		0/6				3–14		1/13[c]	11/13, 1/13 (1.75×[d])
138	*LOAlop*	0/6		1/9[e]			1/1[e]	13–33		1/20[c]	19/20
156	*LOAlop*	0/3		0/3				10–14			4/4
143	*LOAlop*	0/6		0/9				5–11			8/8
118	*LOAlop*	0/3		0/3				12			13/13
144	*LOAlop*	0/3		0/3				8			7/7
134	*loaLOP*	3/3	3/3	30/6	26/26			5–12	12/39	27/39	
134 × P36	*loaLOP*								7/30	22/30	1/30 (1.25×[f])
146	*loaLOP*	3/7	3/3	2/20	2/2			19		7/8	1/8[f]
165	*loaLOP*	0/3		2/3	2/2			1–3	1/4	3/4	
152	*loaLOP*	0/3		1/4	1/1			0–3		3/3	
135	*loaLOP*	4/5	4/4	17/9	9/9			4–9	1/13	12/13	
124	*loaLOP*	2/6	2/2	6/17	1/1			0–3		1/1	
115	*loalop*			0/6							
115 × P36	*loalop*							4–9		29/29	
168	*loalop*	0/3		0/3				3–8		4/4	

[a]Stigmas and anthers above the ovaries were removed by cutting with a razor blade to prevent cross-pollination. This may damage florets.
[b]Plants pollinated with sexual P36 are indicted.
[c]Predicted to result from fertilization of a meiotically derived egg.
[d]Predicted to result from pollination of an unreduced egg with an aberrantly reduced pollen grain (Krahulcová *et al.*, 2004; Fehrer *et al.*, 2007b).
[e]Predicted to result from accidental cross-pollination.
[f]Predicted to result from alterations in ploidy during female meiosis in the 35 chromosome plant or pollination with an aneuploid/unreduced pollen grain.

Cytological analysis of reproduction in 11 of the hybrid progeny (1.5×) derived from a cross between *LOAlop* mutant 179 and apomictic A35 identified eight progeny with the *LOAlop* phenotype and three with the apomictic phenotype *LOALOP* (Table 3). Unreduced gametophytes lacking the autonomous seed formation function can therefore be fertilized with pollen carrying the *LOP* factor to restore functional apomixis.

These analyses reveal several important features of *LOP* activity. *LOP* function is required for fertilization-independent seed formation. Loss of *LOP* function does not impair the ability to form unreduced aposporous embryo sacs, and causes a partial reversion to sexual reproduction such that fertilization is required for seed formation. Finally, genetic information at the *LOP* locus is not essential for the fertilization-induced events of embryo and endosperm formation.

Loss of *LOA* function results in sexual female gametophyte development and gametophytic segregation of autonomous seed formation (*LOP*)

Six mutants that had lost *LOA*-linked markers and retained most *LOP*-linked markers (*loaLOP* 134, 146, 165, 152, 135 and 124; Figure 2) were unable to form AI cells (Figure 1k). MMCs differentiated and meiosis occurred, as did megaspore selection and the subsequent mitotic events of sexual female gametophyte development (Figure 1l), leading to formation of a mature embryo sac (Figure 1m). In the absence of pollination, embryo and endosperm formation initiated in approximately half of the gametophytes of these mutants once the egg and central cells formed (Figure 1i and Table 1). The eggs and central cells were quiescent in the remainder (Figure 1m and Table 1). These data suggest that, in the absence of AI formation, sexual female gametophyte formation is functional, and that *LOP* segregates gametophytically amongst meiotically reduced gametophytes to induce autonomous seed formation (Table 1).

Functional sexual female gametophyte development and segregation of *LOP* function in the gametophytes of these mutants was confirmed following analysis of the progeny arising from them in the presence and absence of pollination. All of the progeny from unpollinated *loaLOP* mutants had half (0.5×) of the relative ploidy of the R35 parent (Table 2), and were smaller in stature (Figure S3a). SCAR markers linked to *LOA* were found to segregate in the 0.5×

