Characterising genetic loci associated with loss of apomixis in *Hieracium*

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Andrew S. Catanach

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Abstract of a thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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by Andrew S. Catanach

Most plant species strictly utilise sexual reproduction to generate genetically diverse seed to ensure adaptation of their descendents to the changing demands of their environment. Some species, however, have largely dispensed with sexual reproduction, opting instead to propagate clonally via apomixis, and maintain genotypes that are presumably already sufficiently adapted. Researchers of apomixis have long been attracted to the phenomenon as a biological curiosity, but more significant investigative attention is now being paid to it due to its ability to fix heterosis and therefore enable the economic production of high yielding hybrid varieties of the world's major crops.

Despite the strong motivation to integrate apomixis into seed-production systems, previous attempts to introgress the trait from wild apomictic relatives, or to induce it via mutagenesis, have yet to produce commercial apomictic varieties. It now appears likely that the successful transfer of apomixis into sexual crops will first require the elucidation of the molecular mechanisms, employed by native apomicts, that enable the avoidance of key components of sexual reproduction that otherwise serve to generate genetic diversity.

The Apomixis Programme at Crop & Food Research, Lincoln, aims to elucidate the genetics and molecular mechanisms of apomixis in *Hieracium* subgenus *Pilosella*. Two major deviations from sexual reproduction are required: the avoidance of meiosis, or apomeiosis, and the avoidance of fertilisation, or parthenogenesis. Segregating populations demonstrate independent segregation of apomeiosis and parthenogenesis. However, conventional mapping approaches towards determinants of apomixis in other species have often encountered significant difficulties posed by suppressed recombination at their loci. Alternative genetic resources of *Hieracium* were therefore generated using T-DNA and transposon mutagenesis, and deletion mutagenesis.

The present research focused on identifying and generating molecular maps of apomixis loci by screening deletion mutant panels of two genotypes, *H. glaciale* and *H. caespitosum* with secondary digest amplified fragment length polymorphism (SDAFLP). Identified loci

were verified by their associations with apomixis in segregating populations, and SDAFLP markers were sequenced and converted into sequence characterised amplified regions (SCARs). The utility of the SCARs for the future isolation of BAC clones was determined by their presence or absence in key mutants.

The identification and characterisation of three loci whose loss was associated with loss of parthenogenesis in *H. glaciale* are described in Chapter 3. One locus transmitted to hybrid progeny as a determining locus and the other two transmitted as modifying loci. A T-DNA mutant of the *H. glaciale* background, which was included in the mutant panel, was found to carry a deletion at the determining locus. Findings that indicate that T-DNA insertions are not linked to the deletion are set out in Chapter 4, and somaclonal variation is suggested as an alternative cause of the deletion. Chapter 5 describes the use of deletion mutagenesis to identifiy two loci in *H. caespitosum*: one is associated with loss of apomeiosis (*LOA*) and the other with loss of parthenogenesis (*LOP*). Key mutants were screened with SDAFLP to obtain high densities of markers at *LOA* and *LOP* and markers that were predicted to be nearest the determinants were sequenced and converted into SCARs. One sequenced marker at *LOP* is likely to partially code for a regulatory gene. *LOA* and *LOP* segregated independently among hybrid progeny in strong association with apomeiosis and parthenogenesis respectively. Segregation distortion was characteristic of both loci, while recombination did not appear to be suppressed. Chapter 6 discusses how the findings of this research may be used to investigate the evolution of apomixis and to isolate its genetic determinants. It also discusses some challenges that might be encountered in the future during the engineering of apomixis in commercial crop species.

Keywords: Apomeiosis, apospory, amplified fragment length polymorphism (AFLP), deletion mutagenesis, embryogenesis, mapping, meiosis, molecular markers, parthenogenesis.

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Chapter 1 Introduction

1.1 Overview

Apomixis, or clonal seed formation, although not common, is a character widely distributed throughout the plant kingdom. A notable apomict is *Taraxacum officinale*, the common dandelion, found throughout New Zealand, but very likely originating from a single apomictic accession. Other apomicts include many cultivated varieties of citrus, mango and blackberries. Although apomixis is found in a number of species of the grass family Poaceae, it is present in no cereal crops of economic significance. Several authors have proposed that the introduction of apomixis into important crop species would have profound benefits to crop production worldwide (Koltunow et al., 1995a; Toenniessen, 2001). The commercial use of high-yielding hybrid crops is currently economically prohibitive for many cereal, forage grass, fibre and forestry crops due to the expense of existing hybrid seed production systems. Apomixis would enable hybrid lines with fixed heterosis to be grown through successive generations without any need to control pollination. Furthermore, apomixis may allow the commercial production of seed from one-off sports with useful attributes, leading to more rapid and economic development of new varieties.

Species that undergo an apomictic mode of reproduction are diverse, and, to some extent, so too are the known processes of apomixis. Sporophytic apomixis is the formation of somatic embryos directly from ovular tissue outside of the embryo sac, and is utilised by *Citrus sinensis* (Koltunow et al., 1995b). The endosperm, which is derived from the embryo sac, is necessary for the maturation of the adventitious embryos. This can result in their competing against zygotic embryos for this resource. The more common gametophytic apomixis is characterised by apomeiotic formation of unreduced embryo sacs which then develop into embryos without fertilisation. Gametophytic apomixis, which is the major focus of this review, is most prevalent in the families Poaceae, Asteraceae and Rosaceae. It is under investigation mainly in genera of Poaceae, including *Poa* (Matzk, 1991; Barcaccia et al., 1998), *Tripsacum* (Leblanc et al., 1995a), *Brachiaria* (Lutts et al., 1994; Pessino et al., 1997) and *Pennisetum* (Dujardin and Hanna, 1984; Ozias-Akins et al., 1993), and in genera of Asteraceae including *Taraxacum* (Richards, 1970; Tas and van Dijk, 1999; van Dijk et al., 1999), *Erigeron* (Noyes and Rieseberg, 2000) and *Hieracium*

(Bicknell and Borst, 1994; Bicknell et al., 2001). Other well documented apomicts include *Hypericum* (Matzk et al., 2001) and *Ranunculus* (Nogler, 1984).

Gametophytic apomixis can be further categorised into diplospory and apospory. Both of these forms of gametophytic apomixis include the development of unreduced apomeiotic embryo sacs. The derivations of the embryo sacs, however, differ in each. In diplosporous systems, megaspores that have avoided meiotic reduction develop into embryo sacs with unreduced ploidy. Apospory is characterised by the formation of one or more aposporous initial cells derived from nearby nucellar tissue, which develop into unreduced aposporous embryo sacs displacing the reduced sexual megaspore. A minority of gametophytic apomicts, including *Hieracium*, autonomously produce endosperm but most, including those of the family Poaceae, require fertilisation of the central cell for endosperm development.

The effect apomixis has on progeny was observed by Mendel, and was documented at least as early as the first decade of the 20th century (Ostenfeld, 1906; Rosenberg, 1907; Nogler, 2006), but the phenomenon generally remains poorly understood. More recently, with its promise to revolutionise seed production, and to some extent due to the capabilities offered by molecular biology, apomixis has moved from a biological curiosity to an important topic of mainstream research undertaken from a number of different approaches. Attempts to introgress the trait into maize (Savidan, 2001) and pearl millet (Dujardin and Hanna, 1989) via wide crossing with apomictic relatives were undertaken with only limited success. Another approach is to induce apomixis in non-apomicts by mutation. Genes were found in *Arabidopsis*, that, when mutated, enabled aspects of seed development to proceed without fertilisation (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998), but only to abort later. These genes play key roles in sexual as well as apomictic reproduction, and additional key regulators are likely to be required for apomixis. However, the mutant *Arabidopsis* lines were not apomictic *per se*. It is apparent, therefore, that for apomixis to be successfully introduced into commercially important crop species, the gene sequences and genetic mechanisms utilised by native apomicts need to be revealed. Histological investigations into gene expression differences between sexual and apomictic ovules in *Arabidopsis* and *Hieracium* indicate that the two modes of reproduction are essentially parallel processes (Tucker et al., 2003). There are, however, two key deviations: avoidance of meiosis and avoidance of fertilisation. Regulatory

elements uniquely expressed in apomicts at these points in embryo development are yet to be identified.

Attempts to isolate regulatory elements for apomixis via gene expression studies (Vielle-Calzada et al., 1996; Leblanc et al., 1997; Chen et al., 1999; Pessino et al., 2001; Rodrigues et al., 2003) have typically yielded low numbers of verifiable expression differences, providing further evidence that apomixis and sexuality share developmental pathways. Differences that were found in these studies are yet to be shown to play critical roles in apomixis; instead they may be genes whose expression is regulated downstream from expression of major determinants. A number of attempts towards the isolation of genetic determinants of native apomicts have also been made via linkage mapping. Almost all of these mapping studies revealed apomixis determinants to be resident at large non-recombinant loci. Finer genetic mapping of non-recombinant loci using segregating populations is not possible, and more novel approaches are required to isolate the residing genetic determinants.

The aim of this research was to identify genetic loci associated with apomixis using deletion mapping. Deletion breakpoints for molecular mapping are analogous to recombinational breakpoints, and their utility for mapping genomic regions with suppressed recombination in plants has been previously demonstrated (Marais, 1992; Liharska et al., 1997). Two panels of gamma-irradiation induced *Hieracium* deletion mutants with loss of apomixis were generated. Secondary digest amplified fragment length polymorphism (SDAFLP) (Knox and Ellis, 2001) was applied as a molecular comparative screening technique to the panels alongside wild type control plants, to identify DNA markers that were frequently absent in association with loss of apomixis. Progeny analyses demonstrated co-segregation of the identified markers with apomixis, or with the components of apomixis, apomeiosis and parthenogenesis. The focus was placed on gaining DNA sequence as close as possible to genetic determinants involved in apomixis, while characterising the genomic nature of the apomixis loci, and their respective effects on aspects of the trait.

1.2 Literature Review

1.2.1 Apomixis in *Hieracium* in the context of sexual reproduction

Species of the genus *Hieracium* subgenus *Pilosella* (referred to more simply as *Hieracium* in this thesis) are dicotyledonous perennials, of which most are polyploid and apomictic.

There are, however, some diploid and polyploid sexual species and many hybridise readily with their apomictic counterparts. Most apomictic *Hieracium* are facultative, that is, they undergo some level of sexual reproduction (Koltunow et al., 1998). Furthermore, sexual development proceeds alongside apomictic processes at a low level, and, as outlined above, many developmental genes and processes are shared between the two pathways (Tucker et al., 2003). Apomictic development may therefore be viewed in the context of sexual reproduction. The flower of *Hieracium* consists of a capitulum made up of multiple bisexual florets arranged radially as a flattened disk on a floral receptacle. A mature floret, as discussed in detail by Koltunow et al. (1998), is composed of an inferior and terminal ovary, and, protruding from an external tube of fused petals, a style terminating with a stigma surrounded by a fused ring of lineate stamens (Fig. 1.1).

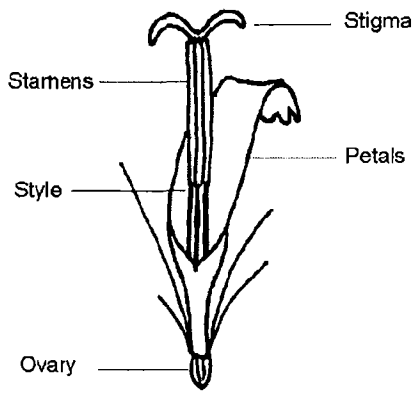


Fig. 1.1 A single floret of a *Hieracium* capitulum (adapted from Koltunow et al., 1998).

During early stages of floral development a single ovule is formed from undifferentiated cells of the ovary wall and a megaspore mother cell differentiates within. The integument of the ovule grows, surrounding the nucellar lobe that contains the megaspore mother cell. During this time the ovule becomes anatropous and the megaspore mother cell undergoes meiosis, resulting in a megaspore, and three micropylar spores (Fig. 1.2 a) which degenerate. The megaspore undergoes the mitotic divisions of megagametogenesis (Fig. 1.2 b) and differentiation into a mature embryo sac of a polygonum type (Fig. 1.3). The development of the reproductive structures of different florets of a single capitulum is initially largely synchronous, but loses synchronicity as the florets mature, with those of the outer whorls of the flower opening first.

Apomictic *Hieracium* utilise apospory and are autonomous with no requirement of fertilisation for endosperm development. The process has been described histologically in detail in two species of the genus, *H. aurantiacum* and *H. piloselloides*, and compared with embryo development of sexual *H. pilosella* (Koltunow et al., 1998). Development of

apomictic gametophytes is similar in the three species. In each, sexual embryo sacs initially develop before being out-competed by aposporous initial cells derived from nucellar tissue (Fig.1.4).

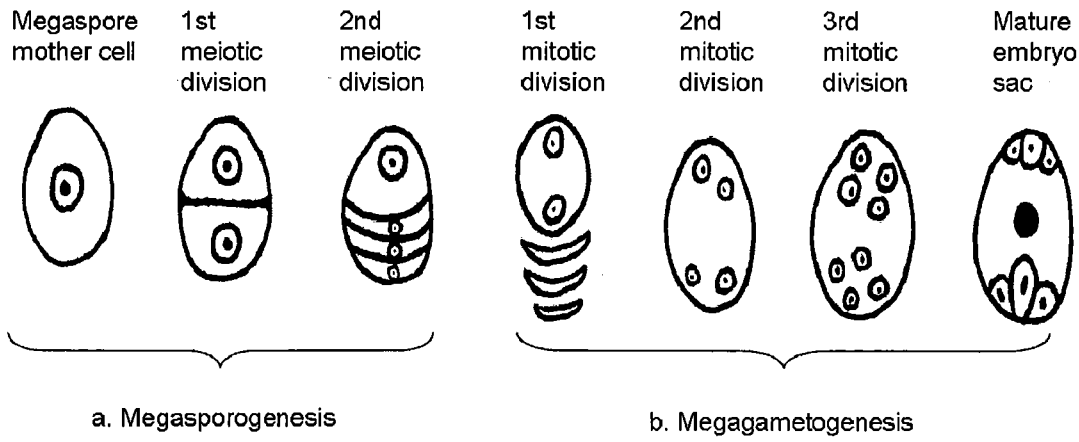


Fig. 1.2 Megasporogenesis (a) and megagametogenesis (b) of the development of a sexual embryo sac.

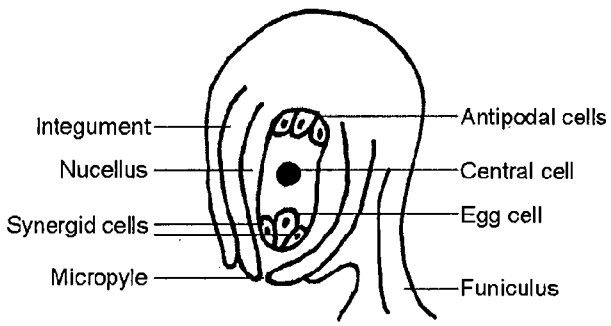


Fig. 1.3 Stylised ovule containing a mature embryo sac of the polygonum type.

Details of the timing of initiation of aposporous development, and the extent to which sexual development proceeds, vary in each species studied. In *H. aurantiacum*, aposporous initial cells form early in ovule development; therefore meiotic structures are rarely observed. By contrast, sexual embryo sac development in *H. piloselloides* proceeds similarly to that of sexual species, and meiosis is observed before degeneration of most meiotic embryo sacs with the later appearance of aposporous initial cells. These expanding cells develop into unreduced embryo sacs occupying the space that would otherwise be occupied by a meiotic embryo sac. Embryogenesis and endospermy then proceed

autonomously but otherwise analogously to sexual meiotic embryo sacs that have undergone fertilisation.

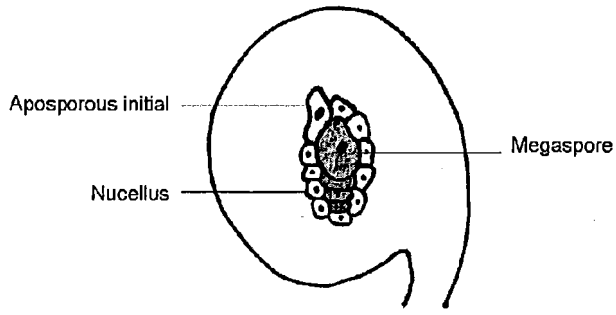


Fig. 1.4 Appearance of an aposporous initial during megaspore development (meiotic tetrad is shaded) in apomictic *H. piloselloides* (adapted from Koltunow et al., 1998).

Apomixis is not the only way that *Hieracium* can reproduce asexually. Plants typically have a low lying sprawling habit, and many members reproduce readily from stoloniferous growth. *Hieracium* is very successful at utilising asexual reproduction and can form large clonal populations, having become a pernicious weed throughout high country of New Zealand and North America. However, it is likely that the on-going success of *Hieracium*, and many apomicts, is dependent to some extent on low levels of sexual reproduction to retain population diversity and to enable adaptation when required. Sexually derived progeny may result from fertilisation of both reduced meiotic and unreduced apomeiotic embryo sacs. Using terminology that is now most prevalent in apomixis literature (Harlan and De Wet, 1975), progeny derived from reduced and unreduced embryo sacs are termed n+n and 2n+n hybrids respectively. Hybrid progeny may be apomictic upon the transmission of genetic control elements of apomixis via reduced or unreduced embryo sacs, or via pollen.

1.2.2 Genetics of apomixis: Allelism, genes involved, and their relationship with sexual processes

The inheritance of apomixis has been investigated in both monocotyledonous and dicotyledonous species, usually by crossing an apomict with a sexual relative and observing transmission of the trait in F1 progeny. Inheritance studies require the homologous pairing and crossing-over of chromosomes during meiosis. Apomixis itself overrides meiotic megasporogenesis; therefore obligate apomicts cannot be used as maternal parents. Facultative apomicts may be used as maternal parents, but this requires a method to distinguish meiotically derived hybrid progeny (n+n), that are required for analysis, from a background of clonal (2n+0), and less commonly, non-meiotically derived

hybrid (2n+n) progeny. Apomicts are often uncompromised in their ability to produce meiotically reduced pollen. Inheritance studies have therefore focused largely on the transmission of the trait through pollen via one-way crosses using a sexual relative as the pistillate parent. This strategy has been used to determine transmission of a wide variety of apomictic processes within diverse lineages, including diplospory in dicotyledonous *Taraxacum officinale* (van Dijk et al., 1999) and monocotyledonous *Tripsacum dactyloides* (Leblanc et al., 1995b), and apospory in dicotyledonous *Ranunculus* (Nogler, 1984), and monocotyledonous *Pennisetum squamulatum* (Ozias-Akins et al., 1993).

Inheritance studies have indicated that in many species the trait segregates as a single dominant locus. After hybridising a sexual and an apomict, followed by successive back-crossing to the sexual parent, Nogler (1984) concluded that apospory in *Ranunculus* resides as an allele of a gene that is possessed by sexual plants. Nogler further postulated that the gene may ordinarily activate a cascade of genes required for the differentiation of the embryo sac from the megaspore, while the allele that gives rise to apomixis could act as an activator of similar genes in a cell of nucellar origin. Consistent with this hypothesis is the direct association of the penetrance of apospory in *Ranunculus* with the proportion of alleles that confer the trait. The proportion of alleles for apospory is in turn affected by the ploidy. For example, a triploid that is simplex for the apospory allele A+ (A+A-A-) has greater penetrance of apomixis conferred than a tetraploid that is simplex (A+A-A-A-), which in turn has greater penetrance conferred than a pentaploid (A+A-A-A-A-). This implies that while apospory is dominant over the sexual condition, the allele that confers sexuality has a suppressing or competing effect. Nogler observed histologically a reflection of inter-allelic competition in the timing of aposporic development relative to meiosis in this species. Early induction of aposporous processes results in a greater suppression of meiotic embryo sac development resulting in a greater proportion of ovules undergoing aposporous development.

It is possible that apospory in *Hieracium* is initiated by alternative alleles of one or more regulatory genes that ordinarily induce embryo sac development in a megaspore mother cell. Such alternative alleles may result in developmental competition between aposporous and sexual processes. Observations in *Hieracium* have indicated a dilution of penetrance in plants with higher ploidy levels (Bicknell, 1997). Furthermore, it appears that two variable aposporic mechanisms, each utilised by different species, are allelic, a conclusion drawn from a 5:1 segregation ratio of the trait among hybrids of triploid apomictic parents that

were simplex for the trait (Bicknell et al., 2000). This has important implications for future research as it raises the possibility of using markers isolated in this study to help locate components of apomixis in other *Hieracium* species.

Whether key determinants of apomixis and sexuality are allelic in *Hieracium* is not certain. Analysis of genes involved in sexual reproduction, however, indicate that instead of utilising alternative regulatory pathways, apospory shares functional expression of at least some genes that are important for sexual processes (Tucker et al., 2003). Genes investigated included the *FIS* (*FERTILISATION INDEPENDENT SEED*) class genes, that were identified from mutations of *Arabidopsis* plants that exhibited features of apomixis. The *FIS* class genes, *MEDEA* (Grossniklaus et al., 1998), *FIS2* (Chaudhury et al., 1997) and *FIE* (*FERTILISATION INDEPENDENT ENDOSPERM*) (Ohad et al., 1996) are of the *Polycomb* group and are believed to form a multimeric repressor complex. The expression of these genes within embryo sacs, embryos and endosperm of both sexual and aposporous derivation were spatially and temporally equivalent. Another gene investigated was *SERK* (somatic embryo receptor kinase) discovered as a marker gene expressed in carrot competent and embryogenic cells (Schmidt et al., 1997). In *Arabidopsis*, *AtSERK1* has a role in the transition to somatic embryogenesis in culture, and is expressed during megasporogenesis, in the megaspore, and during embryogenesis until the heart stage (Hecht et al., 2001). Expression of a homologous *Hieracium SERK*-like (*HpSERK-L*) transcript marks competency of somatic cells, both meiotic and apomeiotic, to form embryo sacs (Tucker et al., 2003). *SERK* genes from different species show a high level of homology with respect to one another, and *HpSERK-L* is likewise strongly similar.

An apomeiotic embryo sac of *Hieracium* may accept the entry of a pollen tube and the transfer of sperm nuclei to participate in a hybridisation event, as if it were meiotically derived. This suggests that apomeiotic embryo sac formation is uncoupled from parthenogenesis, and that apomixis may therefore be determined by more than one gene. Apospory (apomeiosis) and parthenogenesis were uncoupled in F1 progeny at low levels in *Poa pratensis* (Albertini et al., 2001). More recent evidence indicates that five major genes play a role in apomixis in that species, including determinants that initiate apospory and parthenogenesis with modification from genes that are dominant in sexual genotypes that prevent each of the two components (Matzk et al., 2005). The uncoupling of diplospory and parthenogenesis was similarly evident in *Taraxacum*, and an incidence of autonomous endospermy without parthenogenesis indicates further uncoupling of those components of

the trait (van Dijk et al., 1999). In *H. caespitosum* apomeiosis and parthenogenesis segregate independently indicating that each resides at a different locus.

In *Pennisetum*, no separation of the components of apomixis has been found (Ozias-Akins et al., 1993; Ozias-Akins et al., 1998). This may be a reflection of linkage between determinants of the different components of the trait. It remains possible that apomixis may be under multigenic control by complex non-recombinant loci in many species in which the trait appears to be monogenic. While in *H. caespitosum* apomeiosis and parthenogenesis segregate as unlinked determinants, the genetics of the two components of apomixis in other *Hieracium* species is less clear.