Table 3 Progeny arising from selected mutants with and without cross-pollination

Plant	Cross	Capitula (n)	Germination per capitulum n (%)	Flow cytometry		Progeny examined[b] (n)	Phenotype (n)
				n	ploidy[a]		
R35	*LOALOP* (UP)	7	24 (53)	20	1.0	15	*LOALOP* (15)
179	*LOALop* (UP)	6	0 (0)	0	0	0	0
179	*LOALop* × P36	3	4.1 (10)	ND	ND	ND	ND
179	*LOALop* × A35	7	9.2 (10)	11	1.5	11	*LOALOP* (3)
							LOALop (8)
				1	1.0	1	*LOALop* (1)
				1	1.75[c]	1	*LOALop* (1)
134	*loaLOP* (UP)	7	4.4 (10)	28	0.5	15	Male and female sterility[d]
134	*loaLOP* × P36	4	9.8 (23)	22	1.0	22	*loalop* (22)
				7	0.5	7	Male and female sterility[d]
				1	1.25[e]	1	*loaLOP* (1)
134	*loaLOP* × A35	7	7.8 (18)	27	1.0	23	*LOALOP* (4)
							loaLOP (4)
							LOALop (7)
							loalop (8)
				12	0.5	12	Male and female sterility[d]
115	*loalop* (UP)	6	0 (0)	0	0	0	0
115	*loalop* × P36	3	9.9 (24)	29	1.0	11	*loalop* (11)

UP, unpollinated.
[a]Ploidy relative to R35 parent with 35 chromosomes with a value of 1.0×.
[b]Cytological analysis by ovule clearing.
[c]Predicted to result from pollination of an unreduced egg with an aberrantly reduced pollen grain (Krahulcová *et al.*, 2004; Fehrer *et al.*, 2007b).
[d]Predicted to be *loaLOP*, sterility made phenotyping difficult.
[e]Predicted to result from alterations in ploidy during female meiosis in the 35-chromosome plant or pollination with an aneuploid/unreduced pollen grain.

progeny obtained from the unpollinated 134 *loaLOP* mutant, but all retained the LOP 380 marker (Table S2).

When *loaLOP* mutant 134 was pollinated, seed initiation was observed in most ovules, instead of only half in the absence of pollination (Table 1). Pollination of *loaLOP* mutant 134 and the other *loaLOP* mutants also resulted in two progeny classes: progeny with a ploidy of 0.5× relative to the apomict parent and hybrid progeny with a relative ploidy reflecting that of the apomict parent (1×; Table 2). Most of the 0.5× progeny examined (10/11) contained the LOP 380 marker, irrespective of whether they were pollinated by a sexual or an apomict plant, while most (13/15) of the hybrid progeny (1.0×) did not contain the marker (Table S2). Thus, *LOP* appears to segregate gametophytically in the *loaLOP* mutants, promoting autonomous seed formation.

These data led us to conclude that *LOA* acts early in the tissues of the ovule to stimulate AI cell formation. Loss of *LOA* function results in loss of AI cell formation and the re-emergence of sexual female gametophyte development. Thus, *LOA* has a role in suppressing this part of the sexual pathway. Loss of *LOA* function also leads to segregation of *LOP* in meiotically reduced gametophytes, where it

functions to stimulate autonomous seed formation. *LOP* can therefore activate seed formation in both unreduced gametophytes formed via apospory and in meiotically reduced gametophytes.

Gametophytes carrying *LOP* in R35 avoid fertilization

Phenotyping of the 23 hybrid plants obtained from a cross between *loaLOP* mutant 134 and the apomict A35 recovered four functional apomicts (*LOALOP*; Table 3). Therefore, meiotically reduced gametophytes are receptive to fertilization and the assimilation of genetic factors that enable restoration of *LOA* function in the progeny. It was not possible to determine from this cross whether the gametophytes inheriting *LOP* were as receptive to fertilization as those that did not inherit *LOP*. For example, the four plants phenotyped as *LOALOP* may have arisen from fertilization of a female gametophyte lacking *LOP* with A35 sperm containing both apomictic loci (Table 3). We examined progeny arising from a cross between *loaLOP* mutant 134 and sexual P36 to determine the receptivity of *LOP*-carrying female gametophytes to fertilization. Surprisingly, the 22 hybrid progeny arising from this cross were all sexual in phenotype (*loalop*; Table 3). They therefore arose from fertilization of female

gametophytes lacking functional *LOP* activity, indicating that gametophytes carrying the dominant *LOP* allele do not participate in fertilization.

We examined the frequency that eggs carrying the dominant *LOP* allele can be fertilized by pollinating R35 with a diploid sexual accession (O18) containing a single copy of a green fluorescent protein (*GFP*) marker gene. Thirteen hybrids containing the *GFP* marker were recovered from over 2000 seedlings, and all had a ploidy equating to a value of 0.75× relative to the R35 parent. None of the hybrids contained the LOP 93 marker. This marker is tightly linked with the gametophytic trait of autonomous seed formation, as all 244 polyhaploid plants (0.5× relative ploidy) generated from wild-type R35 using a previously established protocol (Bicknell *et al.*, 2003) possessed it. *LOP*-carrying gametophytes in *H. praealtum* R35 are thus rarely fertilized.

Components stimulating embryo and endosperm formation are tightly linked at the *LOP* locus

If two independent components at the *LOP* locus were required for autonomous embryo and endosperm formation and were not tightly linked, they would be expected to segregate with high frequency as individual components soon after seed initiation in developing gametophytes of unpollinated *loaLOP* mutants. Cytological analyses of seed initiation in unpollinated *loaLOP* mutants indicated that this was not the case (Table 1). Seeds with cellular-like endosperm tissue were observed at low frequency, as found in the parental apomict R35. This was considered to be related to embryo abortion in these seeds, because a corresponding category of seeds containing only embryos was not observed (Table 1). Thus the determinants at the *LOP* locus required for autonomous embryo and endosperm development are tightly linked in *H. praealtum* (R35).

Sexual reproduction is the default mode in apomictic *H. praealtum* R35

Catanach *et al.* (2006) identified 25 mutants that required fertilization to produce hybrid progeny. Two mutant plants cytologically examined here, 115 and 168, underwent the events of sexual female gametophyte development and were unable to initiate autonomous seed formation (Figure 1o–r and Table 1). After pollination with apomict A35 or sexual P36, hybrids were produced that had the same relative ploidy as the R35 parent (Tables 2 and 3 and Figure S3). Analysis of the progeny of plant 115 crossed with sexual P36 revealed that all 11 plants analyzed underwent sexual reproduction (Table 3), with no evidence of apomictic reproduction. Loss of both *LOA* and *LOP* function in R35 therefore leads to loss of apomixis and complete reversion to sexual reproduction, indicating that sexual reproduction appears to be the default reproductive mode in apomictic *H. praealtum*.

The sexual events of megasporogenesis are required for initiation of apomixis

The initiation of sexual reproduction precedes the initiation of apomixis in all apomictic subgenus *Pilosella* species we have studied to date. We examined whether the sexual pathway was required for apomixis initiation by disturbing the events of meiosis. Sexual P36 and apomicts D36 were transformed with a chimeric cytotoxic *SPOROCYTE-LESS:BARNASE* (*SPL:BARNASE*) gene. These plants were selected for this experiment because our previous work indicated that the Arabidopsis *SPL* promoter directed low levels of linked *GUS* gene expression to the MMC in P36 and D36 ovules (Figure 3g). Expression was not observed in the AI cell, aposporous embryo sacs or other parts of the plant except the anther, in which expression was high in pollen mother cells (Tucker *et al.*, 2003).

Partial penetrance of the *SPL:BARNASE* gene was observed in three sexual and three apomictic hemizygous plants examined, indicating that meiosis was not completely inhibited in anthers and ovules. Pollen development was defective in plants containing the chimeric *SPL:BARNASE* gene. In contrast to the typical development of meiotic tetrads (Figure 3a) and free pollen grains (Figure 3b) in anthers of control plants, meiosis aborted at the first meiotic division and/or during tetrad formation in transgenic plants (Figure 3c–e). Mature pollen grains were found at a frequency of 2–5% in transgenic sexual and apomictic plants relative to untransformed controls (Figure 3f).

SPL:BARNASE expression in ovules resulted in meiotic arrest at various stages, enabling examination of the correlation between the ability to form an AI cell at a particular stage of meiosis in the apomict. AI cells were not observed in any of the analyzed sexual transgenic or control plants at any stage of development (Figure 3h and Table 4). Seed germination per capitulum provided a measure of the impact of the transgene on developmental success relative to controls (Table 4).