1.2.3 Gametophytic apomixis has a close association with polyploidy

Despite a variety of forms and its presence in diverse lineages of angiosperms, gametophytic apomixis is rarely found in diploid plants. Exceptions to this rule are limited primarily to experimentally derived plants, including accessions of an apomictic *Ranunculus* hybrid (Nogler, 1984) and an accession from *H. aurantiacum* (Bicknell, 1997). Both of these examples were polyhaploids derived from polyploids and were likely products of parthenogenetic development of meiotically reduced embryo sacs (n+0). Polyhaploids are often less vigorous than their polyploid counterparts and have other perturbations such as male sterility and low seed set, although Nogler (1984) reported examples of *Ranunculus* polyhaploids that were male fertile and effectively set seed.

While polyploidy is not an absolute requirement for apomixis, there is little evidence of diploid apomicts that have thrived and competed effectively against polyploid counterparts in the wild. The reason for the close association of apomixis with polyploidy is unclear and is a topic of some debate. It is hypothesised that apomixis may have evolved as an escape from sterility - caused by a breakdown of synchrony in gene expression necessary for sexual reproduction - after hybridisation between distant taxa (Carman, 2001). That many apomicts are allopolyploids from inter-specific hybridisation (Asker and Jerling, 1992) lends support to this hypothesis. There are, however, a number of examples in which the use of apomixis cannot be explained purely as an escape from sterility. One such example is the allotetraploid apomict *Hyparrhenia diplandra*, a facultative apomict, which produces low levels of sexual seed (Durand et al., 2000). Furthermore, some well-characterised apomicts show tetrasomic inheritance and therefore appear to be autopolyploids (Haldane, 1930). Apospory segregates by tetrasomic inheritance in *Pennisetum* (Ozias-Akins et al.,

1998); no markers were found linked in repulsion to the ASGR, and tetrasomic inheritance was observed in the diplosporous *Tripsacum* (Grimanelli et al., 1998b), indicating that these species are autopolyploids.

Apomixis has advantages over sexual reproduction in some respects as there is no cost of meiosis and well-adapted populations can quickly prevail. However, models suggest that if apomixis were to be active at a diploid as well as at a polyploid level, a population would be rapidly invaded by apomixis and sexuality would disappear (Marshall and Brown, 1981). Apomixis would effectively lead a species to an evolutionary dead end. Dysfunction of apomixis in diploid plants may be an adaptive mechanism that ensures that sexuality at a low level can occur. Nogler observed that the determinant for apomixis could only be transferred in sperm nuclei in a heterozygous and diploid or polyploid state. Polyhaploid *Ranunculus* could produce fertile pollen but none contained the determinant for apospory. Likewise, apospory was only transmitted maternally through unreduced embryo sacs that developed parthenogenetically (2n+0) or as hybrids (2n+n). Haploid gametes produced only sexual progeny. Nogler suggested that apomixis is gamete-lethal in the haploid state. Apomicts of *Ranunculus* were therefore incapable of forming apomictic diploid hybrids, and their ability to produce maternal seed was diminished compared to their polyploid counterparts. Nogler did, however, suggest that occasional diploid apomicts might feature in the microevolution of polyploid apomicts, as they can hybridise to form 2n+n or 2n+2n hybrids, providing a mechanism of genetic reassortment.

A similar elimination of the genomic region of diplospory from haploid gametes of maize-*Tripsacum* hybrids is also postulated to protect sexual diploid populations from the invasion of apomixis (Grimanelli et al., 1998a). The authors propose a mechanism whereby the transmission of apomixis to reduced female gametes is restricted by a lethal factor that acts in *trans* and is incompletely penetrant, features consistent with a meiotic drive mechanism. A recessive lethal factor appears to cause segregation distortion of apospory in female gametes of *Pennisetum* (Ozias-Akins et al., 1998), and to cause gametophytic selection against parthenogenesis in *Erigeron* (Noyes and Rieseberg, 2000). Diplospory appears to be excluded from haploid gametes of *Erigeron* (Noyes and Rieseberg, 2000) by univalent inheritance, whereby segregation of the chromosome with diplospory into a diploid gamete is strongly favoured. Alternative models, such as incomplete penetrance of apomixis in diploids due to insufficient dosage (Mogie, 1988),

and sterility induced by allelic ratios higher than 0.25 (Noirot, 1993), are not commonly supported by current molecular marker data.

The mechanisms that have evolved to preserve sexuality in diploid populations may be many and varied. Lethality and meiotic drive appear to be common mechanisms in the apomicts investigated so far. In the case of an *Hieracium* apomictic polyhaploid, both pollen and female sterility were demonstrated (Bicknell, 1997), suggesting the presence of a mechanism that prevents further introduction of apomixis into sexual diploid populations. The mechanism that prevents apomixis from invading diploid populations appears to be zygotic lethality in *Hieracium*, rather than gametic lethality. Factors for apomixis were found to be transmitted effectively by haploid gametes to contribute towards functional triploid zygotes (2n+n). By contrast, functional diploid zygotes (n+n) were not seen to develop (Bicknell et al., 2000).

1.2.4 Molecular mapping: what do apomixis loci look like and how have they evolved

Early efforts towards mapping apomixis loci were conducted on apomictic grasses using populations generated while introgressing apomixis into related crop species. Cosegregating restriction fragment length polymorphisms (RFLP) markers were identified from an F1 population of maize-*Tripsacum* hybrids (Leblanc et al., 1995c). RFLP and randomly amplified polymorphic DNA (RAPD) markers were traced through backcross generations of pearl millet carrying apospory introgressed from *Pennisetum squamulatum* (Ozias-Akins et al., 1993), and two markers were found to strictly co-segregate with the trait. The mapping of markers cosegregating with apomixis and components of apomixis has since progressed using F1 segregating progeny in members of the Poaceae including *Poa pratensis* (Barcaccia et al., 1998), *Brachiaria decumbens* (Pessino et al., 1997; Pessino et al., 1998), and members of the Asteraceae including *Erigeron annuus* (Noyes and Rieseberg, 2000), and *Taraxacum officinale* (van Dijk and Bakx-Schotman, 2004).

All species of known apomictic grasses used for mapping are pseudogamous. Fertilisation of the central cell via pollination is therefore required for endosperm development, and segregating progeny are frequently scored for apomixis using cytoembryological analysis to detect apomeiotic structures. By contrast, endospermy is autonomous in *Hieracium* and most other apomictic members of the Asteraceae; apomixis may therefore be scored by assessing the production of germinable seed in the absence of fertilisation. Markers often demonstrate strict cosegregation with apomixis indicating that determinants commonly

reside at large non-recombinant regions of genomic DNA. This is invariably the case for all grass species investigated. By contrast, however, while the locus associated with diplospory in the asterid *Erigeron annuus* is believed to reside on a non-recombinant univalent chromosome, no significant suppression of recombination was reported at the locus associated with parthenogenesis (Noyes and Rieseberg, 2000). Similarly, no suppression was evident at the *DIPLOSPORY* locus of *Taraxacum officinale* (Vijverberg et al., 2004).

A continued effort to map apospory in *P. squamulatum* resulted in the identification of 22 linked RAPD markers, of which 11 were converted into sequence characterised amplified regions (SCARs) (Ozias-Akins et al., 1998). Five of the 11 SCAR markers and one sequence tagged site, ugt197, identified from previous work (Ozias-Akins et al., 1993), hybridised as low copy number sequences. Four of the six low copy markers were found to be hemizygous within apomictic progeny (and completely absent in sexual progeny). This reflects partial hemizygosity of this region, and implies that it is non-allelic to any region of a homologous chromosome. This partially hemizygous region was termed the apospory-specific genomic region (ASGR).

Loci for apospory in related grasses demonstrate a high level of conservation. Two markers linked to the ASGR in *P. squamulatum*, the SCAR marker OPC04 and the sequenced tagged site ugt197, are both linked to apospory respectively in a total of 3 and 8 out of 11 diverse aposporous species of *Pennisetum* surveyed. Neither marker showed hybridisation to DNA of any of 8 sexual species (Lubbers et al., 1994). Furthermore, five of the 12 markers tightly linked to the ASGR in *P. squamulatum* were also tightly linked to apospory in *Cenchrus ciliaris*, a related apomict, and one other marker segregated from the trait at a low frequency. That markers linked to apospory are shared between *Pennisetum* species indicates that they share a common genomic region specific for apospory. The partial hemizygosity shared by the loci of different species is not consistent with apospory evolving repeatedly on multiple occasions. Therefore, the common genomic region for apospory implies a single ancestral origin. If this was the case, apomixis has not precluded interspecific hybridisation events between apomictic species and between apomicts and sexuals. Retention of physically distant markers during hybridisation required that the region be transmitted through many generations as non-recombinant DNA, which is consistent with the non-recombinant natures of the loci that are seen today.

Unexpectedly, the SCAR marker OPC04 is also linked to apospory in *Brachiaria brizantha* (Pessino et al., 1997). Whether the similarity between the apospory loci of *Pennisetum* and *Brachiaria* is more extensive indicating common origins is not known. However, more general sequence similarity and synteny among grass species enabled the use of RFLP probes from maize to generate markers linked to apospory in *Brachiaria.* Sexual and apomictic bulks of F1 progeny were probed with 90 genomic and cDNA clones of maize. The approach proved successful with a total of 61 of the probes hybridising successfully. Probes from a duplicated linkage group on maize chromosomes 1L and 5S were found in linkage with apospory.

Diplosporous *Tripsacum* is the closest known apomictic relative of maize. RFLP probes from maize were therefore ideally suited to identify molecular markers linked to diplospory. Three probes that detected RFLPs linked to diplospory are from a duplicated linkage group on maize chromosomes 6L and 8L (Leblanc et al., 1995c; Leblanc et al., 1995b; Grimanelli et al., 1998a). Intriguingly, one probe from maize chromosome 5, which had detected polymorphisms associated with apospory in *Brachiaria* (Pessino et al., 1997), mapped to the chromosome carrying diplospory in *Tripsacum.* However, no additional sequence similarities between the two modes of apomixis were apparent.

It may be speculated that diplospory and apospory evolved as mutations of ancestral genomes. Under this scenario it is possible that such mutated genes are recessive lethals in their simplex or homozygous form. Pessino et al., (1997) suggested candidate genes located within identified linkage groups in maize that may conceivably, in mutated forms, be determinants of apospory. However, lack of success in inducing apomixis in sexual plants complicates the explanation of determinants as alleles of sexual genes. Furthermore, hemizygosity of markers in *Pennisetum* suggests that if allelism through mutation did occur, significant divergence that has since occurred has resulted in a molecular basis of the trait of far greater complexity.

Apomixis has evolved multiple times in a range of species as a very successful reproductive strategy. It is likely that it has occurred by exploiting different processes, and produced variable advantages, including an escape from hybridisation-induced sterility. Some species may possess genetic and physiological predispositions that enable the ready adaptation to an apomictic way of life. In *Hieracium,* allelism between apomixis loci of different species (Bicknell et al., 2000) indicates either that apomixis evolved once and radiated through speciation, or that it arose repeatedly from multiple events, with the genus

being genetically and physiologically amenable to the reproductive transformation, and able to exploit the advantages that apomixis brings.

1.2.5 Difficulties of mapping apomixis determinants at very large non-recombinant loci

The common suppression of recombination of apomixis loci places them alongside similar notable examples including the self-incompatibility (S) locus of *Brassica* (Boyes et al., 1997) and the extreme case of the mammalian Y chromosome. Similarly, the univalent chromosome of *Erigeron* on which the determinant of diplospory resides is expected to be under suppression of recombination to the extent of the whole chromosome. Suppressed recombination of less extensive regions may involve the presence of the trait on a mini-chromosome, a centromeric region, or a hemizygous region with localised suppressed recombination.

Map based cloning strategies towards isolating molecular determinants for apomixis have encountered significant difficulties due to suppressed recombination. In genomic regions where recombination readily occurs, molecular markers that show complete cosegregation with a trait are often a relatively small physical distance from a determining gene. However, when clusters of markers show no segregation from each other or from the trait, the utility of meiotic recombination for the mapping of linked markers is markedly reduced. The SCAR marker OPC04, which is shared by *Pennisetum* and *Brachiaria*, was apparently found to segregate at a low frequency from apospory in *Brachiaria* (Pessino et al., 1997), but no recombination between that marker, or any other linked marker, and the trait, was detected in *Pennisetum* (Ozias-Akins et al., 1998).

The lack of meiotic recombination at the ASGR in *Pennisetum* necessitated a unique approach towards the isolation of genetic determinants of the ASGR. It was reasoned that genetic determinants may feature as regions of high conservation of the ASGR that are retained between related species (Roche et al., 2002). To identify regions of high conservation, the microcolinearity or microsynteny between bacterial artificial chromosome (BAC) contigs spanning the ASGR from two sources was investigated. Two BAC libraries were generated: one from genomic DNA of a polyhaploid derived from a cross between the sexual *P. glaucum* and the apomictic *P. squamulatum*, and the other from the close relative *Cenchrus ciliaris*. The probing of each library with six low copy SCAR markers yielded 28 positive BAC clones from each library, which were arranged into 12 contigs of between two and six clones each, with seven singletons remaining. None

of the identified clones or contigs carried two markers. On this basis, it was estimated that the ASGR was at least several hundred kilobase pairs (kbp) in size. The true extent of the ASGR, however, was revealed by means of fluorescence *in situ* hybridisation of the isolated BAC clones to pachytene chromosomes. It was found to be approximately 50 megabase pairs (Mbp) (Akiyama et al., 2004). The ASGR is therefore extensive, approximately a quarter of a chromosome, making the tasks of assembling a BAC contig, annotating the sequence, and identification and testing of candidate genes quite formidable. However, this process may be assisted by the discovery of microsynteny between the ASGRs of *P. squamulatum* and *C. ciliaris*, and the rice genome, which may be used as a reference and a source of markers to assist the physical mapping of BAC contigs. Furthermore, if apospory did evolve as a mutation of a gene or genes involved in sexual reproduction, annotated sequence of the rice genome may provide candidate genes (Gualtieri et al., 2006).

The microsynteny based approach of positional cloning of apomixis determinants in *Pennisetum* provides a possible alternative to recombination-based mapping of the ASGR. In the light of the difficulties encountered by this programme, further alternative approaches have been suggested that include insertional mutagenesis and deletion mutagenesis (Ozias-Akins et al., 1998) and radiation hybrid mapping (Goel et al., 2003), to identify the smallest unit required for functional apospory.

1.3 Background to the methodology

1.3.1 Generation of deletion mutants and deletion mapping

Deletion mutagenesis has proved to be a very effective technique to generate genetic resources for breeding programmes and to identify molecular markers linked to genes of interest. It has been used successfully in a variety of plant species including wheat (Marais, 1992) and *Arabidopsis* (Vizir et al., 1994), and specifically to map a centromeric region with little recombination in tomato (Liharska et al., 1997). Deletion mutagenesis was therefore seen as an alternative means to generate resources to map apomixis loci. In addition to its potential to provide a resource to map regions with suppressed recombination, its use in an apomict was seen as is particularly suitable; mutants are of an isogenic background, therefore marker screening is simplified. Assessing phenotypes may also be simplified as no complicating factors are brought in from a sexual parent, an inevitable consequence of a conventional mapping approach.

There are two commonly utilised forms of ionising radiation used for generating deletions in genomic DNA; gamma irradiation from a ^{60}Co or ^{137}Cs source (Marais, 1992; Vizir et al., 1994), and irradiation from fast neutrons (Okubara et al., 1994). Ionising radiation in higher plants causes internal or terminal deletions (Vizir and Mulligan, 1999), and sometimes inversion or translocation of chromosomal segments (Grosovsky et al., 1986). Gamma irradiation of *Arabidopsis* diploid pollen was found to cause deletions of an average size of 160 kb to 280 kb (Vizir et al., 1994).

To screen for suitable mutants from mutagenesis using pollen, the female parent needs recessive morphological markers linked to the trait of interest. The phenotypes of these markers become pseudodominant when the dominant allele of irradiated pollen is lost (Vizir et al., 1994; Liharska et al., 1997; Vizir and Mulligan, 1999). Alternatively, high-throughput assays may be utilised to isolate mutations in irradiated pollen from a male parent that is homozygous for a dominant allele of the trait. This technique was used to generate mutants for loss of downy mildew resistance in lettuce (Okubara et al., 1994).

While the irradiation of pollen presents an ideal means of generating genomic deletions and apomixis is transmitted through pollen, homozygosity is required for loss of the trait through deletion in the M1 to be distinguishable from the sexual condition through segregation. No *Hieracium* genotypes are known that are homozygous for determinants of apomixis. *Hieracium* genotypes were instead selected in which determinants for apomixis were believed to be in simplex. In the simplex condition, loss of critical determinants on irradiation of seed results in readily observable phenotypes. By contrast to the irradiation of pollen, however, the irradiation of seed results in chimeric plants due to the multicellular nature of the embryo. Loss of apomixis, detected as empty seed heads, was expressed by some flowers but not by others of the same mutant plant. Paradoxically, the chimeric nature of mutants resulting from irradiation of seed presents an advantage, as each mutant carries multiple opportunities to express loss of apomixis on any inflorescence. To gain a plant of unicellular origin, subtending tissue of mutant flowers was taken and regenerated via tissue culture.

1.3.2 A model of deletion-based mapping of loci associated with apomixis

As determinants of apomixis in *H. glaciale* and *H. caespitosum* appear to segregate as dominant loci in simplex, a model predicting how deletions of the locus are represented within the mutant panel may be developed. The model is based on the assumption that

gamma-induced deletions occur between random double strand breaks. Only deletions (rather than inversions, translocations and point mutations) are considered, and the locus and its associated deletions are assumed to be internal rather than terminal. Deletions of any size up to a maximum are assumed to occur at the same frequency. A maximum deletion size of 10 "deletion units" is imposed on the basis that few individuals are expected to survive large deletions. Polyploid plants may have greater tolerance of deletion than diploids. However, lethality from gamma irradiation is expected to be the result of extensive deletion or chromosome loss. Therefore, the model allows for a deletion to lie across the locus, or from the locus centre to anywhere up to 10 deletion units either side.

Within these assumptions, a panel of virtual "mutants" is generated. For each deletion of each mutant two parameters based on arbitrary physical (not genetic) units are randomly (but conditionally) set; length (l), the length of the deletion, and distance (d), the distance that the centre of the deletion is from the determinant whose deletion causes the mutant phenotype. The parameters are generated by the following equations or functions:

- Equation 1: $l = \text{RAND}() \times (10 - 1) + 1$
  $= \text{RAND}() \times 9 + 1$
- Equation 2: $d = \text{RAND}() \times ((l/2) - (-l/2)) + (-l/2)$
  $= \text{RAND}() \times l - l/2$

where RAND() is a function that returns a randomly distributed number between 0 and 1. Equation 1 generates a deletion which has a length of between one and 10 units. Equation 2 places the centre of the deletion at a location between $-l/2$ and $l/2$ units from the determinant (at "0" units), which fulfils the requirement that the determinant that is critical to the function of interest is deleted. When these equations are run 100 times to reflect a mapping panel of 100 deletion mutant plants, the results can be arranged into a 100 X 20 matrix (100 columns of mutant panel members, and 20 rows of chromosomal locations or "markers" at the locus). The frequencies at which different locations are deleted within the mutant panel can be calculated and represented as a histogram (Fig. 1.5).

Frequent loss of a DNA marker by members of the panel indicates that it is close to the controlling locus. Conversely, a marker lost less frequently is more distal from a controlling locus. Markers can therefore be ordered from those positioned centrally (most frequently lost), outwards towards distal markers that are occasionally lost. Whether two occasionally lost markers are on the same side, or on opposite sides, of the central marker depends on the frequency of deletion breakpoints between them. For example, if two

markers A and B are considered, the frequency of plants that have lost marker B in addition to marker A is expected to be related to the physical distance between A and B. This is analogous to conventional mapping, in which two molecular markers are more likely to segregate if they are physically more distant.

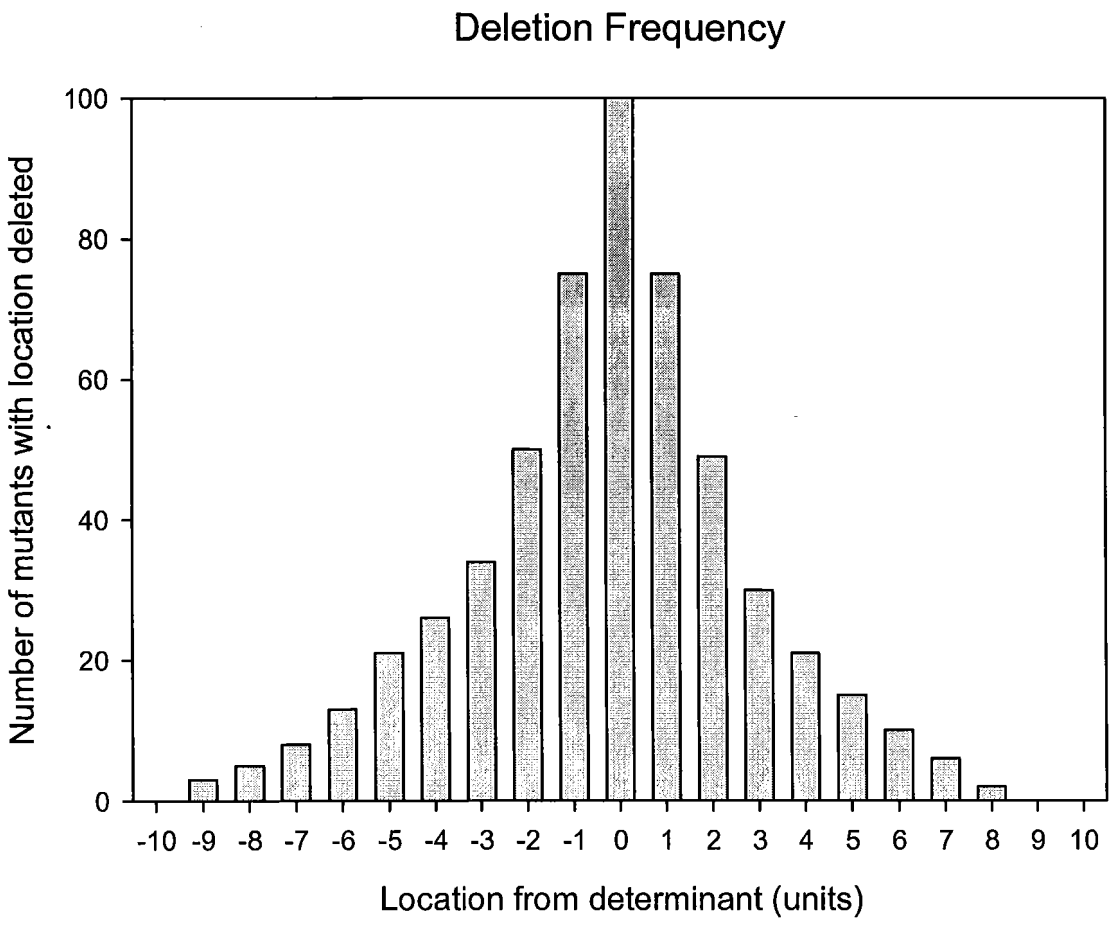


Fig. 1.5 Frequencies of deletion of chromosomal locations neighbouring a genetic determinant, as predicted by a model of deletion based mapping using a "virtual" panel of 100 mutants. The model generates a binomial distribution of cumulative deletions over the region of DNA, with those locations most proximal to the determinant being most commonly deleted.

1.3.3 Molecular analysis of mutants

The strategy of irradiating clonal seed ensures that deletions are detected against a uniform genetic background. Two strategies may be employed to detect deletions in genomic DNA: one is genomic subtraction and the other is systematic screening of molecular markers. The former was originally developed to isolate probes corresponding to the human Y chromosome (Lamar and Palmer, 1984). An excess of randomly-sheared DNA from a

female was used to drive hybridization of *Sau*3A-cleaved DNA from a male; male DNA formed heteroduplexes with female DNA if it came from any chromosome other than the Y chromosome. DNA from the Y chromosome self-annealed, forming *Sau*3A overhangs which could be selectively cloned into a vector with a *Sau*3A acceptor site. A variation on this approach was made for isolating DNA corresponding to deletion mutants (Straus and Ausubel, 1990), by repeatedly annealing melted biotinylated DNA from a mutant to melted non-biotinylated DNA from a wild type individual, and using avidin coated beads to extract the bound DNA. Unbound DNA of the wild type, which should reflect DNA absent in the mutant, was cloned. Representational difference analysis (RDA) is a more recently developed technique based on similar principles (Lisitsyn et al., 1993; Lisitsyn et al., 1994). The mutant is referred to as the driver and the wild type as the tester. Tester DNA has adaptors ligated prior to melting and annealing with excess melted DNA from the driver. Only fragments of tester DNA that have reannealed and have adaptors at both ends are amplifiable by PCR.

Subtraction between wild type and mutant pools of cDNA could be employed to isolate genes whose expression is lost in mutants. This technique has the potential to provide enlightening data; however, there are two significant reasons why it can only be employed with significant risk. The first is that the timing of mRNA capture from developing floral buds would be critical for success. The second reason is that the primary cause of mutation may be the loss of expression of a gene that occurs in the wild type at only low levels, such as a repressor. Such a mutation may be very difficult to detect, particularly if it results in many changes of expression of downstream secondary genes. Investigation of well-characterised mutants using cDNA subtraction at a later date may be more appropriate, for dissection of biochemical pathways of the trait.

While subtraction techniques theoretically provide a rapid route to cloned sequences, they are not ideal as a method for characterising deletions associated with the loss of apomixis in *Hieracium.* A bulked sample of a number of mutants of the panel is unsuitable, as different deletions of different mutants will likely complement each other so none will be detectable. Cloned differences between single deletion mutants and their wild type counterpart will include sporadic deletions that are alternative to loci of interest, and these would only be distinguishable through further investigation. RDA effectively detects only large deletions of unique DNA; deleted sequence of the mutants may be well represented elsewhere in the genome, making the deletions difficult for RDA to detect. Furthermore,

any markers that are successfully obtained from genomic subtraction techniques will require further downstream analysis to determine marker order and relative proximity to genetic determinants, effectively negating the potential expediency of the technique.

A systematic screen to isolate molecular markers that were commonly lost among the mutants was therefore used. By generating markers present in control samples but absent in mutants, the deletions within each mutant were molecularly characterised, enabling plants with the most informative deletions to be identified. The most informative deletions were usually small as their breakpoints lay near putative determinants. Those mutants carrying informative deletions were selected for further analysis to more finely map the region. A number of different marker classes were available for a systematic marker screen. Some such as RFLP are labour intensive and, as with the use of microsatellites, require prior knowledge of the genome. Two classes of markers, random amplified polymorphic DNAs (RAPDs) (Williams et al., 1990) and amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995), are more recently developed classes of markers that do not require any prior sequence information of the genome. RAPD analysis was used to successfully identify and isolate markers linked to the ASGR of *P. squamulatum* (Ozias-Akins et al., 1998). The technique has a low labour requirement and produces markers that are easily isolated and of a reasonable length (300-1200 bp). Isolated markers in this size range are more likely to be useful for downstream analysis, and, for this reason, RAPD analysis was an option for this research.

The labour required for AFLP is well rewarded with the generation of approximately 170 scorable markers per reaction, and it is a readily automatable technique using a DNA analyser. However, preliminary screens of deletion mutant panels using conventional AFLP by other members of the apomixis programme gave confounding results, probably due to variable methylation states of their genomic DNA. Conventional AFLP is susceptible to the effects of methylation, due to the methylation sensitivity of the restriction endonuclease *Pst*I which is used to digest genomic DNA simultaneously with the restriction endonuclease *Mse*I. Using the alternative technique of SDAFLP (Knox and Ellis, 2001), genomic DNA is initially digested with the methylation insensitive *Mse*I. Following digestion, *Mse*I adaptors are ligated followed by PCR amplification between *Mse*I adaptors. The resulting amplicons, now free of methylation, are able to be digested consistently by *Pst*I, which is followed by ligation of *Pst*I adaptors, and subsequent amplification between *Mse*I and *Pst*I adaptors.

1.4 The Apomixis Programme at Crop & Food Research

The Apomixis Programme at Crop & Food Research, Lincoln was initiated by Dr Ross Bicknell in 1992. I joined the programme early in 1996 and it has since been the main focus of my professional career. Investigations have focussed largely on *Hieracium*, with some peripheral work on other species. More recently a formal collaboration has been established with Prof. Anna Koltunow's group of CSIRO Plant Industry in Adelaide, Australia. Research in the programme has progressed from establishing suitable apomictic and sexual species to investigate the genetics of the trait, through to conducting strategies to isolate linked or implicated gene sequences. Work prior to this doctoral research focussed on two broad streams: the development and introduction of an *Activator/Dissociation* (*Ac/Ds*) transposable element system, and the generation and characterisation of segregating populations. Both of these approaches resulted in resources that continue to be integral in the research of the programme. The generation of *Ac/Ds* insertion lines has produced mutants whose characterisation have been a recent focus of mine in the programme, and segregating populations continue to be used to assess the different components of apomixis and associated molecular markers.

1.4.1 *Hieracium* as a model apomict

Hieracium species have haploid genome sizes of approximately 1.8 X 10^9 bp spanning nine chromosomes. Some key species have been described regarding their mode of reproduction, ploidy, and transmission of apomixis, and utilised in experiments and breeding schemes. Sexual biotypes of the species *H. pilosella* occur in both diploid and tetraploid forms. A tetraploid form, P4, is used extensively as a pistillate parent in segregation studies. Accessions of the apomict *H. aurantiacum* have been studied including aneuploid (A3.4) (Bicknell et al., 2000) and tetraploid forms (A4). Other apomicts utilised are the triploid *H. piloselloides* (D3) (Bicknell et al., 2000), the pentaploid *H. glaciale* (G5), and the tetraploids *H. caespitosum* (C4D) and *H. praealtum* (R4) (Bicknell et al., 2001).

While possessing fundamental features of suitable model species such as easy propagation and a short generation time, *Hieracium* has further characteristics that are beneficial for crossing experiments. Sexual species are largely self-incompatible; therefore the emasculation of florets prior to pollination is not necessary. Furthermore, apomictic species are autonomous so fertilisation is not necessary for endosperm development, and parthenogenesis can be readily scored. Stamens and stigmas of unopened flower heads can

be easily removed, which precludes any opportunity of sexual gamete fusion while allowing maternally derived seed to develop (Koltunow et al., 1995a). By contrast, apomictic grasses under study are invariably pseudogamous and the trait is scored more laboriously with cytoembryological analysis (Ozias-Akins et al., 1993), test-crossing (Ozias-Akins et al., 1998), and flow-cytometric analysis (Matzk et al., 2000).

Most species of *Hieracium* can be transformed by T-DNA integration (Bicknell and Borst, 1994). Transformation has expedited genetic investigations and enabled the use of an insertional mutagenesis strategy towards tagging genes involved in apomixis. In the future it is envisaged that transformation will enable attempts to induce apomixis by the transfer of candidate genes, or to knock out the phenotype with RNA interference (RNAi). Use of the negative selectable marker *codA*, that confers sensitivity to the nucleotide analogue 5'-fluorocytoseine, enabled the detection and measurements of meiosis as a function of elimination of the gene through reduction (Bicknell et al., 2003). More recently, selection under *codA* has enabled the isolation of a population of meiotic polyhaploids from apomictic *H. caespitosum*.

1.4.2 Locating apomixis loci: insertional mutagenesis

Insertional mutagenesis by integration of a transposable element is a strategy that has resulted in the successful isolation of gene sequences in both animal and plant species including *Drosophila* (Cooley et al., 1988), zebrafish (Gaiano et al., 1996), maize (Athma et al., 1992), and *Arabidopsis* (Grevelding et al., 1992). This strategy was attractive for gene tagging of apomixis determinants in *Hieracium* as tagging could bypass difficulties associated with a possible lack of recombination within the apomixis locus. The chosen system for an insertional mutagenesis approach was the *Ac/Ds* system from maize, which has been utilised successfully for gene tagging in the past of many heterologous species, including *Arabidopsis* (Grevelding et al., 1992) and rice (Izawa et al., 1997).

1.4.2.1 The T-DNA mutant *loss of apomeiosis 1* (*loa1*)

Loa1 is a mutant from an early population of transformants generated by Ross Bicknell to assess *Ds* transposition in A3.4. This plant has lost almost all ability to produce apomictic seed from a cut head; only 0.02% of seeds germinated to form unreduced maternally derived progeny, and 0.09% of seeds germinated to form polyhaploid progeny. However, on fertilisation, 3.39% of seed resulted in hybrid progeny (Okada et al., 2007). Cytoembryological analysis of this plant revealed that the mutant retains processes of

sexual reproduction found in wild type A3.4. Differentiation of a megaspore mother cell within an ovule, followed by its usual degradation prior to meiosis was observed, as previously reported by Koltunow et al. (1998). Aposporous initial cells of this mutant demonstrate a loss of directional growth towards the sexual apparatus and show features of reduced megaspores, including possession of a callose wall and expression of the *HDMC1* (*Hieracium* homologue of disrupted meiotic cDNA 1), which is specifically expressed in meiotic cells (Takashi Okada, CSIRO Plant Industry, pers. comm.). As this plant was derived from a transformation experiment, I investigated whether T-DNA integration was the cause of the mutation by isolating and sequencing genomic DNA neighbouring the right borders of two T-DNA insertions. A complex rearrangement of integrated T-DNA, which segregates as a single linkage group, is now well described, but no evidence of any knock-out of a gene was found (Okada et al., 2007).

1.4.2.2 Transposon tagging in *H. glaciale*

For insertional mutagenesis of determinants of apomixis, Ross Bicknell and I constructed two vectors for each component of the system (Bicknell et al., 2001). The first vector, pAC7, contains a *Ds* element with a reporter gene that expresses on integration close to a gene promoter or an enhancer (see Chapter 4, section 4.1.1 for details). The *Ds* element is capable of excision and reinsertion only on introduction of the second vector that expresses transposase. We generated 218 transformants from tissue of *H. glaciale* and assessed them for T-DNA copy number by Southern analysis. As *Ac/Ds* elements preferentially excise and reintegrate to linked locations, we planned to isolate transformants that contained T-DNA inserts that mapped close to key loci. To develop probes to find T-DNA inserts linked to components of apomixis, junctions between T-DNA right borders and genomic DNA from 22 members of this population were isolated by thermally asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) and sequenced for use as probes to detect linkage between a T-DNA insert and apomixis.

1.4.2.3 The T-DNA mutant *loss of parthenogenesis 1* (*lop1*)

The T-DNA mutant, *lop1*, carries three copies of pAC7, and had lost the ability to produce parthenogenetic seed. No genomic sequences bordering T-DNA inserts showed significant homology to candidate gene sequences, although one showed homology to a polyprotein of a retrotransposon. Whether the mutations of *loa1* and *lop1* reflect losses of two distinct components of apomixis was tested via the possibility of complementation upon

hybridisation of the two mutants. *Loa1* was used to pollinate *lop1* and a population of 43 progeny was generated. Many of the progeny were found to be 2n+n hybrids, but 14 were found to be n+n hybrids, which were used for the analysis. Three progeny plants that demonstrated restoration of apomixis were recovered.

This mutant was included in the G5 mutant panel for deletion screening. The cause of loss of parthenogenesis in *lop1* was later found to be deletion of a critical determinant of the trait, which is described in Chapter 3. Sequence data of T-DNA/genomic DNA junctions enabled PCR tests for segregation of each insert within the progeny, and investigation of their role in causing the deletion. This is described in Chapter 4.

1.4.2.4 A common genomic junction sequence flanks T-DNA right borders

T-DNA integration in *Arabidopsis* and rice occurs in genomic locations semi-randomly, with some bias towards gene rich areas (Chen et al., 2003). On this basis, T-DNA/genomic DNA junctions of transformed *Hieracium* would be expected to be unique. However, five T-DNA/genomic DNA junctions of the *H. glaciale* T-DNA insertion panel, including one of *lop1*, were found to have genomic regions with a high degree of sequence similarity, but the precise points of T-DNA integration varied within a 30 base pair (bp) region. Beyond the 30 bp region of integration, genomic regions showed 100% identity between three of the five T-DNA junctions. Genomic regions of the other two junctions showed 90% identity. PCR analysis of the remainder of the T-DNA insertion panel revealed a further three plants that possessed similar T-DNA/genomic DNA junctions. The eight plants in total that carry these similar DNA junctions amount to almost 4% of the population.

Southern analyses of *Hin*dIII digested DNA of *H. glaciale* and *H. praealtum* indicated that 15-20 copies of the common genomic sequence are present in these species, and PCR analysis indicated its presence in *H. aurantiacum* and *H. pilosella*. While the common genomic sequence appeared to be present in all four *Hieracium* species tested (*H. glaciale*, *H. praealtum*, *H. aurantiacum* and *H. pilosella*) it was not detected in the relatives *Taraxacum officinale* and *Hypochoeris radicata*.

TAIL-PCR was used to determine upstream sequence displaced on T-DNA integration in the wild type genome of *H. glaciale*. Two unique sequences were amplified from *H. glaciale*. The presence of these two upstream regions was confirmed by PCR in the other three species of *Hieracium* tested: *H. praealtum*, *H. aurantiacum* and *H. pilosella*. However, the juxtaposition of the upstream regions with the common genomic region was

restricted to *H. glaciale*. Interestingly, the points at which the upstream sequences started were within the approximately 30 bp region within which T-DNA borders were found. Therefore, wild type genomic sequences bordered the common genomic region in a very similar way that T-DNA was found to.

As this sequence bordered one of the T-DNA inserts of the mutant *lop1*, it was investigated further. Association of a specific region with T-DNA integration has not been reported in the literature and it remains possible that it is involved with the trait. The present data suggests that different juxtapositions of the common genomic region with other genomic regions are unique to individual species, and at least one is in simplex in *H. glaciale*. Therefore, species-specific markers, and potentially mapping markers, can be designed based on the common genomic region. It is striking that this region is associated with distinct regions within *Hieracium* genomes of different species. It appears to attract new regions, such as T-DNA, to integrate nearby and to recombine with native sequences to form new genomic arrangements. It may be a sequence of hyper-recombination. Alternatively it may be a transposable element in its own right, exploiting the process of integration of T-DNA for integration of itself.

1.4.3 Segregating populations and molecular mapping

The acquisition of a collection of different species and individuals of *Hieracium* with different modes of reproduction has enabled analysis of the trait through segregating F1 populations (Bicknell et al., 2000; Bicknell et al., 2003). Emasculation prior to hybridisation is not normally necessary due to the high degree of self-incompatibility and hybrid progeny are relatively easily identified by unique morphological characteristics of the male parent. Two segregating F1 populations were used for this doctoral research for marker verification and to assess the segregation behaviour of components of apomixis. Both were resources that were previously developed by Ross Bicknell. A small population of 28 segregants was generated from multiple pollinations of an inbred sexual accession of P4 by the apomictic G5 (termed "PG"). A significant problem encountered when generating this population was a high incidence of selfing, likely to be due to the breakdown of the sporophytic self-incompatibility system caused by a mentor effect (Mraz, 2003) by the aneuploid pollen of G5. A second population of approximately 500 progeny was generated from routine crosses between P4 and C4D (termed "PC"). A subset of 101 segregants was selected for closer analysis. PC progeny demonstrate the independent segregation of the two components of apomixis, apomeiosis and parthenogenesis.

1.4.4 Generation of deletion mutant panels and preliminary analysis

Two panels of *Hieracium* mutants based on G5 and on C4D were generated by Suzanne Lambie, Ellen Podivinsky and Sylvia Erasmuson at C&FR. Preliminary analysis of the G5 mutant panel by Sylvia Erasmuson established common loss of markers among 22 deletion mutants and *lop1* when compared to wild type control samples. The current research commenced with the resources of the deletion mutant panels and the segregating populations established.

Chapter 2 Materials and Methods

2.1 Plant Material

The apomictic *Hieracium* species under investigation were the pentaploid *H. glaciale* (G5) and the tetraploid *H. caespitosum* (C4D). Other accessions used in this study were the apomictic tetraploid *H. aurantiacum* (A4) as a pollen parent to produce hybrid progeny from sexual plants, and the sexual *H. pilosella* (P4), used as a female parent for the production of segregating populations. The T-DNA mutants *loa1* and *lop1* are derived from the aneuploid accession of *H. aurantiacum* (A3.4) and from G5 respectively.

2.1.1 Plant propagation and maintenance

All plants were maintained in a greenhouse at between 16° C and 28° C in summer and 12° C and 20° C in winter, on gravel beds with seasonally adjusted automated watering. Flowering of mature plants was encouraged during winter months by supplementary lighting between the hours of 2 am and 9:30 am. Pollinations were made by gently brushing stigmas of open flowers with open flowers of the pollen parent. Seeds were collected from mature heads and dried for at least one week before they were surface sterilised by shaking in sterilising solution (0.96% sodium hypochlorite and approximately 2% TWEEN® 20 (BDH)) for 50 minutes, rinsed with sterile dH_2O and sown on agar-solidified rooting medium containing MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), and 3% sucrose. Seedlings were raised at 21-23° C with a daily light period of 16 hours, and were ex-flasked at approximately the four leaf stage into a mist unit for approximately 3 days before placing in the greenhouse.

Plant accessions were propagated via single rosette cuttings placed in potting mix and allowed to take root in a mist unit. Alternatively, for *in vitro* storage, young leaves were picked and sterilised with sterilising solution for 10 minutes before rinsing with sterile dH_2O and placing on agar-solidified regeneration medium containing MS salts, B_5 vitamins, 3% sucrose, 2 mg/l benzyladenine (BA), 0.5 mg/l indole-3-butyric acid (IBA), and 200 mg/l glutamine. Regenerated shoots were placed on agar-solidified rooting medium.

2.2 Mutant panels

Two mutant panels were utilised for this research: a panel of 77 mutants derived from G5 and a panel of 79 mutants derived from C4D. Dry collected seeds from emasculated heads of propagated material were exposed to 400 gray of γ-irradiation from a ^{60}Co source at the National Radiation Laboratory (108 Victoria Street, Christchurch, New Zealand). Irradiated and non-irradiated control seeds were surface sterilised and germinated on agar-solidified rooting medium and grown as M_1 plants to flowering in the greenhouse as described above. Assessment for loss of apomixis was made by visual detection of reduced seed set on sectors of M_1 plants. To generate stable non-chimeric mutants, tissue subtending seed heads expressing reduced seed-set was harvested and surface sterilised, and placed on agar-solidified regeneration medium as described above. Regenerated shoots were placed on agar-solidified rooting medium to form roots prior to ex-flasking to a greenhouse and growing to flowering. Mutants that continued to demonstrate reduced seed-set of a non-chimeric nature were selected for on-going analysis.

2.3 Segregating populations

Two segregating populations, PG and PC, were generated prior to this research by Dr Ross Bicknell. A population of 28 PG segregants was generated from pollinations of an inbred sexual accession of P4 by the apomictic G5. Further crossing was attempted to enlarge the PG population but no further hybrids were obtained. A second population of approximately 500 PC segregants was generated from pollinations between P4 and C4D. A subset of 101 segregants was selected for closer analysis.

2.4 Phenotype assessment

Plants subject to phenotype assessment were first scored for parthenogenesis and then for apomeiosis. To assess seed production and parthenogenesis of a plant, seed was harvested from mature seed heads and left in open Wheaton vials to dry at room temperature for between 7 and 21 days. *Hieracium* is generally self-incompatible but to ensure that a plant was scored correctly as parthenogenetic, stigma and anthers of at least one capitulum destined for seed harvest and scoring, were removed at approximately stage five when floral heads are well developed but prior to anthesis (Koltunow et al., 1998). Stigmas and anthers were removed by transversely excising the top half of the unopened floral head. Dark seed, associated with successful development of endosperm, (Koltunow et al., 1998) were counted. Seed was sown on rooting medium and assessed for germination, evidenced

by formation of a seedling or of callus, at approximately five weeks after sowing. Plants that produced seed with no fertilisation were scored as parthenogenetic.

Apomeiosis was assessed as a function of nuclear DNA contents of progeny relative to those of their parents. Plant nuclei were released by finely chopping leaf tissue from between one and six seedlings in 0.4 ml extraction buffer (21 g/l citric acid, 0.5% TWEEN® 20), stained by adding 0.6 ml DAPI staining solution (4', 6-diamidino-2-phenylindole at 2.25 mg/l in a saturated solution of dibasic sodium phosphate), and filtered using a 30 μm CellTrics® disposable filter (Partec). DAPI fluorescence, as a function of nuclear DNA contents, was measured using a Partec PAII flow cytometer. Parthenogenetic progeny were scored as 2n+0 or n+0, being derived from unreduced and reduced female gametes respectively (Harlan and De Wet, 1975). Hybrid progeny, obtained after pollination of non-parthenogenetic plants with A4 as the pollen donor, were similarly scored as 2n+n or n+n, being derived from unreduced and reduced female gametes respectively. Plants were scored as positive for apomeiosis if they produced any progeny from unreduced megagametes and negative for apomeiosis if they consistently produced progeny from reduced megagametes. For most plants, at least six progeny were tested. Some plants produced low numbers of progeny suitable for analysis from multiple heads. To be certain of the origin of progeny from parthenogenetic plants, only maternal progeny were used for the scoring of apomeiosis, despite the low numbers of maternal progeny available in some instances. The possibility remains that some plants are falsely scored as negative for apomeiosis, but it is unlikely that any plants are falsely scored as positive for apomeiosis.

2.5 Histology

Histology was conducted on ovaries of mutants of the *H. glaciale* mutant panel. Floral capitulae were collected, prepared and serially sectioned as previously described (Koltunow et al., 1998). Capitulae were collected at stages 2, 3, and 4 to investigate forming aposporous initials, stage 6 to view fully formed embryo sacs, and stages 8 and 10 to view embryogenesis. Capitulae were halved (or quartered if at stages 8 and 10) longitudinally and fixed in fresh (made within 48 hours) 3.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) under vacuum overnight at room temperature. The buffer was replaced the following day, in which tissue was stored at 4° C until use.

Except for stage 2 capitulae, stigmas, stamens and petals were removed so that only a small section of stamen filaments and associated structures remained attached to ovaries, which were then dissected from the floral receptacles. To assist with dehydration and infiltration of ovaries at stage 5 or higher, the ovary walls were carefully pierced with a sharp pin. Dissected ovaries were placed in sieves and dehydrated using a graded series of 20-30 minute acetone washes at room temperature, starting at 15% and ending at 100% with gradations of approximately 15%, and then infiltrated with 1:1 acetone/Spurr's resin (Spurr, 1969) overnight under vacuum at -70 kPa at room temperature. Ovaries were then placed in 100% Spurr's resin in molds which were allowed to polymerise overnight at 65-70° C. Blocks were trimmed and serially sectioned at 2 μm using a Reichert Jung microtome. Sections were stained in 0.1% toluidine blue in 0.02% sodium carbonate and were photographed under bright field optics using a Leica DM R microscope.