In apomictic D36 plants, AI cells begin forming when meiotic tetrads are evident, which is also the case for R35 (Figure 1b and Table 4). In transgenic D36 plants containing the *SPL:BARNASE* construct, AI cell formation and growth were not evident in ovules containing arrested MMCs or dyads (Table 4). AI formation and enlargement primarily correlated with the appearance of a tetrad of megaspores (Figure 3i and Table 4). This trend was observed over several capitula stages, during which ovules containing arrested MMC and dyad cells in addition to ovules with degenerated megaspores and AI cells were identified in the same capitulum (Table 4). AI cells continued to enlarge and underwent nuclear division coinciding with degeneration of all four megaspores (Figure 3j). These data indicate that the meiotic events of megasporogenesis are required for initiation of apospory.
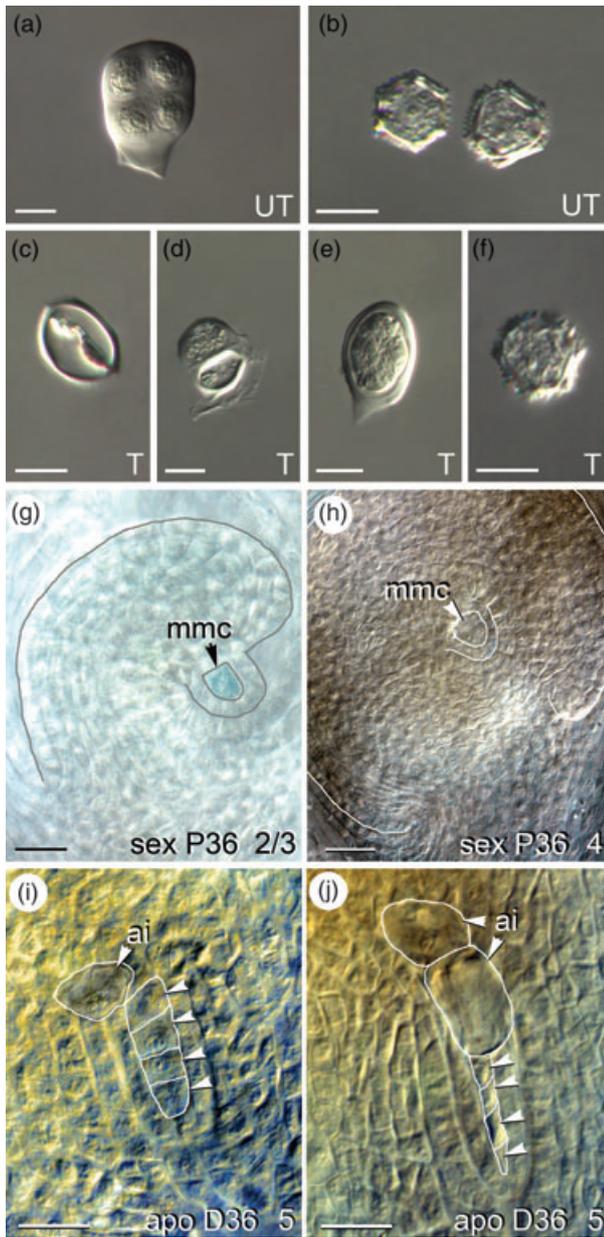
Figure 3. The *SPOROCYTELESS:BARNASE* construct results in meiotic arrest at various stages in the anther and ovule of sexual *H. pilosella* (P36) and apomictic *H. piloselloides* (D36).

(a, b) Pollen formation in untransformed (UT) control plants, showing a tetrad and free pollen, respectively.

(c–e) Pollen arrest at various stages occurred in transgenic (T) plants.

(f) Rare mature pollen grain in a transgenic (T) plant.

(g) *SPOROCYTELESS:GUS* expression in sexual P36; the same pattern is observed in apomict D36.

(h) Cleared ovule of a transgenic sexual P36 plant showing developmental arrest of meiosis.

(i) AI cell near arrested meiotic tetrad (arrowheads) in apomict D36.

(j) AI cells in a transgenic D36 ovule at the same stage as (i) undergoing megaspore degeneration.

Capitula stages are indicated at bottom right. ai, aposporous initial cell; mmc, megaspore mother cell. Scale bars = 25 μm.

DISCUSSION

Apomixis is superimposed upon a default sexual pathway in subgenus *Pilosella*

We have shown that initiation of sexual reproduction in ovules is required before apomixis can begin in subgenus *Pilosella* by using a construct to inhibit the events of meiosis. Developmental analysis of apomixis mutants has shown that the dominant *LOA* locus is required for AI cell formation and suppression of the adjacent sexual pathway, while the dominant *LOP* locus is required for autonomous embryo and endosperm formation. The consequence of loss of function at either *LOA* or *LOP* loci in an apomict is partial reversion from the apomictic mode of reproduction to sexual reproduction. Loss of function in both loci in an apomict leads to re-emergence of sexual reproduction. Collectively, these data are consistent with the conclusion that sexual reproduction is functional and is the default reproductive mode in the apomict (Figure 4). Thus, the dominant *LOA* and *LOP* loci are unlikely to contain determinants that are essential for the sexual program function. Instead, it appears from our analyses that *LOA* and *LOP* interact with the default sexual pathway and function to suppress it (Figure 4b). Our current understanding of the functions of the *LOA* and *LOP* loci and their interactions with the default sexual pathway in apomicts is discussed further below.

Sexual cues are required for initiation of apomixis and activation of *LOA* function

We have demonstrated that sexual cues enabling meiotic tetrad formation in ovules are required for AI cell formation. By inference, these cues promote the activity of the dominant *LOA* locus, which functions to stimulate AI cell formation (Figure 4b). Sexual female gametophyte development in Arabidopsis and maize is known to be both independent of and inter-dependent on the events in surrounding sporophytic ovule tissues (Gasser *et al.*, 1998; Tucker and Koltunow, 2009; Garcia-Aguilar *et al.*, 2010; Olmedo-Monfil *et al.*, 2010). Whether the sexual events of meiosis provide signals that activate expression of determinants at the *LOA* locus, or whether *LOA* determinants are already expressed and completion of meiosis and megaspore selection provides an interacting factor to enable apomixis is not clear. The sexual cues may be hormonal, as transgenically induced alterations in ovule structure related to auxin perception have previously been observed to influence the frequency and timing of apomixis initiation in *Hieracium* (Koltunow *et al.*, 2001). Alternatively, they may have an epigenetic basis. Epigenetic pathways appear to be involved in restricting sexual female gametophyte formation to a single cell in both Arabidopsis and maize (Garcia-Aguilar *et al.*, 2010; Olmedo-Monfil *et al.*, 2010). Mutations in Arabidopsis

Table 4 Analysis of AI cell formation in ovules of transgenic plants arrested at various stages of megasporogenesis

Plant	Stage 2				Stages 3 + 4						Stages 5 + 6						Germination	
	ON	MMC/AI	DY/AI	Tetrad[a]/AI	ON	MMC/AI	DY/AI	Tetrad/AI	MSS/AI	MSD/AI	ON	MMC/AI	DY/AI	Tetrad/AI	MSS/AI	MSD/AI	CN	%/capitulum[b]
P36 sexual	87	84/0	3/0	0/0	113	0/0	5/0	32/0	71/0	0/0	99	0/0	0/0	0/0	78/0	21/0	2	62 (D36)
P36 Spl:RNase (6)	67	67/0	0/0	0/0	92	1/0	9/0	40/0	34/0	8/0	83	0/0	1/0	25/0	49/0	8/0	2	0
P36 Spl:RNase (10)	69	68/0	1/0	0/0	93	20/0	7/0	31/0	24/0	11/0	94	13/0	9/0	39/0	20/0	13/0	2	21 (R35)
P36 Spl:RNase (11)	72	71/0	1/0	0/0	80	20/0	11/0	11/0	6/0	32/0	68	17/0	0/0	0/0	9/0	42/0	2	0 (A35)
D36 apomict	105	103/1[c]	2/0	0/0	81	1/1[c]	6/0	21/12	5/5	48/43	100	0/0	0/0	0/0	5/5	95/73	5	64
D36 Spl:RNase (1)	102	101/0	1/0	0/0	95	39/1[c]	18/0	28/4	0/0	10/4	120	14/1[c]	8/0	10/4	10/4	78/54	5	32
D36 Spl:RNase (4)	99	99/0	0/0	0/0	102	5/0	11/0	28/3	15/11	43/28	109	4/0	4/0	28/6	18/5	55/21	7	22
D36 Spl:RNase (6)	104	102/0	2/0	0/0	92	10/1[c]	15/0	22/9	11/8	34/27	117	9/0	7/1[c]	20/8	9/8	72/37	11	40

ON, ovule number; MMC, megaspore mother cell; AI, aposporous initial cell, undergoes nuclear division at stage 4–5 in wild-type; DY, dyad meiosis stage; MSS, megaspore selection. In sexual plants, these undergo nuclear division at stage 5; MSD, megaspore degeneration and embryo sac formation; CN, capitula number.
[a]Tetrad (quartet of megaspores).
[b]Parentheses indicate pollen parent; the apomict was unpollinated.
[c]Slightly enlarged cells were seen near sexual cells but they did not have the large round AI cell phenotype.

ARGONAUTE 9, a gene involved in a non-cell-autonomous small RNA pathway, result in specification of multiple megaspore like-cells resembling AI cells, but these do not appear to progress further and the sexual pathway continues (Olmedo-Monfil *et al.*, 2010). In maize, inactivation of a DNA methylation pathway results in proliferation of multiple aposporous-like embryo sacs that do not undergo functional apomixis (Garcia-Aguilar *et al.*, 2010). It remains to be determined whether epigenetic pathways contribute to the promotion of *LOA* function or whether such epigenetic elements are the functional determinants at the *LOA* locus in *Hieracium*.