2.6 SDAFLP analysis

Total DNA was isolated from approximately 100 mg of fresh young *Hieracium* leaves, either from greenhouse or *in vitro* stocks, using a DNeasy Plant Mini Kit (Qiagen). DNA was quantified using a GeneQuant RNA/DNA calculator (Pharmacia) and by comparing with known standards on an ethidium bromide-stained 1% agarose gel. Preparation of SDAFLP template was based on the method previously described (Knox and Ellis, 2001). Adaptors and primers for template preparation are listed in Table 2.1. Approximately 0.5 μg of genomic DNA was digested with 5 U of *Mse*I (New England Biolabs) in 50 μl reactions containing 1X restriction/ligation (RL) buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM DTT) and 100ug/ml bovine serum albumen (BSA, New England Biolabs) at 37° C for 16 hours. The digested genomic DNA was diluted with 10 μl of ligation mixture containing 1X RL buffer, 50 pmol of annealed *Mse*I adaptor 1 and *Mse*I adaptor 2, 12 nmol of ATP and 1 U of T4 DNA ligase (Roche) and incubated at 37° C for 4 hours, followed by incubation at 4° C for 16 hours. The resulting template was diluted with 440 μl of T0.1E (10 mM Tris, 0.1 mM EDTA) pH 8.0 and 2 μl was used in 20 μl PCR reactions containing 1X supplied reaction buffer (Roche), dNTPs at 200 μM, 7.5 pmoles of *Mse*I adaptor 1 as non-selective *Mse*I primer, and 1 U of *Taq* polymerase (Roche). PCR conditions (hereby referred to as AFLPPRE) were as previously described (Vos et al., 1995) for primers with no or one selective base (20 cycles of the following profile: 94° C for 30 seconds (s), 56° C for one minute, 72° C for one minute) using an Eppendorf Master Cycler. Following amplification, 5 μl of

preamplification product was digested with 20 U of *Pst*I (New England Biolabs) in 50 µl as described above for *Mse*I digestion, followed by dilution with 10 µl of ligation mixture containing 5 pmol of annealed *Pst*I adaptor 1 and *Pst*I adaptor 2, as described above for *Mse*I adaptor ligation. Following ligation, template was diluted with 440 µl of T0.1E pH 8.0 and 4 µl was amplified by PCR in 25 µl reactions containing 1X supplied reaction buffer (Roche), each dNTP at 200 µM, and primers *Pst*I+N and *Mse*I+N (where N represents A, C, G or T as selective bases) at 2.5 ng/µl, using the cycling regime AFLPPRE. As many as 16 preamplified SDAFLP templates were generated for each sample, each with a different combination of the selective bases.

Table 2.1 The oligonucleotide adaptors and primers used for the preamplified SDAFLP template preparation.

Oligonucleotide name	Oligonucleotide Sequence
*Mse*I adaptor 1	GACGATGAGTCCTGAG
*Mse*I adaptor 2	TACTCAGGACTCAT
*Pst*I adaptor 1	CTCGTAGACTGCGTACATGCA
*Pst*I adaptor 2	TGTACGCAGTCTAC
*Mse*I+N*	GACGATGAGTCCTGAGTAAN*
*Pst*I+N*	GACTGCGTACATGCAGN*

* A, C, G or T

2.6.1 SDAFLP selective amplification and visualisation using [γ-^{33}P] ATP

*Pst*I+N oligonucleotide primers were end-labelled at a concentration of 5 ng/µl in reactions containing 1X supplied kinase reaction buffer, [γ-^{33}P] ATP (Amersham) at 1.5 µCi/µl and 0.2 U T4 polynucleotide kinase (New England Biolabs) incubated at 37° C for 1 hour. Templates generated by amplification with the respective *Pst*I+N and *Mse*I+N were used in 12 µl reactions essentially as previously described (Vos et al., 1995), with some minor modifications. Amplification reactions contained 1X supplied PCR reaction buffer (Roche), each dNTP at 200 µM, 0.75 µl of end labelled *Pst*I+N reaction, 1.25 ng of unlabelled *Pst*I+N, 1.5 ng of *Mse*I with three selective bases (*Mse*I+NNN, either GACGATGAGTCCTGAGTAANNN or GATGAGTCCTGAGTAANNN) and 0.75 U *Taq* polymerase. A modified cycling profile (AFLPSELE) for selective AFLP reactions was based on that previously described for AFLP selective amplifications (Vos et al., 1995), as follows: 94° C for 2 minutes, 9 cycles of 94° C for 30 s, 65° C for 30 s (reducing by 1° C per cycle), 72° C for 1 minute, followed by 24 cycles of 94° C for 30 s, 56° C for 30 s and 72° C for 1 minute, with a final strand extension of 72° C for 10 minutes.

Amplified selective SDAFLP products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. The gel matrix solution was made by dissolving urea to a final concentration of 7.67 M in 6% acrylamide/bisacrylamide (29:1, Biorad) and 1X TBE. 50 ml of gel matrix solution at 4° C was mixed with 50 µl of N,N,N',N'-tetramethylethylenediamine (TEMED, Biorad) and 500 µl of freshly made 10% ammonium persulphate and cast in plates of a S2 gel rig (Life Technologies) between 0.35 mm spacers with the smaller gel plate coated in Sigmacote® (Sigma). After polymerising overnight, the gel was pre-run with parameter maxima set at 120 Watts, 75 mAmps, 2400 Volts and 45° C. Once the gel plate reached 45° C, SDAFLP products were mixed with 6 µl STR loading buffer (95% formamide, 10 mM sodium hydroxide, 0.05% bromophenol blue and 0.05% xylene cyanol), denatured at 95° C for 4 minutes before quenching on ice. Approximately 5 µl of each sample was loaded into a 64-lane comb. The gel was run with the parameter maxima described above, until the dye fronts had run the optimal distance, dependent on the length of the marker(s) in question. The gel apparatus was dismantled so that the gel remained attached to the large plate. A piece of 3MM paper was lightly pressed onto the damp gel which adhered to the paper so that it could be lifted from the plate. The gel was dried under vacuum for 2 hours at 80° C and exposed to auto-radiographic film for 3 days followed by additional exposure to new film for 10 days. Exposed film was developed using an Agfa Curix 60 automatic developer.

2.6.2 SDAFLP selective amplification and analysis using the DNA analyser

Products of SDAFLP selective amplification were generated in 12 µl reactions containing 1X supplied reaction buffer (GeneCraft), each dNTP at 200 µM, 15 ng of one of the fluorescently labelled primers *Pst*I+A+FAM, *Pst*I+G+FAM, *Pst*I+C+HEX, or *Pst*I+T+HEX (GeneWorks), 18 ng of one of *Mse*I+NNN selective primers, 1.2 µl of the respective *Pst*I+N, *Mse*I+N template, and 0.6 U of *Taq* polymerase, using the cycling profile AFLPSELE. Profiles were visualised by adding 1 µl of labelled reaction to 10 µl of HiDi formamide (Applied Biosystems), denaturing as described above, and running on an ABI PRISM® 3100 Genetic Analyser fitted with a 36 cm array filled with POP4 polymer. Data was analysed by the software package GeneMapper™ (Applied Biosystems) and scorable markers were identified visually.

2.6.3 SDAFLP selective amplification and visualisation using silver staining

Products of SDAFLP selective amplification were generated in 12 µl reactions as described for generating products for visualisation using the DNA analyser, except non-labelled *Pst*I+N primers were used. Products were separated by electrophoresis on a 6% denaturing polyacrylamide gel, as described above, except the larger plate was coated with 6 µl of methacryloxypropyltrimethoxysilane (Sigma M-6514) in a solution of 2 ml of 0.5% acetic acid in 95% ethanol. Following electrophoresis, the gel plates were dismantled with the gel firmly attached to the larger plate. With the gel on the underside, the plate was submersed in four litres of the following solutions in a photographic development tank, rotating at approximately 30 rpm. First the gel was fixed in 10% acetic acid for 30 minutes. It was then rinsed three times with dH_2O for 2-3 minutes each rinse. The gel was then stained for 40 minutes in silver stain (1 g/l $AgNO_3$, 0.05% formaldehyde). This was followed by a very brief (3-5 seconds) submersion in dH_2O, followed by immediate transfer to fresh developer at approximately 6° C (30 g/l $NaCO_3$, 0.05% formaldehyde, 2 mg/l sodium thiosulfate ($Na_2S_2O_3.5H_2O$). Development proceeded for 5-10 minutes until the desired level of band intensity was achieved. The gel was fixed in 10% acetic acid for 5-6 minutes, rinsed in dH_2O for 2-3 minutes, and air dried overnight. Scoring of bands was made either directly from the gel on a light box or from an image on exposed and processed APC film (Promega).

2.6.4 Marker identification and data analysis

The criterion for a putative marker associated with loss of apomixis was its absence in three or more mutants. For the *H. glaciale* mutant panel, putative markers were given two letter identifiers, starting with Aa, then Ab etc. Markers of the *H. caespitosum* mutant panel were identified using a genetic analyser and were given identifiers with the locus and the marker size. Commonly lost markers were scored in mutant-by-marker matrices with 0 for absence, 2 for presence and 1, if necessary, for ambiguous presence. The markers were scored for loss and clustered by sorting in Microsoft Excel. Ordering of markers was conducted empirically within clusters into the order that best reflected the expected continuous nature of deletions as previously described in Chapter 1, section 1.3.2. Markers that were found to be commonly lost in the mutant panel, and that were able to be easily identified in the hybrid background, were scored for their presence or absence in members of the the PG segregating population.

2.7 Band isolation and analysis

Bands corresponding to markers of importance were isolated, sequenced and characterised, and converted into SCAR markers.

2.7.1 Mini-sequencing of markers

Mini-sequencing was conducted essentially as described previously (Brugmans et al., 2003). SDAFLP amplification products of mutant and control samples were diluted 1:120 in ddH_2O, and re-amplified in 12 reactions in which the *Mse*I selective primer was replaced by one of 12 mini-sequencing primers. Each of the 12 mini-sequencing primers contain between three and five degenerate bases followed by an additional selective base at the 3' end (Table 2.2). Mini-sequencing products were visualised using polyacrylamide gel electrophoresis (PAGE) or the DNA analyser, as described above.

Table 2.2 *Mse*I mini-sequencing primer sequences.

Oligonucleotide name	Oligonucleotide Sequence
*Mse*I+3N+A	CGATGAGTCCTGAGTAANNNA
*Mse*I+3N+C	CGATGAGTCCTGAGTAANNNC
*Mse*I+3N+G	CGATGAGTCCTGAGTAANNNG
*Mse*I+3N+T	CGATGAGTCCTGAGTAANNNT
*Mse*I+4N+A	CGATGAGTCCTGAGTAANNNNA
*Mse*I+4N+C	CGATGAGTCCTGAGTAANNNNC
*Mse*I+4N+G	CGATGAGTCCTGAGTAANNNNG
*Mse*I+4N+T	CGATGAGTCCTGAGTAANNNNT
*Mse*I+5N+A	CGATGAGTCCTGAGTAANNNNNA
*Mse*I+5N+C	CGATGAGTCCTGAGTAANNNNNC
*Mse*I+5N+G	CGATGAGTCCTGAGTAANNNNNG
*Mse*I+5N+T	CGATGAGTCCTGAGTAANNNNNT

2.7.2 SDAFLP using *Mse*I primers with extended selective bases

Extended *Mse*I primers were designed composed of the core sequence 5'-ATGAGTCCTGAGTAA-3', followed by the three selective bases of the *Mse*I primer from which the marker was generated, and then by two or three bases detected by mini-sequencing. Only bases that were mini-sequenced with good confidence were included in these extended primers. Profiles were generated and visualised as described above.

2.7.3 Marker band excision and cloning

Bands were isolated from SDAFLP profiles that were generated by amplification using labelled or unlabelled *Pst*I+N primers and standard *Mse*I+3N primers, mini-sequencing primers, or *Mse*I primers with extended selective bases. Profiles were separated by PAGE and visualised as described above. Bands of [γ-^{33}P] ATP-labelled profiles were located by aligning common locating holes of the gel and the developed autoradiograph that were punched prior to exposure, enabling accurate excision of the band with a scalpel through the autoradiograph. Alternatively, silver-stained bands were excised directly from the developed gel adhered to the plate.

The gel slice containing a band was suspended in 30-50 µl of 50 mM Tris pH 9.0, and incubated at room temperature for 1-2 hours with intermittent vortexing before being placed at 4° C overnight. Each band was reamplified from 3-5 µl of eluate in 60 µl reactions containing 1X supplied buffer (GeneCraft), each dNTP at 200 µM, 75 ng of oligonuleotide primers and 2.5 U of *Taq* polymerase, using the cycling regime AC55 (94° C for 2 minutes; 40 cycles of 94° C for 30 seconds, 55° C for 30 seconds and 72° C for 1 minute; 72° C for 7 minutes). Products were visualised by electrophoresis of 5 µl on an ethidium bromide-stained 1% agarose gel and either gel purified using a QIAquick® Gel Extraction Kit, or purified directly using a QIAquick® PCR Purification Kit (Qiagen), and eluted in 30 µl of supplied elution buffer, according to the manufacturer's protocol.

Purified products were visualised and quantified on a 1% agarose gel and cloned into pGem®T or pGem®T-Easy (Promega) in 10 µl reactions, according to the manufacturer's protocol, except half of the recommended quantities of vector and T4 DNA ligase were routinely used. Generally, 3 µl of purified product was used as insert DNA. Ligation reactions were incubated at 4° C overnight before transferring 3 µl for transformation of sub-cloning efficiency competent cells (Invitrogen). Transformation proceeded according to the manufacturer's protocol except 25 µl of cells instead of 50 µl of cells were transformed followed by the addition of 475 µl of LB instead of 950 µl. After incubation of transformed cells for expression, the cells were centrifuged at 1500 X g for two minutes and 300 µl of LB was removed. The cells were resuspended and plated onto agar-solidified LB containing 100 µg/ml ampicillin overlaid with 800 µg of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and 800 µg isopropyl thiogalactoside (IPTG), and incubated overnight at 37° C. Four white colonies were picked with a flame sterilised inoculating loop and re-streaked onto agar-solidified LB containing 100 µg/ml ampicillin.

2.7.4 Marker sequencing

Single colonies from re-streaked transformed cells were picked with a 200 µl pipette tip, touched three times onto the bottom of a microplate well containing 15 µl of PCR mix (1 X supplied reaction buffer (GeneCraft), each dNTP at 200 µM, primers M13Forward (5'-GTAAAACGACGGCCAG-3') and M13Reverse (5'-CAGGAAACAGCTATGAC-3') at 600 nM and 0.75 U *Taq* polymerase), and then washed into 700 µl of LB with 100 µg/ml ampicillin. Cloned inserts were amplified using the cycling regime AC55 and the respective LB inoculations were incubated at 37° C overnight rotating at 600 rpm, before adding 300 µl of glycerol for long-term storage at -80° C.

Successful amplification of PCR products of appropriate sizes was confirmed by electrophoresis of 5 µl of each product on a 1% agarose gel. Each product was purified for sequencing by incubating a second 5 µl aliquot with 2.5 U exonuclease I and 0.5 U shrimp alkaline phosphatase in 10 µl reactions, at 37° C for 30 minutes, and then at 80° C for 15 minutes (Werle et al., 1994). Extension products were generated using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), with some adjustments to the manufacturer's protocol; 3 µl of purified product was added to 7 µl containing 0.5 µl Ready Reaction Premix, 1.75 µl 5X buffer, and 25 ng primer, and amplified using the cycling regime ACSEQ50 (96° C for 1 minute, followed by 50 cycles of 96° C for 10 seconds, 50° C for 5 seconds, and 60° C for 4 minutes). The products were purified using the Ethanol/EDTA precipitation method, according to the manufacturer's protocol, and sequenced on an Applied Biosystems ABI PRISM 3100 Genetic Analyser fitted with a 36 cm array filled with POP4.

2.7.5 Marker sequence characterisation

To accurately reflect sequence of genomic origin, marker sequences were trimmed of primer sequence and bases were added to restore genomic marker restriction sites. Potential open reading frames (ORFs) were predicted using the online ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Predicted ORFs that occupied a significant proportion of the sequence or that were bounded by one or both of the sequence margins, and stop codons were noted. Exons were predicted using the GENSCAN web server (http://genes.mit.edu/GENSCAN.html).

Genomic Markers were tested for similarity with known sequences of GenBank using the algorithms blastn, blastx and tblastx (http://www.ncbi.nih.gov/BLAST) (Altschul et al.,

1990). Any sequences from which significant matches were detected were further tested for similarity against the "non-redundant" (nr) peptide sequence database of GenBank using the algorithm blastx. All searches were made using the default parameters, except that the low complexity filter was disabled and any influence of low complexity on the significance of the result was determined manually.

2.7.6 SCAR marker design

For conversion of SDAFLP markers identified from the *H. glaciale* mutant panel into SCAR markers, oligonucleotide primers for PCR were designed from each sequenced marker with melting temperatures (Tms) of approximately 60° C from sequenced SDAFLP markers. For SCAR markers of the *H. caespitosum* panel, many primers were designed using the web-based programme Primer3 using SDAFLP marker sequences as input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The parameters used for primer design were those given by default, with the exceptions of the Tm requirements set at a minimum of 60° C and a maximum of 67° C, with an optimal Tm of 64° C, and the primer length to be between 20 and 27 bases with the optimal length set at 24 bases. Primers were only accepted if they lay internal to the SDAFLP primers inclusively. Other primers were designed to exploit the regions of polymorphism that generated SDAFLP markers (*Pst*I and *Mse*I restriction sites, followed by the variable bases of the selective primers). These primers therefore contained either *Pst*I or *Mse*I restriction sites at their 5' ends, followed by marker sequence to a length that gave each primer a Tm of between 64° C and 66° C.

2.7.7 SCAR marker amplification

The SCAR markers were tested for their utility as single copy markers by PCR using approximately 100 ng of genomic *Hieracium* DNA as template in 15 µl reactions containing 1 X supplied reaction buffer (Geneworks), each dNTP at 200 µM, each oligonucleotide primer at 600 nM and 0.6 U of *Taq* polymerase. Oligonucleotide primers of SCAR markers derived from SDAFLP markers of the *H. glaciale* mutant panel were tested using the cycling profile of AC58 (94° C for 2 minutes; 40 cycles of 94° C for 30 seconds, 58° C for 30 seconds and 72° C for 1 minute; 72° C for 7 minutes), AC64 and AC68, which had primer annealing temperatures of 64° C and 68° C respectively. SCAR markers derived from *H. caespitosum* were amplified using the cycling regime AC64. All SCAR markers were run on a 1% agarose gel.

2.8 Data analysis

The inheritances of traits and markers were tested using exact one sample binomial tests (Arimitage and Berry, 1994) under GenStat (GenStat-Committee, 2002).

Chapter 3 Detection of loci associated with parthenogenesis in *Hieracium glaciale*

3.1 Introduction

Apomixis is now widely agreed to utilise sexual processes to generate functional seed, but with up to three key modifying components: apomeiosis, parthenogenesis and, in cases of autonomous apomicts, autonomous endospermy. Only when all components are present is clonal seed successfully formed. Within a typical ovule of *H. aurantiacum* or *H. piloselloides*, sexual processes proceed at least to some extent. However, apomictic processes soon predominate with the formation of apomeiotic aposporous initial cells followed by their expansion towards the nucellar lobe. Sexual structures that were under development normally degrade in deference to aposporous initial cells so that unreduced embryo sacs may form. Possessing the genetic complement of the parent, the unreduced embryo sacs may then undergo parthenogenetic development that often proceeds prior to anthesis (Bicknell, 1997; Koltunow et al., 1998).

Some seed in *Hieracium*, however, is derived from ovules in which sexual processes predominate. The resulting minor progeny classes may develop from combinations of sexual and apomictic processes, or entirely from sexual processes that have escaped subversion by apomictic ones (Bicknell, 1997). The extent of sexuality varies between different *Hieracium* species, and is related in part to the penetrance of apomixis. How effectively apomictic processes predominate is likely to result from delicate interactions, both temporally and spatially, between major determinants of apomixis, genes that play a role in both apomixis and sexuality, and possibly other genetic and epigenetic modifying elements.

The mutation of genes associated with sexual reproduction has produced some tantalising semblances of features of apomixis. However, it now appears that the use of apomixis for fixation of heterosis will require the isolation and characterisation of key genes for the trait followed by extensive characterisation of their action on downstream genes and pre-existing sexual processes. The difficulties of map based cloning using segregating populations associated with lack of recombination were circumvented with a marker screen of the *H. glaciale* (G5) deletion mutant panel. This enabled the identification and characterisation of markers linked to three loci whose loss was accompanied by loss of apomixis. The roles that each locus played in apomeiosis, parthenogenesis, autonomous

endosperm formation and also the suppression of sexuality associated with apomixis were then assessed in *H. pilosella* X *H. glaciale* (PG) segregants.

3.2 Generation of the *H. glaciale* deletion mutant panel and phenotype assessment

The *H. glaciale* deletion mutant panel was generated and assessed for loss of apomixis by other members of the apomixis group prior to this research. The panel of 73 mutants was derived from mutant sectors that showed reduced seed set from approximately 5000 irradiated seed. All mutants showed substantial or entire loss of parthenogenesis, and 44 showed reduced potential for apomeiosis.

3.2.1 Histological evidence of loss of apomeiosis and loss of parthenogenesis

Serial sections of ovules at approximately stage 4 (Koltunow et al., 1998) from two deletion mutants, G5γ20 and G5γ47 were generated. Ovules from these mutants were selected at stage 4 on the basis that meiosis is complete in the sexual P4 and in apomicts, aposporous initials are often visible at at this stage. In the apomict *H. piloselloides*, meiotic tetrads in addition to aposporous initials are visble at this stage (Koltunow et al., 1998).

While G5γ20 and G5γ47 both showed loss of parthenogenesis, they differed with respect to the derivation of hybrid progeny. The testing of nuclear DNA contents of progeny relative to those of the parents indicated that G5γ20 had retained apomeiosis, while G5γ47 demonstrated loss of apomeiosis. Histological evidence of the presence or absence of aposporous initial cells in stage 4 ovules from these mutants was consistent with the phenotypes attributed from the relative nuclear DNA contents of progeny. Aposporous initials were evident in stage 4 ovules of G5γ20 (Fig. 3.1 a). By contrast, meiotic tetrads were visible in ovules of G5γ47 with no evidence of aposporous initial cells (Fig. 3.1 b).

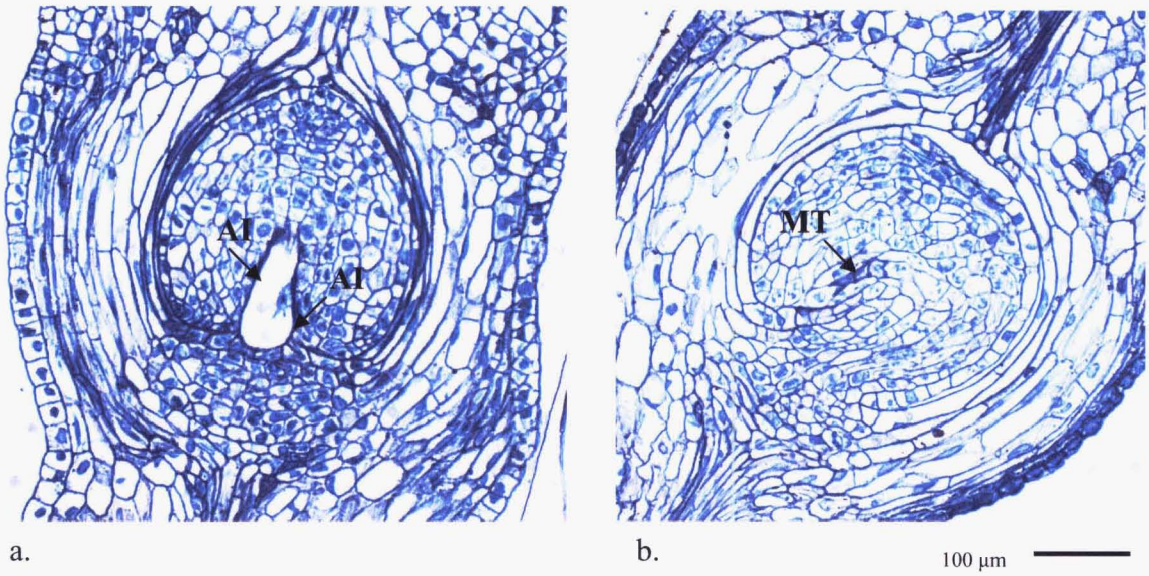


Fig. 3.1 Histological sections of Stage 4 ovules from florets of G5γ20 (a) containing two developing vacuolar aposporous initials (AI), compared with the denser spores of a meiotic tetrad (MT) in G5γ47 (b), a mutant in which apomeiosis is absent.

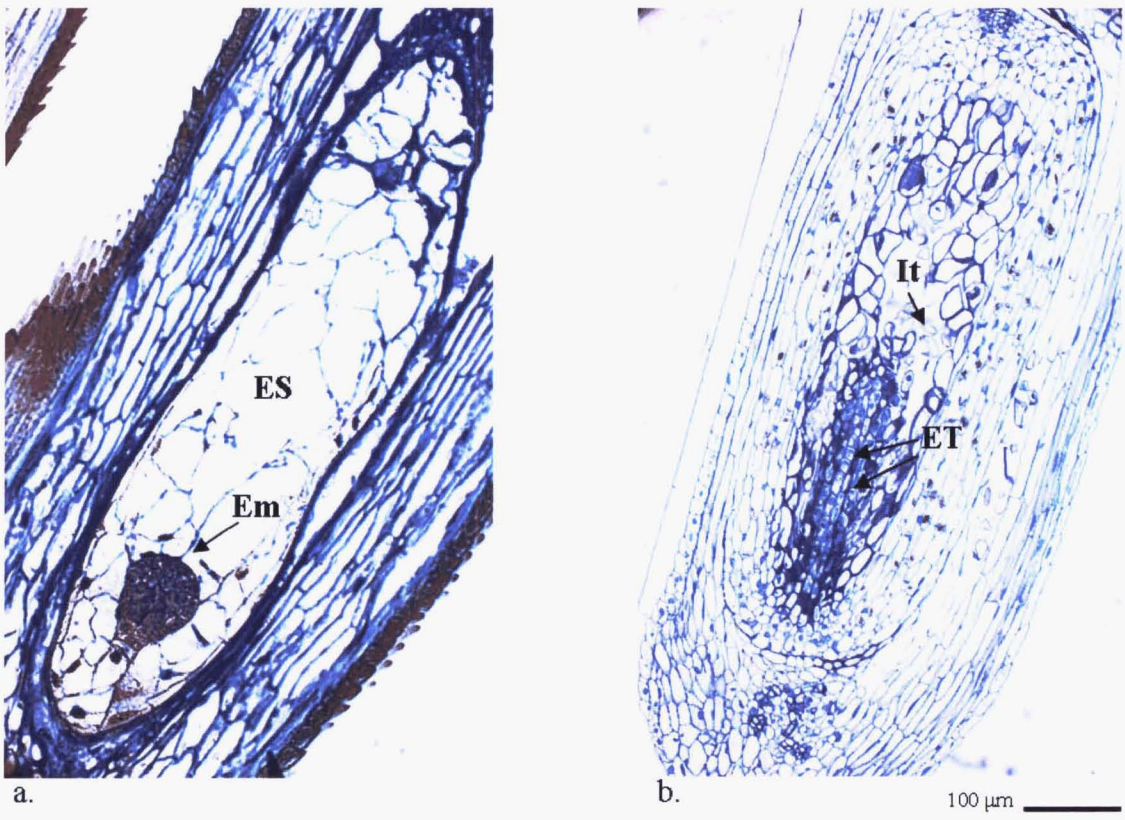


Fig. 3.2 Stage 10 ovules of florets from wild type G5 (a) and G5γ47 (b). The wild type (a) shows successful development of a globular embryo (Em) and formation of the endosperm (ES). In an unfertilised ovule of the mutant (b), the endothelium (ET) of the embryo sac has collapsed and endosperm development has not proceeded. Integument cells (It) appear to be degrading.

Parthenogenetic embryos at the globular stage and endosperm are seen in serial sections of ovules at stage 10 of wild type *H. glaciale*. (Fig. 3.2a). To view the effects of loss of apomixis on embryo and endosperm development, serial sections were generated from unfertilised floral buds of the mutant G5γ47 (Fig. 3.2b). This mutant shows loss-of-apomeiosis and loss-of-parthenogenesis phenotypes but produces 2n hybrid progeny when fertislised (Sylvia Erasmuson, pers. comm.). By contrast to wild-type ovules, there was no evidence of embryos in the mutant. The embryo sac and the surrounding endothelium had collapsed and integument cells appeared to have undergone degradation. A collapsed embryo sac and degrading integument cells was observed at this stage in unfertilised stage 10 ovules of sexual *H. pilosella* (Koltunow et al., 1998).

3.3 SDAFLP analysis of the mutant panel for the detection of commonly lost markers

To test the use of SDAFLP for the detection of commonly lost markers, SDAFLP using the primer *Pst*I+A paired with 16 *Mse*I+CNN and 16 *Mse*I+GNN primers was conducted on an intitial subset of 22 mutants of the G5 mutant panel, chosen due to their clear phenotypes, alongside a wild type control. This work was conducted by Sylvia Erasmuson of the Apomixis Programme at Crop & Food Research. The T-DNA mutant *lop1* was included for SDAFLP analysis due to its expression of a loss-of-parthenogenesis phenotype very similar to those members of the deletion mutant panel. This screen generated 34 commonly lost markers that fell into two clusters that were predicted to be associated with two loci. From this initial screen, seven primer combinations (*Pst*I+A paired with *Mse*I+CCA, CCT, CGA, CGC, CGG, CGT, and CTA) were selected on the basis of the quality and yield of the 18 markers which they detected. The presence or absence of each of the 18 markers was assessed by me in the remaining 51 mutants of the G5 mutant panel, alongside three irradiated controls with no loss of apomixis, and six mutants from the subset of 22 mutants that were previously screened, to assess SDAFLP consistency and to assist with marker identification.

3.3.1 Marker loss associated with loss of apomixis

The SDAFLP profiles showed almost complete consistency within and between batches, demonstrating that the SDAFLP is highly replicable. There were occasional variations in relative band intensities between batches, and bands occasionally showed batch-specific presence or absence which appear to be due to variations in gel-running conditions and

exposure times. All of these outliers were easily distinguished from scorable markers that showed absence across the two batches. Each of the 18 markers that were scored from the first batch were scorable in the second. One further marker, Dz, was identified from the second batch. This marker was scorable in only some members of the first batch. A second further marker, Ea, was identified in the PG segregating population, and was retrospectively scored in both mutant batches. Therefore, a total of 20 commonly lost markers were scored across the complete panel of 73 mutants.

All of the panel except G5γ61 were found to have lost at least one of the 20 commonly lost markers. These markers fell into three clusters that are postulated to be groups of markers linked to three loci. The loci are hereby termed Locus 1, defined by 11 markers, Locus 2 defined by five markers and Locus 3, defined by four markers (Fig. 3.3). Thirty, nine and 13 mutants carry deletions detected at Locus 1, 2 and 3 respectively, and 20 mutants carry deletions in two or more of the three loci. The extent of deletions ranged from the loss of a single marker, to the loss of an entire locus. With the exception of mutants that carry deletions of entire loci, all mutants were included in the analysis to assign marker order. The most parsimonious marker order, based on the principle that deletions are most likely continuous segments of chromosomal DNA, is given in Fig. 3.3. The T-DNA mutant *lop1* was found to be a single locus mutant with loss of all the markers associated with Locus 3 (see Fig. 3.3).

As described in Chapter 1, section 1.3.2, the proximity of each marker to a determining locus is expected to be directly related to the frequency at which each marker is lost within the mutant panel. This principle holds only when deletions that span determinants and therefore cause mutant phenotypes are considered. Those mutants with multiple deletions were therefore excluded from the calculations of marker loss frequencies, as no single deletion carried by these mutants could be confidently attributed as the cause of the mutant phenotype. Based on frequencies of marker loss from single-locus deletion mutants, the central markers are likely to be Cw of Locus 1, Cu and Cy of Locus 2, and Dl of Locus 3. Each of these central markers is absent in all mutants that have single deletions within the locus to which the marker belongs. It remains possible that some mutants classed as single locus mutants might carry additional deletions at other loci that are not detectable at the resolution of this SDAFLP screen. Any failure of detection of small deletions is not expected to have occurred frequently enough to have a profound effect on the interpretation of these data.

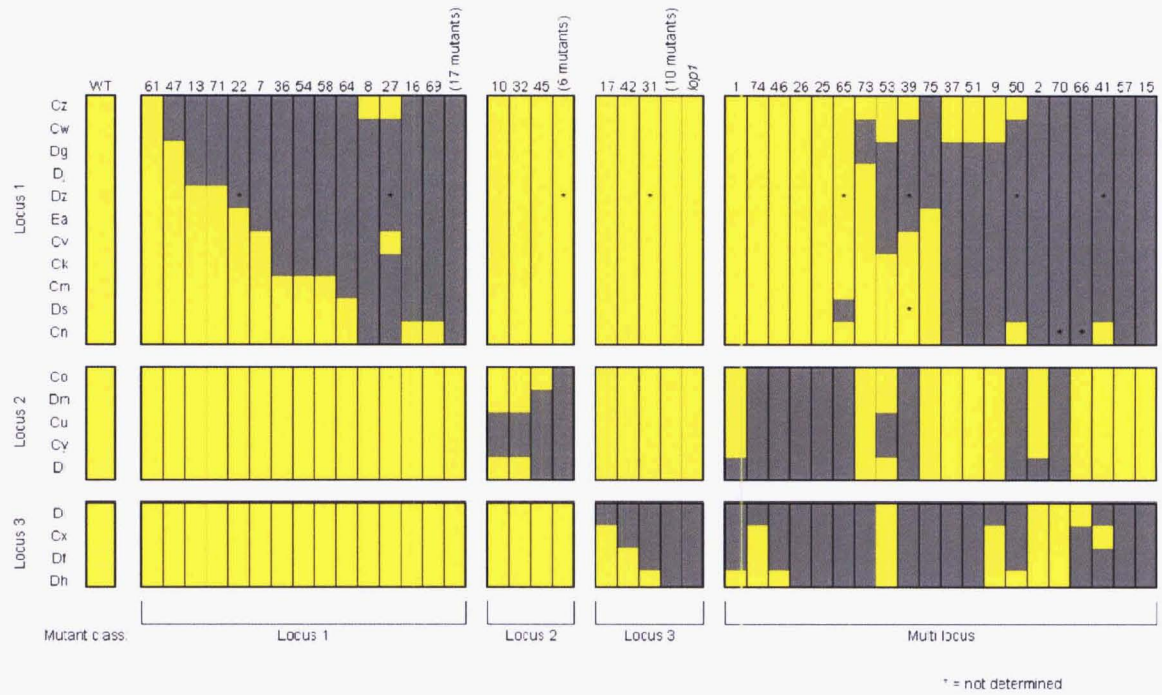


Fig. 3.3 Marker presence and absence scored in a wild-type (WT), 73 *H. glaciale* loss-of-parthenogenesis deletion mutants and the T-DNA mutant *lop1*. Each column represents a different plant while each row represents a different marker. Cells coloured yellow indicate marker presence while those in grey indicate marker absence. The mutants were sorted into three classes depending on three putative loci from which markers are lost: Locus 1, Locus 2, Locus 3, and multi-locus.

The marker order illustrated in Fig. 3.3 is predicted on the basis that deletion breakpoints occur randomly and that determinants lie proximal to markers that are most commonly lost. Under this model, the determinant at Locus 3 has markers flanking only one side. Similarly, the determinant at Locus 1 is flanked by all except one marker, Cz, on one side. The maps of these loci suggest that the determinants lie at termini of chromosome arms. It is possible however that "hotspots" exist near the markers Cz and Dl that incur γ-irradiation-induced breakages at high frequencies. If this were the case, determinants might actually lie nearer the centromeres than the data suggest.

Each SDAFLP primer combination yielded approximately 170 bands. Therefore, as markers were generated from seven primer combinations, approximately 1190 potential markers were estimated to be under consideration. This may be an overestimate, however, as bands that result from the amplification of sequences that are replicated throughout the genome are not potential single locus markers. However, if the estimation of 1190 potential markers is considered, and given a 5X genome size of 9×10^9 bp, a physical distance of approximately 7.6 Mbp between markers may be estimated. The 20 markers at

the three loci are approximately 1.7% of the total markers estimated. Therefore, the mapped regions may collectively be estimated to occupy 1.7% of the total genome.

One mutant, G5γ61, had no discernible deletion, yet was scored as a loss-of-parthenogenesis mutant. It is possible that this mutant carries a deletion that was not detected at the resolution obtained using only seven primer combinations. With this mutant as the exception, the estimation of the large physical distances between markers indicates that most deletions of this mutant panel are substantial. The extent of deletions reported from previous studies in plants varies considerably. Deletions of at least 1.8 Mbp were generated in chromosomal DNA of lettuce seeds using a fast neutron source. More substantial deletions were likely to have been generated as well; however, any that caused significant perturbations in development were selected against by generating M_2 progeny (Okubara et al., 1994). In tomato, terminal deletions of considerable portions of a chromosome arm were generated by irradiation of pollen (Liharska et al., 1997). By contrast, deletions generated by mutagenesis of *Arabidopsis* pollen were believed to average less than 160 kb (Vizir et al., 1994), although it is possible that this relatively small average deletion size represents a wide range of deletions that includes some that are extensive.

The polyploid status of G5 may offer gene redundancy that reduces the likelihood of dominant lethality, making large deletions more recoverable. Large deletions in chromosomal DNA of pollen were found to be more successfully recovered when diploid eggs were fertilised instead of haploid eggs (Vizir and Mulligan, 1999). In summary, it appears that deletions in the G5 mutant panel are substantial, and the limit of the resolution offered by the panel may almost have been reached. A large deletion, however, may in rare cases offer an informative breakpoint that is located proximal to determinants at the locus centre.

3.4 Marker isolation and sequence characterisation

Mini-sequences were obtained from five markers; gel isolation and sequencing was achieved for four of these and one other marker for subsequent characterisation.

3.4.1 Mini-sequencing of markers

Mini-sequence profiles were generated from SDAFLP products of wild type G5 compared with G5γ19, and wild type G5 compared with G5γ39, amplified by the primer pairs *Pst*I+A

and *Mse*I+CCA, and *Pst*I+A and *Mse*I+CGC respectively. G5γ19 had lost all markers of Locus 1 and G5γ39 had lost all markers of Locus 2 and Locus 3. Mini-sequencing of profiles from these two primer combinations enabled the generation of mini-sequences of up to six markers, although the marker Ds was not able to be mini-sequenced due to its low molecular weight.

The profiles given by each mini-sequencing primer contained a subset of the bands of the SDAFLP profiles thereby reducing the risk of contamination of the band of interest from neighbouring bands on excision (see Fig. 3.4 for example). However, the reduction in profile complexity down to 25% was not achieved. The mini-sequencing profiles often contained additional bands that were not visible in the original SDAFLP profile from which they were amplified. This is believed to be due to their low original abundance in the SDAFLP profile; they became more abundant in the absence of competition by more effectively amplified bands.

Mini-sequences were generated for the markers Ck, Cm, Cn, Cy and Cx (Table 3.1 and Fig. 3.4). Three markers, Ck, Cy and Cx, gave ambiguous mini-sequences at one position due to their amplification from multiple selective mini-sequencing primers (see Fig. 3.5 for marker Ck as an example). Ambiguous mini-sequences may be due to non-specific annealing of mini-sequencing primers. An alternative cause is the presence of genomic duplications, either allelic, linked or unlinked, that carry single nucleotide polymorphisms (SNPs) at the ambiguous bases of the mini-sequences, to which alternative mini-sequencing primers can anneal. Given that the *H. glaciale* genome is tetraploid and is known to be rich in retrotransposons and repetitive DNA, this is considered to be a likely cause.

Table 3.1 Mini-sequences of three bases following the selective bases, of markers amplified with primers *Mse*I+CCA and *Mse*I+CGC. Bases in brackets indicate ambiguity at that location of the mini-sequence.

Marker	*Mse*I primer	Locus	Mini-sequence
Ck	*Mse*I+CCA	1	CC(A/C/T)
Cm	*Mse*I+CCA	1	AGA
Cn	*Mse*I+CCA	1	TTC
Cy	*Mse*I+CGC	2	G(A/G)A
Cx	*Mse*I+CGC	3	CG(A/G)

3.4.2 Gel isolation and sequencing of markers

Markers Ck, Cm, Cy, and Cx were successfully isolated from mini-sequence profiles and Di, Dl and Dh from standard SDAFLP profiles, followed by cloning and sequencing. Most sequences were of a high quality and their sequence lengths approximated the expected lengths estimated from gel migration. The exceptions were sequences of Dh and Dl, which required alignments to more than one sequence read to generate composite sequences. These sequences were unlikely to be the correct sequences for these markers because the *Pst*I+A primer sequence was not found on the sequence of Dh and the sequence of Dl appeared to be an amplification product of only the *Mse*I+CGG primer. They were therefore not characterised further by bioinformatic analyses.

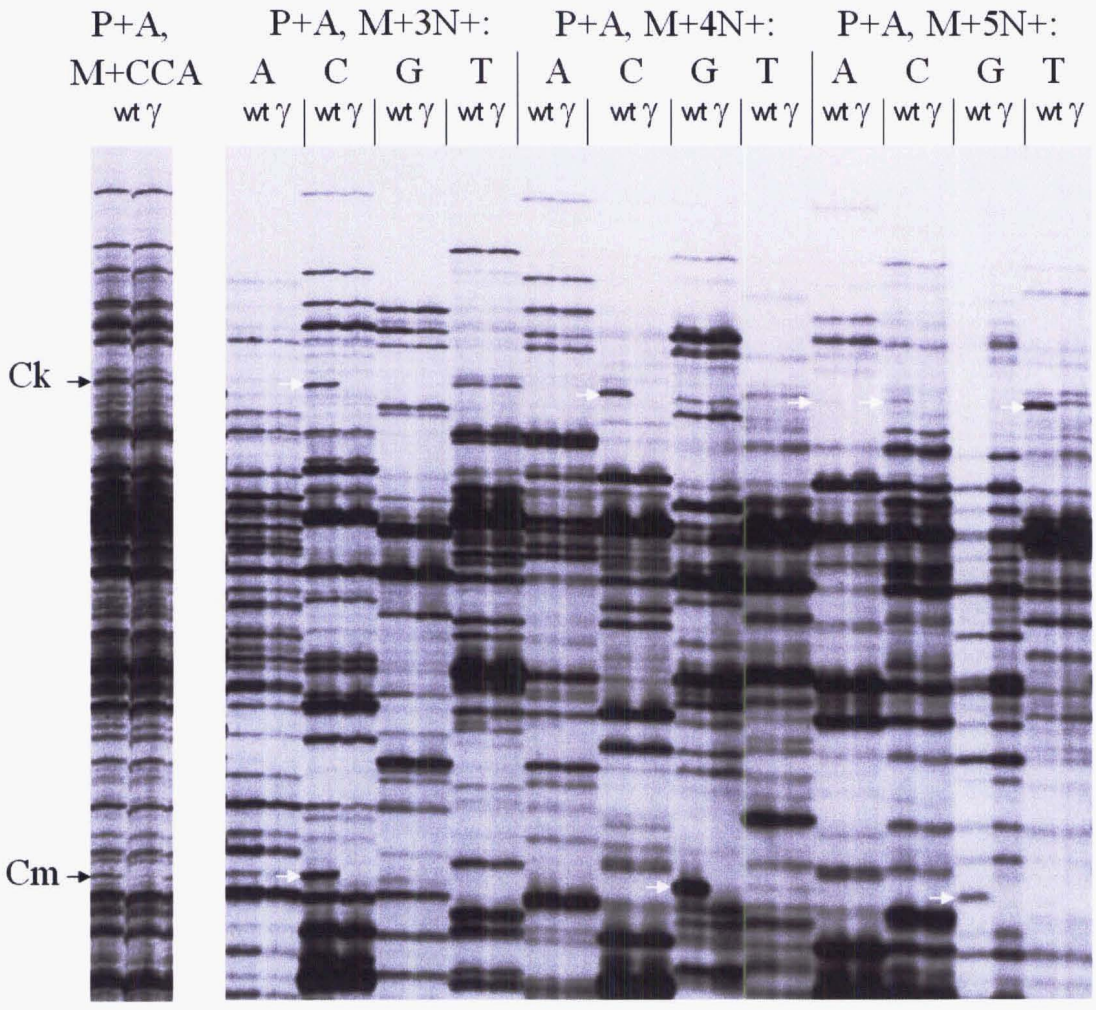


Fig. 3.4 Standard SDAFLP (P+A, M+CCA) and mini-sequence profiles of wild type G5 (wt) and G5γ19 (γ). Arrows indicate amplification of two markers; Ck the lower of a band doublet, and Cm. Amplification of the third base of Ck has occurred from three mini-sequence primers causing ambiguity of the mini-sequence.

3.4.3 Bioinformatic analysis of marker sequences

All marker sequences contained extensive regions that were predicted as open reading frames (ORFs) indicating possible coding sequences (Table 3.2). Only the ORF of Ck was predicted by GENSCAN to be an exon, indicating that GENSCAN is a more conservative predictor of gene characteristics. Notable exceptions were predictions of large exons in Cx by GENSCAN that were not predicted to be ORFs by ORF Finder. These inconsistencies may result from variable codon usage between the two packages. Alternatively, GENSCAN may compensate for potential sequence errors that disrupt an ORF by adding weight to other features such as putative splice sites.

Table 3.2 ORFs predicted by ORF Finder and exons predicted by GENSCAN. ORF Finder outputs orientation, frame, start, and stop codons. GENSCAN predicts the type of exon and orientation and calculates a probability rating. A high probability rating indicates a high chance of a correct prediction.

			ORFs predicted by ORF finder		Exons predicted by GENSCAN	
Marker	**Genomic Sequence Length**	**ORF predictions (frames)**	**ORF length**	**ORF features**	**Exon prediction (strand)**	**Exon type and probability rating**
Ck	568	182-451 (-3) 243-503 (+3) 1-118 (-3) 241-354 (-1)	270 261 118 114	Stop at 182 Stop at 503 Stop at 241	451-182 (-)	Single exon gene, 0.676
Cm	234	2-205 (+2)	204	Stop at 205	None	
Cy	280	2-118 (+2) 173-279 (+2) 1-93 (-2) 1-66 (+1) 1-59 (-3) 218-271(-1)	117 108 93 66 59 54	Stop at 118 Stop at 279 Start at 93 Stop at 66 Start at 271, stop at 218	None	
Di	407	205-330 (-3) 2-127 (-2) 69-182 (+3) 150-257 (-1) 318-392 (-1) 1-63 (-3)	126 126 114 108 75 63	Stop at 205 Start at 127 Stop at 182 Stop at 150 Stop at 318	None	
Cx	291	1-148 (-3) 1-140 (-2)	148 140		223-26 (-) 248-26 (-) 223-39 (-) 248-39 (-) 47-205 (+) 47-209 (+) 223-83 (+) 248-83 (-)	Internal, 0.341 Internal, 0.224 Internal, 0.115 Internal, 0.138 Internal, 0.054 Terminal, 0.023 Internal, 0.037 Internal, 0.043

No markers showed similarity ($E<0.05$) when the blastn algorithm (nucleotide query compared with nucleotide database) was used. On using the tblastx algorithm (translated nucleotide query compared with translated nucleotide database), only Cx showed significant similarity to known genomic sequences. The most significant similarity was to a Ty3-gypsy type LTR retrotransposon sequence of *Stevia rebaudiana*, included as part of

a genomic sequence that contained a kaurene oxidase gene and pseudogene. The marker also showed similarity to Ty3-gypsy type LTR retrotransposon sequences of *Oryza sativa* and *Cicer arietinum* (Table 3.3). A search for peptide sequences with similarity to Cx using the blastx algorithm resulted in a large number of hits with expect values as low as 0.002 to Gag proteins (Table 3.4), which are typical of long terminal repeat (LTR) retrotransposons (Havecker et al., 2004).