LOA is required for AI cell formation, sexual suppression and inheritance of *LOA* and *LOP* in eggs

LOA function is required for AI cell formation (Figure 4b). The functional identity of the AI cell is currently unknown. It does not appear to have an MMC-like identity as it does not possess detectable levels of callose in its cell wall nor does it express various MMC and meiotic markers (Tucker *et al.*, 2001; Okada *et al.*, 2007). Our attempts to determine whether the AI cell has megaspore-like identity have failed because of the absence of suitable markers. For example, the Arabidopsis functional megaspore marker *pFM:GUS* (Huanca-Mamani *et al.*, 2005) is not expressed in the functional megaspore of sexual *Hieracium* or in the AI cell of apomicts, but is expressed in degenerating megaspores (Figure S4). What is apparent is that *LOA* mediates the initiation of mitotic gametophyte development in a somatic cell(s). We have found that the events of AI cell nuclear division and aposporous embryo sac formation share gene expression programs with mitotic events of gametogenesis in reduced embryo sacs of sexual *Hieracium* plants (Tucker *et al.*, 2003). Thus *LOA* may recruit the sexual machinery involved in these mitotic events to enable a similar program in the AI cell.

Suppression of the sexual gametophyte pathway occurs soon after AI cells differentiate, and this may be a direct function of the *LOA* locus encoded by one or more determinants. Alternatively, it may be an indirect effect, for example related to acquisition of nutrients from the degenerating quartet of megaspores or possibly physical factors associated with their physical displacement as the aposporous embryo sac forms. Little is known about the process of megaspore selection, and the AI cell may function as a 'fifth' megaspore, potentially recruiting processes normally involved in megaspore degeneration. Analysis of the T-DNA insertion apomixis mutant *loa1* has suggested that signaling between sexual and AI cells may be required for directional expansion of aposporous embryo sacs towards sexual cells. In *loa1*, AI cells differentiate in positions quite removed from sexually programmed cells. Subsequent aposporous gametophyte development is defective, with either no expansion towards sexual cells
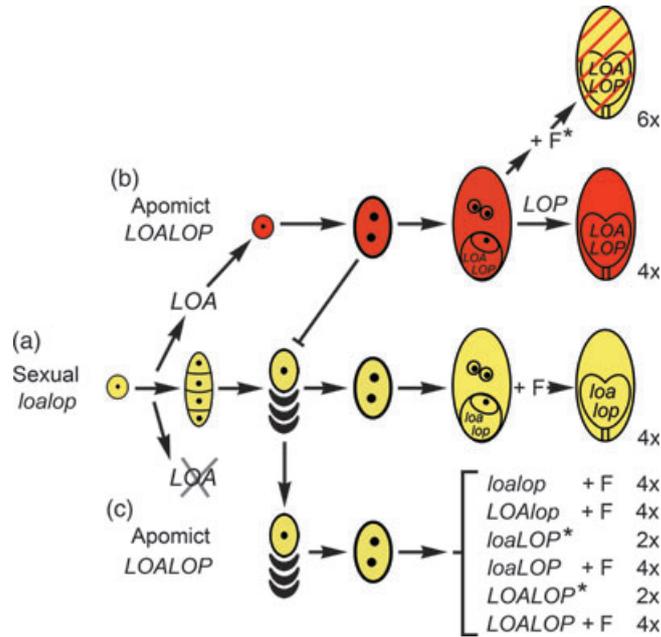
Figure 4. Pathways for the generation of apomictic and sexually derived 'off-type' progeny from a tetraploid apomict containing single copies of *LOA* and *LOP* loci crossed with a tetraploid sexual pollen donor.

(a) The sequence of events comprising the default sexual pathway in the apomict is indicated in yellow.

(b) Timing of *LOA* and *LOP* activity and their interactions with the sexual pathway to generate the apomictic pathway (in red). Fertilization of the unreduced egg in the apomict results in progeny with increased ploidy and a different genetic constitution to the parent (red stripes on a yellow seed).

(c) Generation of 'off-type' progeny. Incomplete penetrance of *LOA* enables completion of meiosis in the apomict as sexual reproduction is no longer suppressed and segregation of *LOA* and *LOP* occurs. Incomplete functional penetrance of *LOP* results in reliance on fertilization to generate viable seed. All progeny in (c) arise from meiotically derived eggs. +F indicates fertilization, and the asterisks indicate gametophytes unlikely to be fertilized in *H. praealtum* R35.

or expansion away from them, and functional aposporous embryo sacs rarely form (Okada *et al.*, 2007).

Another important outcome of *LOA* function is that the *LOA* and *LOP* loci are both inherited in the eggs of the mitotically formed embryo sacs and subsequently the embryos derived from autonomous seed formation. These dominant, unlinked components would normally segregate during the meiotic events of sexual gametophyte development.