The similarity of Cx to a retrotransposon sequence of *Stevia rebaudiana* is intriguing. *Stevia*, like *Hieracium* is of the family Asteraceae, and has apomictic species including *S. rebaudiana* (de Oliveira et al., 2004). It is postulated that retrotransposons in asexual populations become inactivated over time due to host level selection of population members with fewer deleterious effects of the elements (Docking et al., 2006). Conversely, retrotransposons are reported to have accumulated in asexual *Hypericum* species more than they have in sexual species (Matzk et al., 2003). From the data available it cannot be ascertained whether Cx is from an active retrotransposon or from a remnant of an inactive element. The similar retrotransposon sequence of *S. rebaudiana* was not described in the peptide database and, based on the weakness of the similarity it has with other retrotransposons, it appears to be a remnant of an inactive element. However, the conservation at the peptide level between Cx and the sequence of *S. rebaudiana* across most of the marker indicates functional conservation implying that Cx may be part of an active retrotransposon. Active retrotransposons are likely to be abundant in *Hieracium*; however, definitive evidence that Cx is an active retrotransposon within a region of suppressed recombination can only be found from analysis of sequence beyond the margins of Cx.

Table 3.3 Sequences found with similarity to Cx from a tblastx search of nucleotide sequences of GenBank. The E-value is the likelihood that the similarity occurred due to chance alone.

GenBank Accession	Organism	E-value	Gene/region description
AY995178 (17509-17234)	*Stevia rebaudiana*	1e-19	Kaurene oxidase (KO2) and KO pseudogene *
AY995178 (14766-14524)	*Stevia rebaudiana*	1e-19	Kaurene oxidase (KO2) and KO pseudogene *
NM_195999	*Oryza sativa*	4e-4	Putative 22 kDa kafirin cluster; Ty3-Gypsy type (OSJNAb0075K12.33), mRNA
Various, including AC146704	*Medicago truncatula*	0.001	Unannotated sequences of various BAC library clones
Various, including AP006128	*Lotus corniculatus*	0.002	Unannotated sequences of various BAC library clones
CAR411813	*Cicer arietinum*	0.002	Ty3-gypsy like retrotransposon and partial gag gene for gag polyprotein, LTR and PBS, clone 104-20

* similarity shown to a Ty3-gypsy like retrotransposon

Table 3.4 Sequences found with similarity to Cx from a blastx search of peptide sequences of GenBank.

GenBank Accession	Organism	E-value	Gene/region description
AAP53268	*Oryza sativa*	0.002	Putative retrotransposon protein, Ty3-gypsy subclass
ABE89851	*Medicago truncatula*	0.004	Retrotransposon gag protein
AAD27902	*Arabidopsis thaliana*	0.005	Retrotransposon gag protein
ABD28759	*Medicago truncatula*	0.005	Retrotransposon gag protein
CAC44110	*Cicer arietinum*	0.006	Gag polyprotein

3.5 SCAR marker design and amplification

Primer pairs designed from the sequences of four SDAFLP markers, Ck, Cm, Cy and Cx, were tested for their amplification of single-locus SCAR marker derivatives. PCR tests were conducted on DNA from wild type *H. glaciale* and mutant counterparts with deletions of entire loci. Only the SCAR marker of Cm demonstrated utility as a single-locus SCAR marker. This marker was absent in mutants when a primer annealing temperature of 58° C was used. This marker had no mini-sequence ambiguity (see Table 3.1), which is consistent with the possibility that markers with no mini-sequence ambiguity are more likely to be single-locus sequences. The utility of the Cm SCAR marker was demonstrated further by its segregation in eight PG segregants, which was later found to correspond with the segregation of the SDAFLP marker from which it was derived.

The other three SCAR primer pairs gave amplification products from all deletion mutant DNA samples. Amplification from mutant samples also occurred when the primer annealing temperature was raised to 64° C, and ceased from any sample including the wild type control when the primer annealing temperature was raised to 68° C. These observations indicate that for the potential SCAR markers of Ck, Cy and Cx, the primers were annealing to non-deleted genomic duplications, which may be allelic, non-allelic or both, and potentially highly repeated throughout the genome.

The lack of specificity of the SCAR markers is unlikely to be due to the isolation and sequencing of incorrect SDAFLP bands. Most bands were isolated from mini-sequencing lanes in which the band of interest was often of a high intensity and was not in close proximity to potentially-contaminating bands. It remains possible that the SCAR markers contained polymorphisms that could be detected using alternative assays that are more sensitive than agarose gel electrophoresis. Size polymorphisms that are detectable by PAGE, or single strand conformation polymorphisms (SSCPs), may exist in the

amplification products of SCAR primers that enable them to be used as single-locus markers. No attempt was made to exhaust the possible detection of polymorphisms as at this point in the research it was decided that a BAC library would not be constructed for *H. glaciale*. SDAFLP markers were instead used for downstream applications.

3.6 Verification of association of markers with apomixis

The common absence of markers in association with the absence of parthenogenesis was verified by the corresponding presence of the markers in association with the expression of parthenogenesis in a segregating population. Correspondence with other components of apomixis such as autonomous endosperm formation, apomeiosis and the suppression of sexuality were also explored.

3.6.1 The segregating population

Associations of markers with traits were tested in the 28 segregants of the PG segregating population. Attempts to enlarge the numbers of PG (*H. pilosella* X *H. glaciale*) segregants under analysis were made by making crosses using a non-inbred accession of *H. pilosella* as the pistillate parent and *H. glaciale* as the pollen parent. Several crosses were made but they only yielded inbred *H. pilosella* progeny. This is likely to be due to a breakdown of the sporophytic self-incompatibility system caused by a mentor effect (Mraz, 2003), possibly by the aneuploid pollen of *H. glaciale*. A similar mentor effect of aneuploid pollen was seen to cause a similar breakdown of sporophytic self-incompatibility in *Taraxacum* (Tas and van Dijk, 1999).

3.6.2 Assessment of parthenogenesis

All segregants were assessed for parthenogenesis by assessing the ability and frequency of production of dark seed and of germinable seed from at least four seed heads. Dark seed was produced at rates of between 0 and 60 per head. Twelve of the 28 segregants (43%) produced dark seed at rates between 13 and 60 per head, of which between 0.2 and 25.5 per head germinated. Most of the other 16 segregants, which produced no germinable seed, produced dark seed at rates between 0 and 6 per head (Fig. 3.5, Table 3.5). One segregant, PG2, produced dark seed at higher rates with none germinating; however, much of the seed was small and brown in colour and was not likely to contain functional endosperm. The transmission frequency for parthenogenesis in this population was 0.43 which is similar ($P = 0.45$) to the 1:1 transmission ratio expected for a single-locus trait.

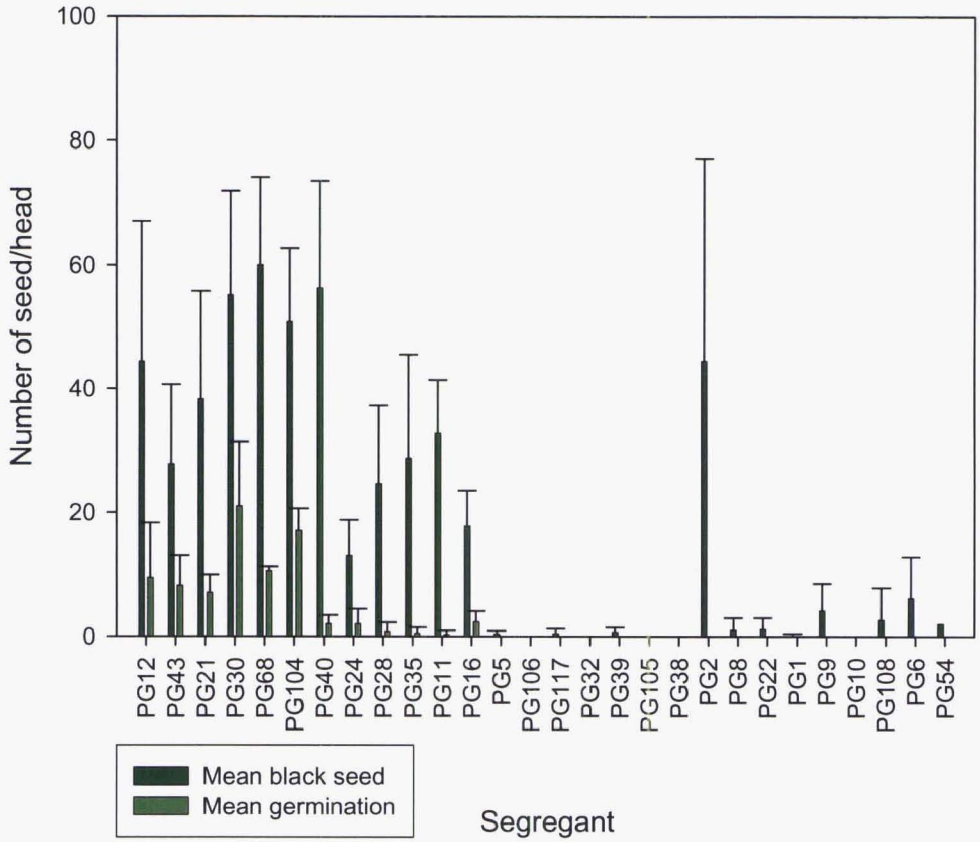


Fig. 3.5 Mean numbers of dark seed production and seed germination per floral head, of seed heads harvested from PG segregants.

3.6.3 Association of autonomous endospermy with parthenogenesis

Endosperm formation in *Hieracium* is autonomous and appears to be associated, either genetically or mechanically, or both, with parthenogenesis. In the PG segregating population, the formation of endosperm without fertilisation was evidenced as dark seed (Koltunow et al., 1998), and offered an initial indication of the plant's parthenogenetic potential. Sexual segregants generally produced low numbers of dark seed, with the exception being PG2 noted above.

The possibility of pseudogamy among segregants was considered but then deemed unlikely. If any segregants scored as sexual, were actually apomictic and pseudogamous, n+0 and 2n+0 progeny after fertilisation would be expected, derived from pseudogamic development of meiotic and apomeiotic eggs respectively. Following pollination of segregants that failed to produce germinable seed spontaneously, a total of 246 seedlings were tested, and none were found to be n+0 as indicated by their relative nuclear DNA

contents. All seedlings had nuclear DNA contents that indicated they were either n+n or 2n+n hybrids.

3.6.4 Assessment of apomeiosis

A total of 19 of the 28 segregants, both parthenogenetic and sexual, were found to produce at least some progeny from apomeiotic embryo sacs (Table 3.6). The frequency of transmission of apomeiosis was therefore 0.68, a ratio that has a low probability of reflecting Mendelian segregation of apomeiosis as a monogenic trait (P = 0.17). It is possible that the trait is conferred by a single gene in duplex or two genes in simplex (P = 0.55). Other more complex gene combinations may have caused a distorted transmission ratio, or alternatively, if apomeiosis is conferred by a single locus, its segregation may be influenced by a meiotic drive mechanism, which has been implicated to act on the segregation of apospory in maize-*Tripsacum* hybrids (Grimanelli et al., 1998a). To ascertain a transmission ratio distortion and what mechanism might be causing it would require a larger number of segregants to be assessed.

3.6.5 Association of reduced sexuality with apomeiosis

The frequency of germinable seed set per head following pollination varied significantly between sexual segregants with means between two and 29. This indicates that a segregant's potential for sexuality is not necessarily restored in the absence of parthenogenesis. Instead, the restoration of sexuality appears to occur to a greater extent in the absence of apomeiosis. Apomeiotic and meiotic segregants of the sexual class set germinable seed on pollination with means at between two and eight per head, and at between four and 29 per head respectively (Fig. 3.6). Thus while apomeiotic embryo sacs are able to be fertilised to form 2n+n hybrids, these data suggest greater fertility within meiotic segregants. The numbers of seed set were highly variable although some relationship between dark seed and germinable seed numbers was apparent within meiotic segregants. On pollination, PG2 produced increased numbers of dark seed from those it produced without pollination, but few seed germinated. Intriguingly, PG10, which produced no dark seed without fertilisation, produced large numbers of dark seed on fertilisation, but few germinated.

Table 3.5 Seed heads of PG segregants, with anthers and stigmas removed, were harvested, and mean numbers and variances (standard deviations, SD) of dark seed and seed germination were scored. Calculations of the percentages of dark seed that germinated were made. Segregants were scored according to whether or not parthenogenesis was detected.

	Seed heads	Dark seeds/head		Germination/head			
PG:	***n***	**Mean**	**SD**	**Mean**	**SD**	**% dark seeds germinated**	**Partheno-genesis detected**
1	9	0.11	0.33	0.00	0.00	0.00%	No
2	6	44.5	32.64	0.00	0.00	0.00%	No
5	3	0.33	0.58	0.00	0.00	0.00%	No
6	8	6.13	6.73	0.00	0.00	0.00%	No
8	4	1.00	2.00	0.00	0.00	0.00%	No
9	7	4.14	4.41	0.00	0.00	0.00%	No
10	5	0.00	0.00	0.00	0.00	n/a	No
11	8	32.63	8.83	0.25	0.71	0.77%	Yes
12	5	44.40	22.60	9.40	8.91	21.17%	Yes
16	7	17.71	5.82	2.29	1.80	12.90%	Yes
21	4	38.25	17.52	7.00	2.94	18.30%	Yes
22	5	1.20	1.79	0.00	0.00	0.00%	No
24	6	13.00	5.76	2.00	2.45	15.38%	Yes
28	10	24.60	12.66	0.70	1.57	2.85%	Yes
30	13	55.15	16.82	21.00	10.46	38.08%	Yes
32	6	0.00	0.00	0.00	n/a	n/a	No
35	14	28.71	16.84	0.43	1.09	1.49%	Yes
38	8	0.00	0.00	0.00	n/a	n/a	No
39	6	0.67	0.82	0.00	0.00	0.00%	No
40	5	56.20	17.31	2.00	1.41	3.56%	Yes
43	6	27.83	12.88	8.17	4.88	29.34%	Yes
54	1	2.00	n/a	0.00	n/a	0.00%	No
68	2	60.00	14.14	10.50	0.71	17.50%	Yes
104	6	50.83	11.86	17.00	3.63	33.44%	Yes
105	13	0.00	0.00	0.00	n/a	n/a	No
106	6	0.00	0.00	0.00	n/a	n/a	No
108	5	2.60	5.27	0.00	0.00	0.00%	No
117	5	0.40	0.89	0.00	0.00	0.00%	No

Table 3.6 Open floral heads of non-parthenogenetic PG segregants were crossed with A4 as a pollen donor and the mean numbers and variances of dark seed and germination per head were scored. Nuclear DNA contents of either maternal or hybrid progeny were measured from which scores for apomeiosis were based.

		Crosses made	Dark seeds/ head		Germination/ head		Seedlings		
PG:	**Parthen-ogenesis:**	***n***	**Mean**	**SD**	**Mean**	**SD**	**Tested**	**Ploidy**	**Apomeio-sis detected:**
1 X A4	No	6	11	5.37	6	3.66	Hybrid	2n, 3n	Yes
2 X A4	No	5	80	12.79	5	3.87	Hybrid	2n, 3n	Yes
5 X A4	No	5	20	10.37	6	6.66	Hybrid	2n	No
6 X A4	No	9	24	12.70	7	6.78	Hybrid	2n, 3n	Yes
8 X A4	No	10	17	14.26	5	4.53	Hybrid	2n	No
9 X A4	No	5	19	15.53	12	10.11	Hybrid	2n	No
10 X A4	No	1	92	n/a	7	n/a	Hybrid	2n, 3n	Yes
11 X A4	Yes	--	--	--	--	--	Maternal	n, 2n	Yes
12 X A4	Yes	--	--	--	--	--	Maternal	n, 2n	Yes
16 X A4	Yes	--	--	--	--	--	Maternal	n	No
21 X A4	Yes	--	--	--	--	--	Maternal	2n	Yes
22 X A4	No	7	27	14.82	4	3.98	Hybrid	2n	No
24 X A4	Yes	--	--	--	--		Maternal	n, 2n	Yes
28 X A4	Yes	--	--	--	--	--	Maternal	n, 2n	Yes
30 X A4	Yes	--	--	--	--	--	Maternal	n, 2n	Yes
32 X A4	No	4	7	6.75	7	6.03	Hybrid	2n, 3n	Yes
35 X A4	Yes	--	--	--	--	--	Maternal	n	No
38 X A4	No	6	26	14.94	19	15.41	Hybrid	2n	No
39 X A4	No	6	26	21.83	4	4.79	Hybrid	2n, 3n*	Yes
40 X A4	Yes	--	--	--	--	--	Maternal	2n, 4n#	Yes
43 X A4	Yes	--	--	--	--	--	Maternal	n, 2n	Yes
54 X A4	No	4	6	2.22	2	1.26	Hybrid	2n, 3n	Yes
68 X A4	Yes	--	--	--	--	--	Maternal	2n	Yes
104 X A4	Yes	--	--	--	--	--	Maternal	2n	Yes
105 X A4	No	4	33	18.45	29	16.27	Hybrid	2n	No
106 X A4	No	1	11	--	8	--	Hybrid	2n, 3n	Yes
108 X A4	No	6	12	6.53	5	3.56	Hybrid	2n	No
117 X A4	No	2	7	0	4	0.71	Hybrid	2n, 3n	Yes

*** Only one 3n was found, all others were 2n. The possibility exists that the sole 3n was derived from pollination of a meiotic embryo sac with 2n pollen. # 4n progeny found, probably the result of endoreduplication.**

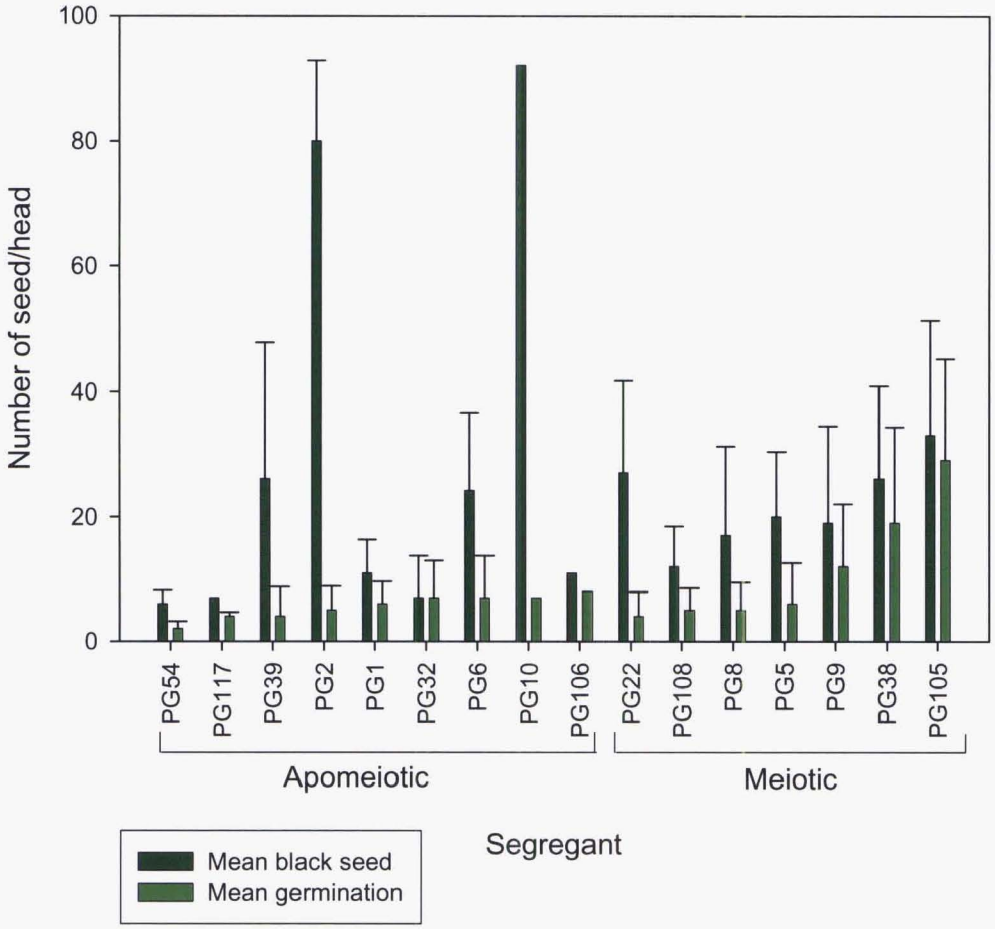


Fig. 3.6 Dark seed and germinable seed set per head of sexual segregants fertilised with pollen from A4, grouped as apomeiotic and meiotic.

The potential for sexual reproduction by parthenogenetic segregants PGs 11, 24, 28 and 35 was assessed from increases in dark seed and seed germination on pollination by *H. aurantiacum* (Table 3.7). These segregants show only low levels of parthenogenetic reproduction ($\leq$ germinable seed per head). All four segregants showed small to moderate increases (1.1–1.97 fold) in production of dark seed per head. PGs 11 and 28, showed small increases in germination per head of 1.27 and 1.66 fold respectively and a moderate 3.34 fold increase was observed from PG24. These three segregants express apomeiosis. The meiotic segregant PG35 showed a 13 fold increase in germinable seed set on pollination. While this observation is limited to one plant, it does lend support to the possibility that female fertility is reduced in the presence of apomeiosis. Pollination was similarly not observed to increase partial seed set in apomictic *Taraxacum*, indicating that the partial seed set is due to partial sterility (Tas and van Dijk, 1999). Thus, apomeiosis may suppress sexuality or induce partial sterility in apomeiotic plants.

Table 3.7 Increases in dark seed and germinable seed per head after pollination. Floral heads of four parthenogenetic PG segregants were fertilised with pollen from A4. The mean numbers of dark seed and germination per head were calculated and the fold increases of dark seed and germination from decapitated heads, and the nuclear DNA contents of seedlings are given.

PG:	Number of heads tested	Mean dark seed per head	Fold increase in dark seed	Mean germination per head	Fold increase in germination	DNA content of seedlings
11	2	40	1.22	0.5	1.27	2n
24	4	25	1.97	7.25	3.34	2n
28	7	27.14	1.1	1.86	1.66	n, 2n
35	5	40.2	1.4	5.6	13.00	2n

Suppression of sexuality in the presence of apomeiosis may simply reflect that meiotic embryo sacs are more predisposed to fertilisation than apomeiotic embryo sacs. Under this scenario, given a lack of parthenogenesis, the fertility of florets that contain apomeiotic embryo sacs is less than those that contain meiotic embryo sacs. In evolutionary terms, it is adaptive for a plant to preserve the integrity of both forms of reproduction, utilising meiotic embryo sacs in the sexual pathway and apomeiotic embryo sacs for the parthenogenetic pathway. Those progeny classes derived from fertilisation of apomeiotic embryo sacs (2n+n), and conversely, parthenogenetic development of meiotic embryo sacs (n+0), have little to contribute to an agamic complex, except in rare circumstances whereby they serve as an intermediate for gene flow into more functional fully apomictic or fully sexual forms.

In *Ranunculus*, suppression of sexuality via the production of aposporous initials was in turn found to be dependent on the timing of the production of aposporous initials relative to meiosis; the less meiosis is allowed to proceed the greater the chance of successful displacement of meiotic structures by those apomeiotic (Nogler, 1984). This is not necessarily the case in *Hieracium*. *H. piloselloides* and *H. aurantiacum* have different timings of appearances of aposporous initials; in the former they appear after the initiation of meiosis whereas in the latter meiosis was never observed. By contrast to Nogler's findings in *Ranunculus,* the *Hieracium* species with early formation of embryo sacs has a slightly lower rate of production of apomictic seed (Koltunow et al., 1998). The timings of the formation of aposporous initials were not investigated in the PG population, but a segregating population's more uniform genetic background may be suitable to investigate any promotion of apomeiotic embryo sac formation by early aposporous initial formation. The preference of mode of development (sexual versus parthenogenetic) of each respective

form of embryo sac (meiotic versus apomeiotic), and how any associated suppression of sexuality imposed by apomeiosis might act, deserve further investigation. This may be implemented by the testing of the derivations of progeny under conditions of emasculation and pollination.

3.6.6 Testing of marker segregation

All markers that were detected by screening the deletion mutant panel were tested on the PG segregating population, along with the parents and G5γ9, 19, 25, 26, 46 and 57 as controls to assist with marker identification. The presence of additional markers transmitted from the female parent precluded the scoring of six markers: Dj, Ck, Ds and Cn of Locus 1, Co of Locus 2 and Cx of Locus 3. For similar reasons, Dz of Locus 1 and Cu of Locus 2 were not scored with high confidence in some segregants. All other markers were scored in characterised segregants with confidence (Fig. 3.7).

In general, marker co-segregation in this population confirmed the pattern of marker linkage inferred from analysis of the deletion mutant panel. There is also evidence of linkage between Locus 2 and Locus 3 indicating that these loci may reside on the same chromosome. Segregation between markers of Locus 1, and of Locus 3 was seen, demonstrating recombination at these loci. All markers of Locus 2 showed complete co-segregation, suggesting that they may be closely linked or that male-meiotic recombination at this locus is suppressed. As previously noted, suppression of meiotic recombination of loci associated with apomixis is commonly reported (Grimanelli et al., 1998b; Ozias-Akins et al., 1998; Noyes and Rieseberg, 2000). The analysis of a larger population is required to ascertain if suppression of recombination is a feature of Locus 2.

3.6.7 Association of the loci with parthenogenesis

A group of six segregants possessed all markers of the three loci. Segregants of this group produced parthenogenetic seed with rates of germination at between seven and 21 seeds per head. A second group of six segregants was characterised by a lack of Locus 1. All contained Locus 2 and at least some markers of Locus 3. Rates of germination of parthenogenetic seed in this group were noticeably lower at 0.25 to 2.29 per head. Furthermore, these plants more frequently showed perturbations among parthenogenetic seedlings such as delayed germination occurring up to 4 weeks after sowing, seedlings being small and weak and seedlings which formed callus. There was also a greater

incidence of polyhaploidy among these seedlings which is discussed in more detail below (see section 3.6.8).

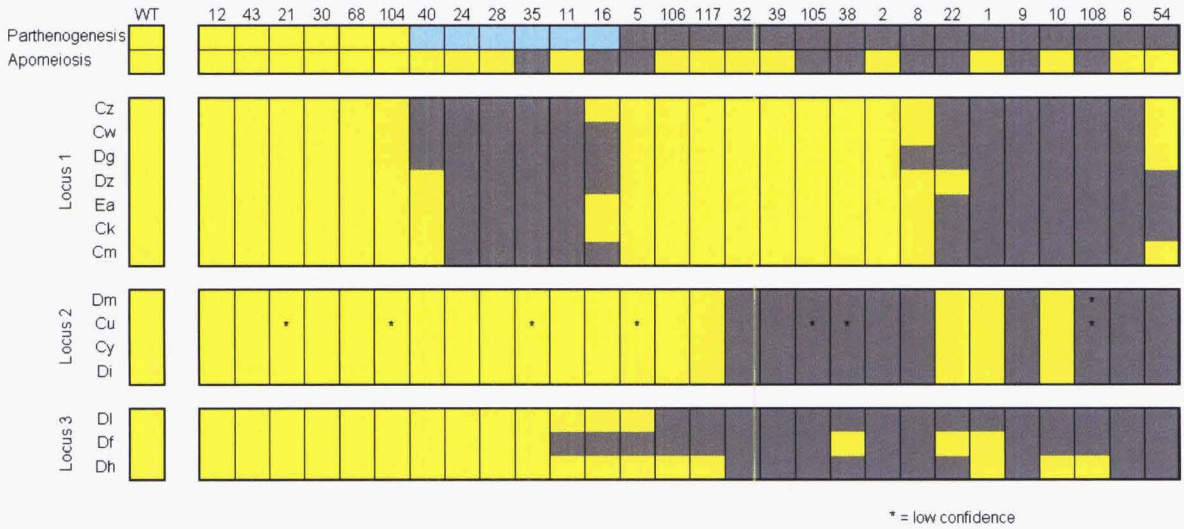


Fig. 3.7 Phenotypes and genotypes of the PG segregating population. Yellow indicates a score of presence and grey indicates a score of absence of either phenotype or marker. Phenotypes for parthenogenesis in blue indicate seed set at very low rates, and in some cases, perturbed growth of seedlings.

The apparent need for all three loci to be present for reasonable levels of parthenogenetic seed to develop indicates that each locus has a role in the expression of the trait in *H. glaciale*. Locus 1 appears to act as a modifying locus. Its presence is not required for apomixis to occur but the parthenogenetic production of germinable seed is significantly reduced in its absence. Further evidence of a modification role of Locus 1 is seen in the total lack of apomixis in segregants that carry Locus 1 but do not carry Locus 2 and Locus 3. The presence of Locus 2 in three segregants (PG5, PG106 and PG117) with no detectable parthenogenesis indicates that Locus 2 is also a modifying locus and not a key enabling locus. The presence of markers of Locus 3 is closely associated with expression of parthenogenesis, indicating that it may be a key enabling locus. Furthermore, only one segregant (PG5) carrying the marker Dl of Locus 3 failed to express parthenogenesis. This segregant shows recombination between markers Dl and Df, indicating that an enabling determinant may lie between these markers. Close linkage of the marker Dl with a determinant at Locus 3 is supported by the deletion of G5γ17. This mutant has lost only the marker Dl, indicating that Dl is the most proximal marker to the determinant of Locus 3.

3.6.8 Association of the loci with apomeiosis

Although the segregation data suggest some role of the three loci in the expression of apomeiosis, the natures of their roles are less clear than they are in the expression of parthenogenesis. Some association of apomeiosis with parthenogenesis is apparent as only two parthenogenetic segregants, PGs 35 and 16, failed to produce apomeiotic eggs. Furthermore, the six segregants that carry all three loci each demonstrated expression of apomeiosis. Which of the loci, however, are directly involved cannot be determined from the current data, although they indicate that the combined presences of Locus 1 and Locus 2, or of Locus 2 and Locus 3, are each insufficient for the expression of apomeiosis. There are no data from this population to deduce any combined effects of Locus 1 and Locus 3. While it is possible that all three loci detected in this study may in combination induce the expression of apomeiosis, it is clear that at least one alternative locus is able to induce this trait. Many segregants that produced no parthenogenetic progeny were still able to produce apomeiotic eggs, including PG6 which possesses none of the three loci.

Locus 1 may have a modifying effect on apomeiosis that is similar to the positive effect it appears to have on parthenogenesis. The locus is not necessary for apomeiosis to occur; however, when it is present in its entirety, both apomeiosis and parthenogenesis together appear to be more penetrant, providing the plant with more fully functional apomixis. Within parthenogenetic segregants of this population, those that carry Locus 1 produced mostly, but not entirely, seedlings derived from apomeiotic eggs. Conversely, segregants that do not carry Locus 1 produced mostly, but not entirely, seedlings derived from reduced meiotic eggs. This observation, along with the increased penetrance of parthenogenesis in segregants that carry Locus 1, implies that the locus may assist in some way with the coupling of the two traits that together promote the effective use of apomixis as a reproductive mode.

The segregant PG40 carries only a recombinant section of Locus 1, with absence of markers Cz to Dg and presence of markers Dz to Cm. The recombination within this locus is accompanied by a low penetrance of parthenogenesis. This observation corresponds with evidence of loss of a modifying determinant for parthenogenesis accompanied by loss of markers Cz and Dg in the deletion mutant panel. While PG40 demonstrates only low levels of parthenogenetic seed production, it demonstrates a high penetrance of apomeiosis, with the seedlings being mostly derived from apomeiotic eggs. Although this observation is restricted to one segregant, it implies that the section of Locus 1 between Dz and Cm may

include a modifying determinant for apomeiosis. If this is the case, any promotion of the coupling of parthenogenesis with apomeiosis by Locus 1 may be mediated by two different modifying determinants, each residing at a different region of the locus and acting separately on the two traits.

3.7 Concluding remarks

The use of deletion mutagenesis in G5 proved successful for the identification of three loci whose loss is associated with loss of parthenogenesis in this species. Deletion mutagenesis proved to be an ideal technique to identify genetic loci of apomicts as abundant quantities of clonal seed are available as material for mutagenesis, and the uniform background between panel members led to the ready identification of commonly lost markers. One marker was successfully converted into a SCAR marker, and may, in principle, be used for BAC clone identification. The distance between markers, however, may be as far as 7.6 Mbp, which may reflect the potential distances between determinants and their most proximal markers. Therefore, while the principle of deletion mutagenesis for the isolation of determinants of apomixis appears sound, taking advantage of this technique requires the analysis of more mutants with more informative deletion breakpoints, so that markers near determinants can be used as starting points to readily build BAC contigs.

The PG segregating population, although small, served to verify the data obtained from the mutant panel, by demonstrating a close association of the three identified loci with parthenogenesis. The population showed variable ranges of phenotypes that indicated that Locus 2 is necessary for the expression of parthenogenesis while Locus 1 and Locus 3 enhanced the levels of parthenogenetic seed produced. Further interpretation of the segregation data, however, could only be made tentatively. Associations between parthenogenesis and autonomous endospermy, and between apomeiosis and suppression of sexuality, are suggested by the data. Whether these associations are the result of genetic linkage or of pleiotropy may only be answered on analysis of far greater progeny numbers.

The two mapping approaches, based on deletion mutagenesis and meiotic segregation, may therefore serve to complement each other. The potential of deletion mutagenesis to avoid any difficulties of conventional mapping of apomixis loci caused by suppressed recombination, and the use of a segregating population for the analysis of the action of each identified locus, were both demonstrated. The use of G5 for the isolation of determinants of apomixis, however, may be limited. Efficient and co-ordinated expression

of both apomeiosis and parthenogenesis appears to occur with the presence of all three loci, but the two components of apomixis otherwise appear to be unlinked in this genotype. The minimum requirements for the expression of apomeiosis are not shown by the current data.

Given the complex genetics of apomixis in G5, the pentaploid genome, and the difficulty generating segregants, further efforts towards isolation of genetic determinants were deferred to the genotype C4D. This genotype is a tetraploid and an efficient pollen parent, whose progeny show clear segregation between apomeiosis and parthenogenesis. With the benefits of this clear segregation, and of experience gained from the G5 mutant screen, the C4D mutant screen was conducted so that mutants with loss of apomeiosis as well as loss of parthenogenesis were identified. The use of the C4D deletion mutant panel and the PC segregating population towards isolation of markers proximal to determinants of apomeiosis and parthenogenesis is described in Chapter 5.

Chapter 4 Chromosomal DNA deletion in the T-DNA mutant *lop1*

4.1 Introduction

Apomeiosis and parthenogenesis together enable facultative apomixis in *H. glaciale* (G5). As seen in Chapter 3, two lines of evidence show that these two components of apomixis are unlinked. First, separation of the two components was evident among F1 progeny of G5. Second, parthenogenesis is able to be mutated with no associated loss of apomeiosis. The T-DNA mutant *lop1*, derived from the G5 background, demonstrates loss of parthenogenesis but retention of apomeiosis. This phenotype is similar to those of many of the mutants of the G5 deletion mutant panel. As *lop1* shares the genetic background of its deletion mutant counterparts, it was easily screened alongside the G5 deletion mutant panel for loss of SDAFLP markers.

Surprisingly, *lop1* was found to have a deletion of Locus 3, a key determining locus for parthenogenesis (see Chapter 3). Deletions and other major chromosomal rearrangements associated with T-DNA integration have previously been found to occur in *Arabidopsis* (Nacry et al., 1998; Kaya et al., 2000) and may in fact be more common than is suggested by the literature. This chapter presents the evaluation of T-DNA insertion as the cause of deletion in the mutant *lop1*. It will begin with a brief introduction outlining prior work during which *lop1* was generated and three T-DNA inserts were identified and partially characterised. The chapter will then describe the utility of the prior work in the investigation, as a component of this doctoral research, of linkage of T-DNA inserts with the deletion of *lop1* at Locus 3.

4.1.1 Generation of the mutant *lop1*

Lop1 is a member of the G5 pAC7 T-DNA insertion panel generated by Dr Ross Bicknell, with assistance from myself and others of the Crop & Food Research Apomixis Programme. The vector used for transformation, pAC7 (Fig. 4.1) was developed jointly by myself and Ross Bicknell. The primary feature of pAC7 is a *Ds* element (Federoff et al., 1983) that is located between a 35S promoter and the selectable marker gene *aada*. On excision of the *Ds* element, the 35S promoter is brought into proximity with *aada* thereby conferring resistance to spectinomycin. An *uida* gene, which confers expression of glucuronidase, with a minimal promoter, was cloned within the *Ds* element. Successful

reintegration of the *Ds* element into the genome at close proximity with a native promoter or enhancer of the host then allows expression of *uida*. The presence of *hpt* driven by a 2'35S promoter on the *Ds* element confers hygromycin resistance, providing a selectable marker for both the presence of the *Ds* element and for transformation. The construct is located between right and left T-DNA borders. The development of pAC7 and the generation of the G5 pAC7 T-DNA insertion panel were made prior to the PhD research.

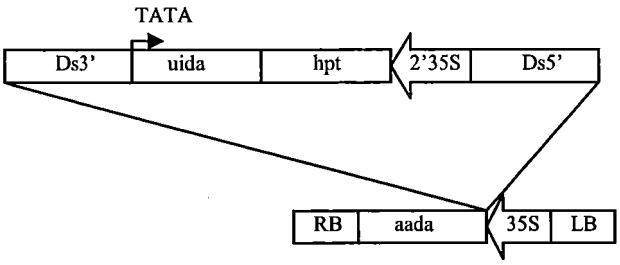


Fig. 4.1 Schematic diagram of the vector pAC7 which was used to generate the *H. glaciale* T-DNA insertion panel. Vector components are not drawn to scale.

4.1.2 T-DNA/genomic DNA junctions of *lop1*

Southern analysis using a probe that spans the right border of pAC7 indicated that *lop1* carries three inserts. To determine if one of the T-DNA fragments had caused the loss-of-parthenogenesis phenotype, TAIL-PCR (Liu et al., 1995) was conducted using primers designed from each border of pAC7 (pApo4RB1, pApo4RB2 and pApo4RB3, see Table 4.1). Three junction fragments were isolated: two derived from right border junctions (pGem33, later extended to include additional sequence, and pGem74) and one from a left border junction (pGem66, see Fig. 4.2). This work was done prior to the PhD research.

4.1.3 Segregating populations

Two populations were generated by Dr Ross Bicknell using *lop1* as the pistillate parent. Each population had different aneuploid accessions of *H. aurantiacum* (A3.4) as pollen parents; one was the wild type A3.4 (*lop1* X A3.4) and the other was the A3.4 T-DNA mutant *loa1* (*lop1* X *loa1*) that carries a complex multiple insert of pSLJ2591 (Yang et al., 1993; Okada et al., 2007). Both n+n and 2n+n progeny formed from these crosses, presumably derived from fertilisation of meiotic and apomeiotic embryo sacs respectively. As apomeiosis results in gametes in which no cross-over has occurred, 2n+n progeny were excluded from the analysis.

4.1.4 Development of PCR markers for T-DNA inserts

PCR tests for the T-DNA inserts were designed using the T-DNA borders and flanking genomic DNA as priming sites. Preliminary screening for the inserts in both *lop1* X A3.4 and *lop1* X *loa1* segregating populations demonstrated cosegregation of the sequences of pGem66 and pGem74, indicating that they are left-border and right-border junctions of the same T-DNA insert or of linked T-DNA inserts. The PCR test of pGem33 showed no co-segregation with other junction PCR tests. Two of the three T-DNA inserts were therefore identifiable by PCR.

Some segregants did not inherit any detectable junction but were positive when PCR tested for *aada*. This indicated the presence of a third insert in *lop1*, as previously indicated by Southern analysis, for which no T-DNA border junction had been identified. However, a PCR specific for the distal region of the left border was found to uniquely identify this insert. The lack of detection of the distal regions of either of the left borders of the other two inserts indicated that they had undergone truncation on insertion. The PCR for the left border of T-DNA could therefore be used to uniquely detect inheritance of this insert. Segregation data indicated that no other T-DNA inserts were present in *lop1*. The three inserts were designated *lop1*A, *lop1*B and *lop1*C, detectable by PCRs that uniquely detected the right border junction sequences pGem33 (using primers pApo4RB2 and pGem33rev), pGem74 (using primers pApo4RB2 and pGem74rev), and a region adjacent to the T-DNA left border (using primers 35SPR1 and Ds5.2), respectively (Table 4.1, Fig 4.2). This work was done by me prior to the PhD research.

Table 4.1 Primers used for the isolation of T-DNA junction fragments of the mutant *lop1* and their subsequent detection. The T-DNA right border primers pApo4RB1, pApo4RB2 and pApo4RB3 were used for TAIL-PCR from the right border of inserts of pAC7 of the mutant *lop1*. The primers pGem33rev and pGem74 rev were designed from genomic DNA sequence of two TAIL-PCR products adjacent to the right borders of two individual inserts, and amplify specific PCR products when paired with a right border primer. The primers 35SPR1 and Ds5.2 amplify a region close to the left border region of pAC7.

Primer name	Primer sequence
pApo4RB1	CCTTAGGCGACTTTTGAACG
pApo4RB2	ACGCGCAATAATGGTTTCTG
pApo4RB3	AGTTCCAAACGTAAAACGGC
pGem33rev	CAATGGTCGGATAATAGAAATGAC
pGem74rev	GCTGAATTAAGTATATTCTTCC
35SPR1	TAAGGGATGACGCACAATCCCACTATCC
Ds5.2	CGTTCCGTTTTCGTTTTTTAC

4.2 Testing for linkage between SDAFLP markers and T-DNA inserts

The following approach described in Section 4.2 was carried out by me as part of this PhD research. To test the possibility of a T-DNA insert being the cause of the deletion of *lop1*, F1 progeny of the *lop1* X A3.4 and *lop1* X *loa1* segregating populations were tested for linkage between the T-DNA inserts and markers linked to the deletion. As *lop1* shows loss of all markers of Locus 3, no markers at this locus were available to test for linkage between T-DNA inserts and the deletion. However, the PG segregating population indicates partial linkage between Locus 2 and 3, so markers Di and Cy at Locus 2 were used as markers putatively linked to the deletion in *lop1*.

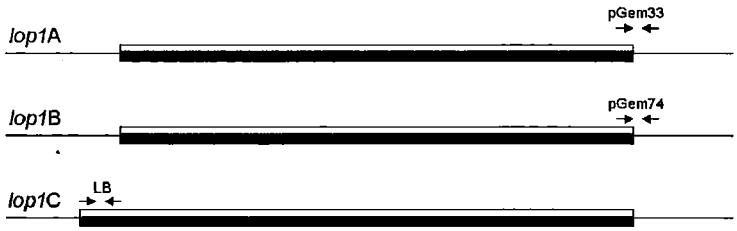


Fig. 4.2 Schematic diagrams of the three T-DNA inserts indicated by Southern analysis that are carried by the mutant *lop1*. The inserts are termed *lop1*A, *lop1*B and *lop1*C. Each insert is detectable respectively by the marker PCRs based on pGem33, pGem74 and left border sequence respectively.

4.2.1 Segregation of T-DNA junctions: variable segregation distortion of T-DNA inserts

A total of 22 *lop1* X A3.4 and 14 *lop1* X *loa1*segregants were tested for the presence of each of the three T-DNA inserts in *lop1*. PCR amplification of the three inserts in *lop1* X A3.4 segregants is illustrated in Fig. 4.3. The segregation of each insert, the SDAFLP markers Di and Cy, and the T-DNA insert of *loa1* (pSLJ2591) among segregants of the two populations, are summarised in Fig. 4.4 a and b. The results indicate that the three inserts of *lop1* (*lop1*A, *lop1*B and *lop1*C) segregate independently and are therefore not linked. Both populations showed some segregation distortion of T-DNA insertions *lop1*A and *lop1*B. The *lop1* X A3.4 population showed significant segregation distortion against inserts *lop1*A (14% inheritance, $P< 0.001$) and *lop1*B (23% inheritance, $P= 0.017$). By contrast, the *lop1* X *loa1* population showed slight segregation distortion towards insert *lop1*A (79% inheritance, $P= 0.057$) and *lop1*B (64% inheritance, $P= 0.424$). Significant segregation distortion of insert *lop1*C was not observable in either the *lop1* X A3.4 population (46%, $P= 0.832$) or the *lop1* X *loa1* population (29%, $P= 0.18$).

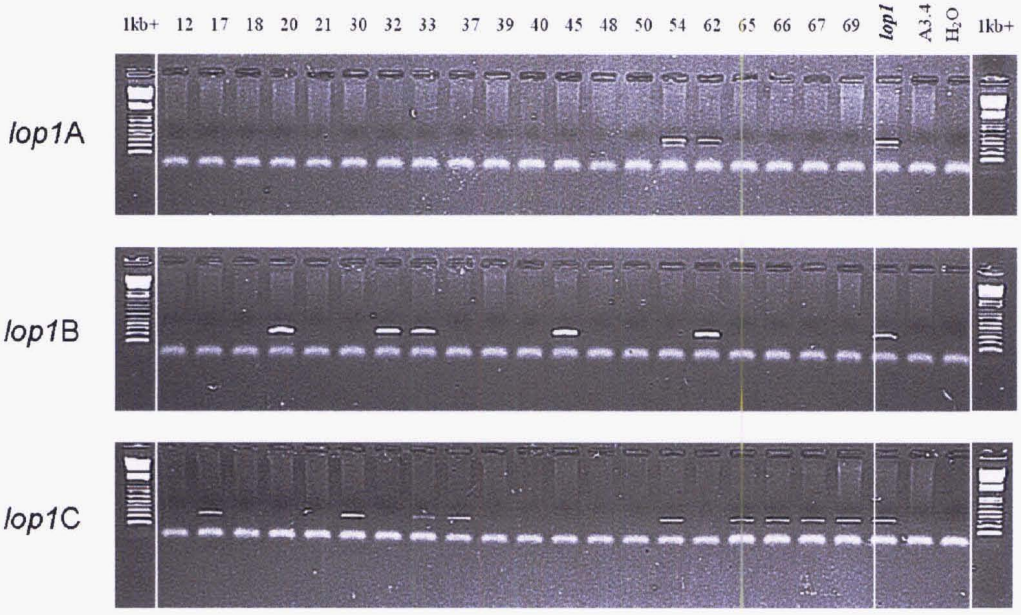
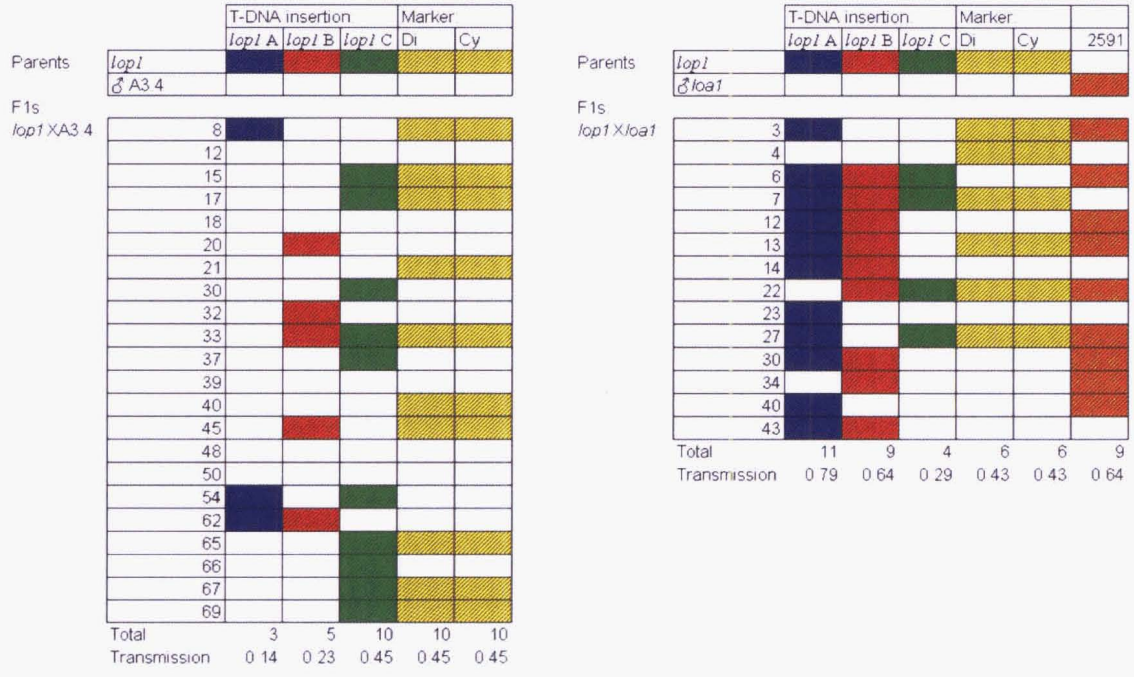


Fig. 4.3 PCR detection of T-DNA inserts *lop1*A, *lop1*B and *lop1*C in a selection of segregants of the *lop1* X A3.4 population.



a **b**

Fig. 4.4 T-DNA insert and marker segregation (coloured) in the *lop1* X A3.4 (a) and *lop1* X *loa1* (b) populations. The presence of the T-DNA insert of *loa1* is indicated by the marker "2591".