LOP enables autonomous seed initiation

LOP is gametophytic in action, and operates to promote fertilization-independent embryo and endosperm formation. Determinants enabling these events appear to be tightly linked at the *LOP* locus in *H. praealtum*. Autonomous embryo and endosperm formation have been reported to be linked in apomictic *Erigeron* species (Asteraceae; Noyes *et al.*, 2007) but unlinked in apomictic *Taraxacum* (Asteraceae; van Dijk *et al.*, 2003). *LOP* function is not dependent on egg cell and central cell ploidy, as both reduced and unreduced gametophytes containing *LOP* give rise to fertilization-independent seeds. Its role may be associated with removal of a repressive block or stimulation of pathways similar to those induced by the products of fertilization.

Members of the Arabidopsis FERTILIZATION-INDEPEN-DENT SEED (FIS) Polycomb group complex are involved in suppression of seed initiation in the absence of fertilization. It has been hypothesized that alterations in the function of *FIS* class genes may give rise to autonomous seed initiation in apomicts (Rodrigues *et al.*, 2008). We have eliminated as a *LOP* candidate the *Hieracium FERTILIZATION INDE-PENDENT ENDOSPERM* (*HFIE*) gene, which is a homolog of one of the core members of this complex. *HFIE* is not linked to the *LOP* locus because it is present in all of the *LOAlop*, *loaLOP* and *loalop* deletion mutants examined (Figure S4). Also, down-regulation of *HFIE* does not result in autonomous endosperm formation in sexual *Hieracium*. However, *HFIE* is essential for the viability of fertilization-induced seeds in sexual plants, and the frequency and viability of autonomously initiated seeds in apomicts (Rodrigues *et al.*, 2008).

The presence of *LOP* in the unreduced gametophytes of apomictic *H. praealtum* (R35) and derived mutants deters the formation of hybrids via fertilization. The mechanism underlying this effect is unknown. It may relate, in part, to the timing of *LOP* action in stimulating embryo and endosperm development prior to flowering, as seed development in R35 begins before the opening of the flower. However, the suppression of hybridization by *LOP* in R35 does not appear

to be typical of all apomictic *Hieracium* subgenus *Pilosella* species. Bicknell *et al.* (2003) noted the formation of higher-ploidy hybrids when apomicts D36 and A36 were pollinated. By inference, the unreduced egg cells in these apomicts presumably carried both dominant *LOA* and *LOP* alleles. Timing of *LOP* action and differences in alleles and the genetic backgrounds of apomicts are likely to affect the potential for *LOP* to suppress or accommodate fertilization of eggs in which it is present.

Pathways generating apomictic and non-maternal 'off-type' progeny

In summary, sexual reproduction is the default reproductive mode in apomicts, and generation of apomictic progeny requires early sexual events to activate *LOA* (Figure 4a), which functions as a positive regulator of AI formation and as a repressor of sexual reproduction (Figure 4b). AI-derived unreduced embryo sacs subsequently require genetically independent *LOP* function to initiate, but not to maintain, fertilization-independent embryo and endosperm formation. Depending on the species, events associated with *LOP* function may interfere with fertilization of unreduced embryo sacs. *LOA* and *LOP* are not essential for sexual function, but they are likely to recruit the sexual machinery to enable apomixis generating a pathway resembling a curtailed sexual pathway in which meiosis and fertilization are absent (Figure 4b).

The dominance of the apomictic pathway over the sexual pathway is likely to be dependent on successful function of dominant *LOA* and *LOP* loci. Formation of sexually derived 'off-type' progeny in apomicts would occur if the *LOA* and *LOP* loci are incompletely penetrant in ovules (Figure 4c). This would result in continuation of meiosis and segregation of the *LOA* and *LOP* loci in gametophytes, and also reliance upon, or an increased opportunity for, fertilization. The progeny types predicted to arise from a combination of these events in a tetraploid apomict with single copies of *LOA* and *LOP* following pollination with a tetraploid sexual plant are shown in Figure 4(c). They exhibit varying reproductive strategies and ploidy states. Additional types would occur if an apomict is used as the pollen donor. Retention of sexual reproduction as the default pathway in apomictic *Hieracium* and the capacity for cross-pollination with other sexual and apomictic *Hieracium* subgenus *Pilosella* species provide mechanisms for variation and speciation to survive environmental changes.

Future prospects

The observation that sexual reproduction is the default reproductive mode in apomictic *Hieracium* subgenus *Pilosella*, upon which apomixis has been superimposed, has encouraging prospects for the installation of apomixis in agronomic crops, providing that *LOA* and *LOP* prove to be functionally transferable to other species. Compelling

questions relate to the identity of the genes present at the *LOA* and *LOP* loci that are required for apomixis. Genomic sequences of *LOA* and *LOP* loci are being isolated using linked markers. The mutants characterized here are useful tools to test candidate genes at each locus by transgenic complementation to establish which genes restore apomixis.

EXPERIMENTAL PROCEDURES

Plant material

Hieracium species were sourced from collections in Europe and from the field in New Zealand. Apomictic *Hieracium praealtum* (Vill.) Zahn R35 ($3x + 8 = 2n = 35$), originally labeled as *H. caespitosum*, was obtained from Dijon, France. Apomicitc *Hieracium caespitosum* Dumort C36 ($4x = 2n = 36$) was obtained from the Krkonoše Mountains in the Czech Republic. Apomictic *Hieracium aurantiacum* L. A36 ($4x = 2n = 36$) was obtained from a field sample in Central Otago, New Zealand, and apomictic *Hieracium aurantiacum* L. A35 ($3x + 8 = 2n = 35$) was obtained from Zürich, Switzerland. Sexual *Hieracium pilosella* L. P36 ($4x = 2n = 36$) was obtained from Caen, France, and another sexual isolate P36 (CR) was obtained from the Czech Republic. *H. piloselloides* Vill. D36 ($4x = 2n = 36$) was obtained from a wild population in Steiermark, Austria. Apomictic *H. piloselloides* D18 ($2x = 2n = 18$) was obtained from an experimental polyhaploid population generated as described by Bicknell *et al.* (2003). The chloroplast *trn*T–*trn*L sequences for A35, A36, R35, P36, D36 and D18 were identified as described by Fehrer *et al.* (2007a), mapped to the chloroplast haplotype network (Figure S1) and submitted to GenBank (accession numbers HM852135–HM852140). A diploid sexual accession of *H. onegense* (O18) was sourced from Bulgaria. This plant was transformed with the *GFP* marker gene using the plasmid pBIN m-gfp5-ER (provided by J. Haseloff, Department of Plant Sciences, University of Cambridge). A transformant with a single copy of the transgene was used as a pollen parent in crosses with the apomict R35. Gamma deletion mutants were generated by Catanach *et al.* (2006).

Plant analyses

The apomict R35 used for irradiation and the 19 irradiation mutants were grown as vegetative explants for cytological analyses of seed formation. All experimental plants used were maintained by micropropagation in culture (Koltunow et al., 1998), and transferred to soil when required. Manual pollinations and seed germination were performed as described by Koltunow *et al.* (1998a, 2000). Emasculations were performed as described by Catanach *et al.* (2006). Developmental staging of capitula and ovary processing were performed as described by Koltunow *et al.* (1998a, 2000). Tissue clearing in methyl salicylate was performed as described by Stelly *et al.* (1984), and observed by Nomarski differential interference contrast (DIC) under a Zeiss Axioskop microscope (http://www.zeiss.com/). Images were captured using a Spot II camera (Diagnostic Instruments, http://www.diaginc.com). DNA loss was determined in mutants by flow cytometry (Bicknell *et al.*, 2003), as was the ploidy of progeny derived from various crosses. The presence of SCAR markers central to the *LOA* and *LOP* loci were examined, and primers and the PCR conditions used are listed in Table S3.

Plant transformation and genetic analyses

The *SPOROCYTELESS:GUS* construct has been described by Tucker *et al.* (2003), and the same *SPOROCYTELESS* promoter region was

inserted into the *BARNASE* cassette described by Koltunow *et al.* (1998b). Plant transformation was performed as described by Bicknell and Borst (1994). Primary transgenic plants containing the inserted genes were maintained by micropropagation in tissue culture. GUS analyses were performed as described by Tucker *et al.* (2003). Polyhaploids were generated from the apomicts as described by Bicknell *et al.* (2003). Analyses of *LOP*-linked marker presence in hybrid populations and polyhaploids were performed as described by Catanach *et al.* (2006).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Chloroplast haplotype network location and karyotypes of *Hieracium* subgenus *Pilosella* plants used in this study.

Figure S2. Defects in the progression of apomixis in mutants with structurally defective ovules.

Figure S3. Morphology of *Hieracium* mutant plants and their progeny.

Figure S4. Expression of *pFM–GUS* in the ovule of apomictic *H. praealtum* R35.

Figure S5. DNA gel-blot analysis of apomicts and irradiation mutants with defective *LOA* and *LOP* loci to examine linkage of *HFIE* to the *LOP* locus.

Table S1. Presence (+) or absence (−) of *LOA*- and *LOP*-linked SCAR markers in plants of various *Hieracium* subgenus *Pilosella* species.

Table S2. Segregation of *LOA* and *LOP* markers in the progeny of the 134 mutant (*loaLOP*).

Table S3. List of primers for SCAR-PCR and PCR conditions.

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