The variable segregation distortions in these populations suggest that the locus of the T-DNA insert *lop1*A acts with linked lethality in combination with the wild type paternal A3.4 genotype, but acts as if under meiotic drive in combination with the mutant *loa1*

paternal genotype. This is possibly the result of embryonic lethality caused by maternal alleles in specific combinations with paternal alleles that are transmitted by wild type A3.4, but not by the mutant *loa1*. While there has not previously been any reason to suspect paternal genes having a role in segregation distortion in *Hieracium*, this possibility has not been thoroughly investigated. The use of enlarged populations would be necessary for a more thorough investigation of these hypotheses, which was not pursued as part of this investigation.

4.2.2 Segregation of T-DNA junctions and SDAFLP markers: no evidence of T-DNA as a cause of deletion

As observed in the PG population, the markers Di and Cy cosegregated in all progeny of the *lop1* X A3.4 and *lop1* X *loa1* populations. The markers showed no segregation distortion in either population. No significant co-segregation of any insert with SDAFLP markers closely linked to the deletion was found. There is therefore no evidence that any of the T-DNA inserts detected in *lop1* were the cause of the deletion. This test, however, does not completely eliminate the possibility. It remains possible that a T-DNA insertion/genomic DNA deletion event was accompanied by translocation of either genomic DNA carrying the SDAFLP markers or carrying the T-DNA involved. An inter-chromosomal reciprocal translocation associated with a significant deletion caused by T-DNA insertion was found in *Arabidopsis* (Nacry et al., 1998). If this occurred in *lop1*, markers at Locus 2 might cease to be linked to a T-DNA-induced deletion of Locus 3. A more extensive chromosomal analysis would be necessary to characterise such an event.

4.2.3 Somaclonal variation as a possible cause of deletion

An alternative cause of the genomic DNA deletion of *lop1* is somaclonal variation. The phenomenon has been implicated as a cause of variation following tissue culture and plant transformation involving de-differentiation of plant tissue. Somaclonal variation is often attributed to epigenetic modification such as genomic DNA methylation (Kaeppler et al., 2000). However, chromosomal changes that include deletions have also been implicated. The extent of DNA polymorphism as a result of *in vitro* processes appears to relate to the transformation method in a manner that is dependent on the length of time of *in vitro* culture (Labra et al., 2001). Transformation of *Arabidopsis* via the floral dip method, which imposes no *in vitro* cell culture, results in little genomic modification, whereas untransformed plants derived from callus show significant variation (Labra et al., 2004).

Previous studies have implicated chromosomal aberrations to be associated with somaclonal variation to the extent of changes in chromosome number and meiotic alterations (Pontaroli and Camadro, 2005), and deletions and duplications (Kharabian and Darabi, 2005).

In conclusion, while the current data do not rule out T-DNA insertion as the cause of the deletion of *lop1*, it is equally likely that the deletion occurred during the callus phase of the transformation process, independent of transformation events.

Chapter 5 Deletion mapping of genetic regions associated with apomeiosis and parthenogenesis in *Hieracium caespitosum*

5.1 Introduction

The successful production of a clonal seed through gametophytic apomixis requires the formation of an unreduced embryo sac via apomeiosis followed by its autonomous development into an embryo via parthenogenesis. Depending on the species, endosperm formation may be autonomous or pseudogamous. It is therefore now widely agreed that gametophytic apomixis is not a simple trait and may instead be viewed as a "composite" trait that universally consists of apomeiosis and parthenogenesis. The genetic nature, however, of each trait is still under debate. Data from molecular marker studies in grass species have suggested that both apomeiosis and parthenogenesis are controlled by a single dominant locus (Pessino et al., 1997; Barcaccia et al., 1998; Grimanelli et al., 1998b; Ozias-Akins et al., 1998). Whether parthenogenesis is a pleiotropic effect of apomeiosis (Mogie, 1988), or the locus is a complex one that carries determinants for both apomeiosis and parthenogenesis (Grimanelli et al., 1998b), has also received some discussion.

More recent data from *Poa pratensis* indicates that determinants of apomeiosis and parthenogenesis may segregate (Albertini et al., 2001; Matzk et al., 2005). Apomictic species of Asteraceae demonstrate regular segregation of apomeiosis and parthenogenesis and are therefore controlled by separate loci (van Dijk et al., 1999; Noyes and Rieseberg, 2000). F1 progeny of the cross between the sexual *H. pilosella* (P4) and the apomictic tetraploid *H. caespitosum* (C4D) show clear segregation between apomeiosis and parthenogenesis. The progeny types therefore fall into four classes: two classes consist of progeny that inherit the parental characters of either sexuality or apomixis, and two intermediate classes consist of progeny that inherit either apomeiosis or parthenogenesis. Segregants that inherit apomeiosis but not parthenogenesis produce progeny that include 2n+n hybrids from apomeiotic embryo sacs that require fertilisation. Conversely, segregants that inherit parthenogenesis but not apomeiosis produce n+0 progeny from parthenogenetic development of reduced embryo sacs. The latter segregant class may be less common; previous observations have indicated cases in which parthenogenesis has a contingency on apomeiosis (Albertini et al., 2001; Noyes, 2005). Interestingly, plants whose reproductive strategies are of the form of either intermediate class of segregants,

that are capable of either apomeiosis or parthenogenesis, but not both, are essentially evolutionary dead ends, either through repeated polyploidisation, or haploidisation.

The independent segregation between apomeiosis and parthenogenesis indicated that C4D possesses an ideal genetic background for a forward genetics approach towards identifying their determining loci. This chapter presents a strategy towards isolating determinants of apomeiosis and parthenogenesis of C4D, which is based on the deletion mutagenesis strategy conducted on the *H. glaciale* (G5) deletion mutant panel described in Chapter 3. The previous screen of the G5 mutant panel was successful for the identification of key and modifying loci for parthenogenesis. However, there was no clear association between the identified loci and apomeiosis. In C4D, the identification of mutants with loss of apomeiosis was facilitated by an associated reduction in seed set. Skeletal maps of two loci whose loss was associated with loss of apomeiosis or with loss of parthenogenesis were generated using a small number of primer combinations to screen a large number of mutants. To generate markers at high densities as proximal as possible to key determinants of the traits, small subsets of the panel were then screened for markers until the maps consisted of markers generated from a total of 256 primer combinations. Selected markers were tested for linkage in the PC segregating population, and proximal markers were isolated and their suitability as SCAR markers and probes of a BAC library was assessed.

5.2 The composition of the *H. caespitosum* mutant panel

The C4D mutant panel was generated largely by the work of Sylvia Erasmuson and Pam Fletcher. The panel comprised of a total of 86 members. Mutant sectors of chimeric M1 plants were initially detected by a visible reduction in seed set when compared to the wild-type. Tissue from mutant sectors was regenerated in tissue culture to obtain non-chimeric mutant members of the panel. Assessment of nuclear DNA contents of parthenogenetic and hybrid progeny of panel members revealed 27 mutants with loss of apomeiosis (Loa-). These mutants were able to produce limited quantities of germinable seed from unfertilised heads but nuclear DNA contents of seedlings indicated that they were derived entirely from reduced meiotic embryo sacs. The decline in seed set associated with the loss of apomeiosis was often accompanied by an apparent restoration of meiosis. Therefore, while low numbers of polyhaploid progeny were produced parthenogenetically, most Loa- mutants readily produced n+n hybrid progeny after fertilisation. A second group of 32 mutants showed loss of parthenogenesis (Lop-), producing no germinable seed from

unfertilised heads. Analysis of nuclear DNA contents of hybrid progeny indicated that these mutants were able to produce 2n+n hybrids on fertilisation, indicating retention of apomeiosis. A third group of 27 mutants showed loss of apomeiosis and parthenogenesis (Loa-/Lop-), producing only n+n hybrids, essentially reverting to being sexual plants producing n+n hybrid progeny.

A panel of 79 mutants including 24 with loss of apomeiosis, 30 with loss of parthenogenesis, and 25 that had lost both traits was selected on the basis of the mutants' clear phenotypes with no mutations characteristic of gametic or zygotic lethality. These underwent molecular characterisation with SDAFLP screening alongside two control samples. One (C4Dγ102) was derived from an irradiated seed which showed no loss of apomixis, and the other (C4D107) was derived from a non-irradiated seed.

5.3 An initial survey of marker loss in the *H. caespitosum* mutant panel

Profiles of fluorescently labelled SDAFLP amplification products in the form of peaks captured by the genetic analyser were found to be very similar to profiles of radio-labelled products separated by PAGE in the form of bands, with respect to the sizes and relative intensities of amplification products. Notwithstanding the similarities, the DNA analyser appeared to generally offer greater sensitivity enabling confident scoring of peaks of low amplitude, although there was evidence of a relative decline in amplitude of high molecular weight peaks. This was likely to be due to a relative inefficiency of electroelution by the genetic analyser of high-molecular-weight products for DNA analysis.

SDAFLP profiles from the entire panel of 79 mutants under analysis, along with the control sample C4Dγ102, were amplified using eight primer combinations, yielding 39 commonly lost markers, all of which grouped into one of two clusters strongly associated with either loss of apomeiosis or loss of parthenogenesis. In accordance with conventional nomenclature of genetic loci identified by mutation, these loci have been termed *LOSS OF APOMEIOSIS* (*LOA*) and *LOSS OF PARTHENOGENESIS* (*LOP*), whose mutant alleles *loa* and *lop* result in the phenotypes Loa- and Lop-.

Markers were tabulated into three mutant-by-marker matrices, each composed of one of the three classes of mutants (Loa-, Lop- and Loa-/Lop-). The most parsimonious marker order of each locus - using the principle that the deletions are most likely to be losses of

continuous segments of chromosomal DNA - is given in Fig. 5.1. The order of two pairs of markers at *LOP* could not be resolved due to a lack of any discerning deletion breakpoints between them. Markers were assigned identifiers according to the locus at which they reside and the estimation of their length in base pairs, given on analysis. The most frequently lost markers, loa300 and lop184, are likely to be the markers most proximal to key determinants that reside at the *LOA* and *LOP* respectively.

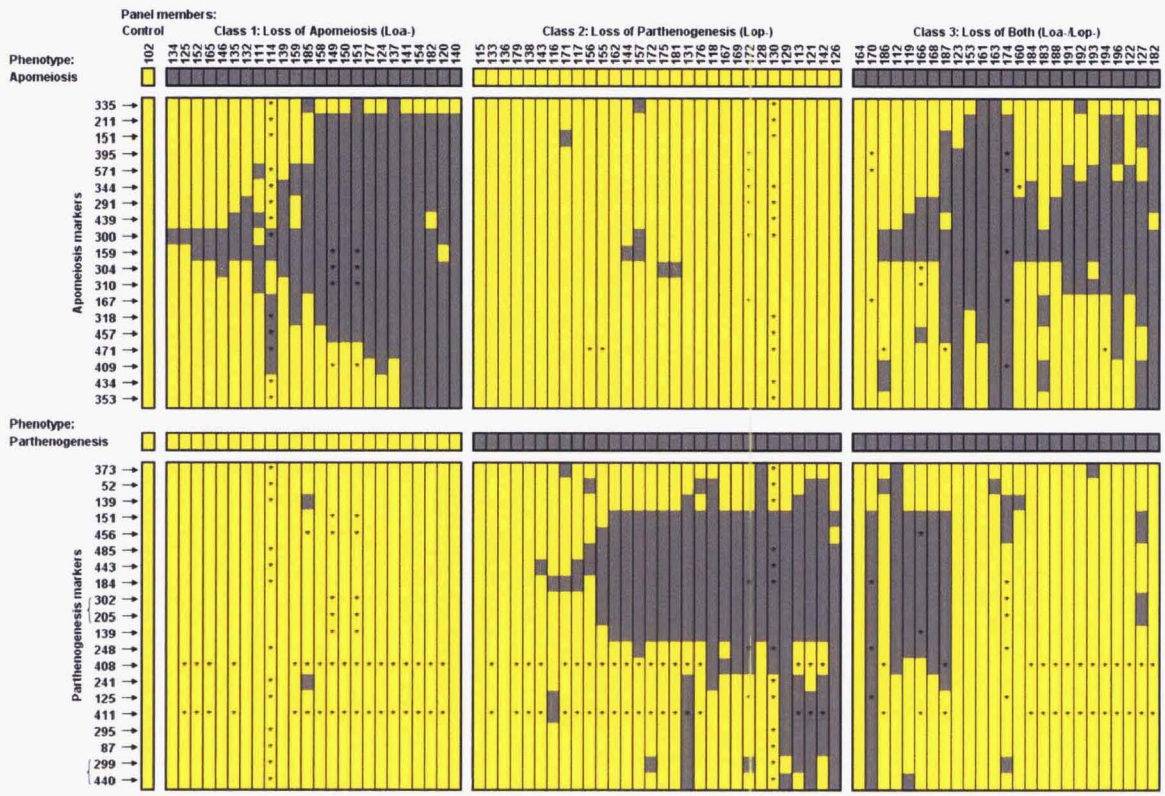


Fig. 5.1 Mutant-by-marker matrices generated by the scoring of markers commonly lost from the C4D mutant panel of 79 mutants. Each column represents either a wild-type control plant (102) or a mutant plant of three classes, Loss of Apomeiosis, Loss of Parthenogenesis, or Loss of Both. Each row represents either a phenotype or a SDAFLP marker. SDAFLP markers were generated by the screening of the mutants with eight SDAFLP primer combinations. Yellow cells represent presence and grey cells represent absence. Asterisks indicate markers whose presence or absence was not determined. Blocks of markers that could not be ordered with respect to each other are in brackets.

Deletions were detected at *LOA* in all 24 Loa- mutants and in 23 of 25 Loa-/Lop- mutants. One Loa-/Lop- mutant showed no deletion. By contrast, five of the 30 Lop- mutants and 12 of the 25 Loa-/Lop- mutants showed no detectable deletions at *LOP*. Another six mutants of the Loa-/Lop- class had deletions that did not span the putative central region of *LOP*. It was possible therefore that loss of parthenogenesis might have had additional causes other than deletions at *LOP*, such as deletions at alternative loci, or in some cases, deletions at

LOA. At this point of the SDAFLP screening, it was assumed that Lop- mutants with no visible deletions possessed small deletions that might be detected when screened with further primer combinations thereby increasing the resolution of the screen.

The number of peaks on an SDAFLP profile averages approximately 170, therefore the yield of the eight primer combinations utilised at this stage of the screen was 1360 potential markers. The 39 markers spanning both loci as a component of the 1360 potential markers may be calculated as representing approximately 2.9% of the genome. Given that C4D has a genome size of 7.2 X 10^9, the physical distance between markers may be estimated at 5.3 Mbp.

5.4 A strategy of further marker generation

A reduced panel of 36 of the potentially most informative panel members was assembled that included nine Loa-, 14 Lop-, and 13 Lop-/Loa- mutants. The reduction in the panel size was achieved by generally eliminating less informative members that possess larger deletions with breakpoints that are distal from the central regions of the loci. SDAFLP screening of this panel alongside the non-mutagenised control C4D107, with a further 20 primer combinations, yielded 13 new commonly lost markers which were added to the map. The mutant-by-marker matrices of this reduced mutant panel are given in Fig. 5.2. Most of the new markers mapped centrally at the two loci, thereby increasing marker density proximal to determinants.

With 20 primer combinations yielding 13 additional markers, the average marker yield at this point was reduced to 0.65 markers for each primer combination. The new markers are, however, expected to be more densely located at *LOA* or *LOP*. Given approximations of 170 scorable bands generated from each primer combination, and a genome size of C4D of 7.2 X 10^9, a total of 4760 potential markers may be estimated to be spaced approximately 1.5 Mbp apart throughout the genome.

The Lop- mutants C4Dγ115, C4Dγ133, C4Dγ136, and C4Dγ179 showed no deletions at *LOP*. Similarly, the Loa-/Lop- mutant C4Dγ164, showed a deletion at *LOP* but not at *LOA*. These mutants were assumed to possess deletions that were not detectable at the resolution of the screen at this time.

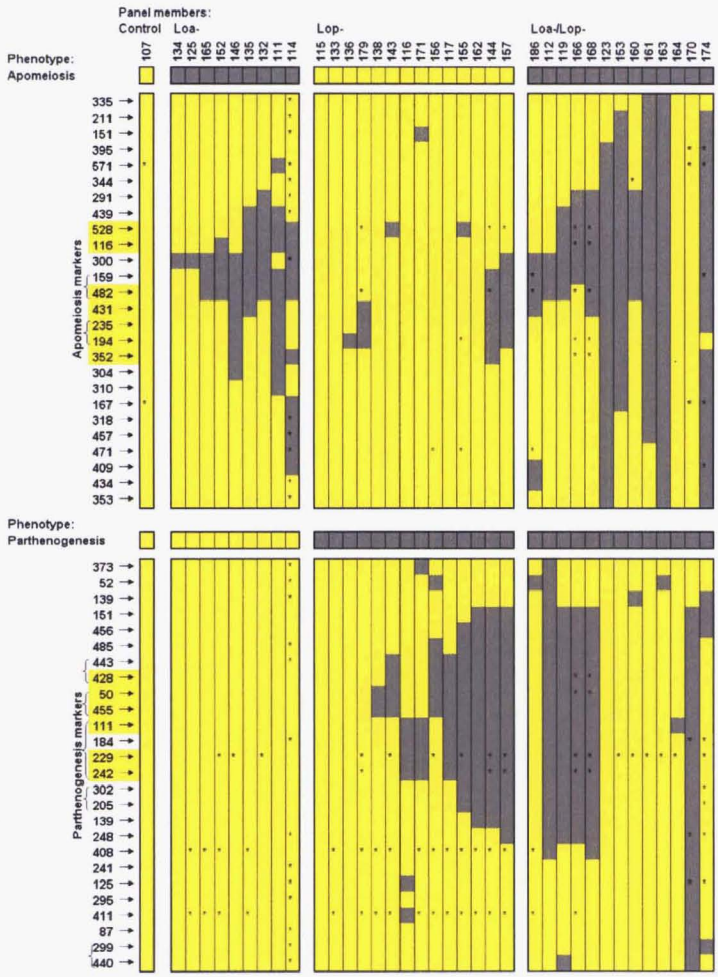


Fig. 5.2 Mutant-by-marker matrices generated by the scoring of markers commonly lost from a reduced panel of 36 mutants, screened with a further 20 primer combinations. The 13 new markers detected from this screen are highlighted in yellow. Asterisks indicate markers whose presence or absence was not determined. Blocks of markers that could not be ordered with respect to each other are in brackets.

5.5 Increasing the densities of marker at *LOP and LOA* and their proximity to determinants

The focus of the SDAFLP screening was maintained on gaining markers at a high density as proximal as possible to the *LOP* and *LOA* determinants. At this point of the marker screening, 28 primer combinations had been used. A total of 256 *Pst*I+N/*Mse*I+3N primer combinations were available. Therefore, a subset of the mutant panel was required to screen the remaining 228 primer combinations for markers. The strategy initially employed was essentially a reiteration of the previous screen, with a reduction of the mutant panel to those members with the most informative breakpoints. A panel of 12 members was selected, composed of 10 mutants with small deletions, one non-irradiated positive control, and, as a negative control to assist to identify potential markers, one mutant with significant deletions in both loci.

While this reduced panel was expected to offer high resolution with respect to the proximity of markers to putative determinants, it was found to have low power with respect to marker detection. This problem might have been compounded by the lack of

duplication of the positive and negative controls. Markers that were present in the positive control, and absent in the negative control, could only be verified against members with small deletions. Therefore only the most proximal markers might readily be detected, resulting in very few markers being generated from this panel. A change in strategy was necessary to prevent any chance of markers being overlooked. The data generated from screening this panel, however, was utilised at a later time to assist with the ordering of markers that were identified using a more powerful panel, as described below.

5.5.1 Raising the throughput of SDAFLP screening with greater marker detection: a "sieve and bin" strategy

The low frequency of detection of markers on SDAFLP screening indicated that the mutants chosen for further screening carried small deletions. Therefore, to ensure the detection of as many potentially useful markers as possible, a panel of mutants was selected that contained members with deletions that spanned a larger region. This panel consisted of 8 samples: two wild type controls, C4Dγ102 and C4D107, the Loa-/Lop- mutants C4Dγ166 and C4Dγ168 which carry moderate deletions at both loci, the Loa- mutants C4Dγ125 and C4Dγ165 which respectively carry small and intermediate deletions at *LOA*, and the Lop- mutants C4Dγ138 and C4Dγ143 which respectively carry small and intermediate deletions at *LOP*, (see Fig. 5.3 a and b for deletion profiles of these mutants). This panel enabled the identification of potential markers (by their presence in C4Dγ102 and C4D107 and absence in C4Dγ166 and C4Dγ168), and, depending on the pattern of their presence and absence in the Loa- and Lop- mutants, their locus and general proximity.

After screening this panel with 96 SDAFLP primer combinations, it was apparent that many primer combinations yielded no markers. To raise the throughput of primer combinations, the panel was split into two mini-panels. The remaining 132 primer combinations were first used to screen a "marker-detection" mini-panel of four samples consisting of the wild type controls C4Dγ102 and C4D107, and the Loa-/Lop- mutants C4Dγ166 and C4Dγ168. Having detected a total of 121 potential markers absent in both Loa-/Lop- mutants (and therefore likely to be positioned within the deletions of C4Dγ166 and C4Dγ168 at either locus, Fig. 5.3 a), each marker was then assessed for presence and absence in a second "marker-location" mini-panel composed of C4Dγ125 and C4Dγ165 (Loa-), and C4Dγ138 and C4Dγ143 (Lop-) (Fig. 5.3 b). Based on the presence and absence

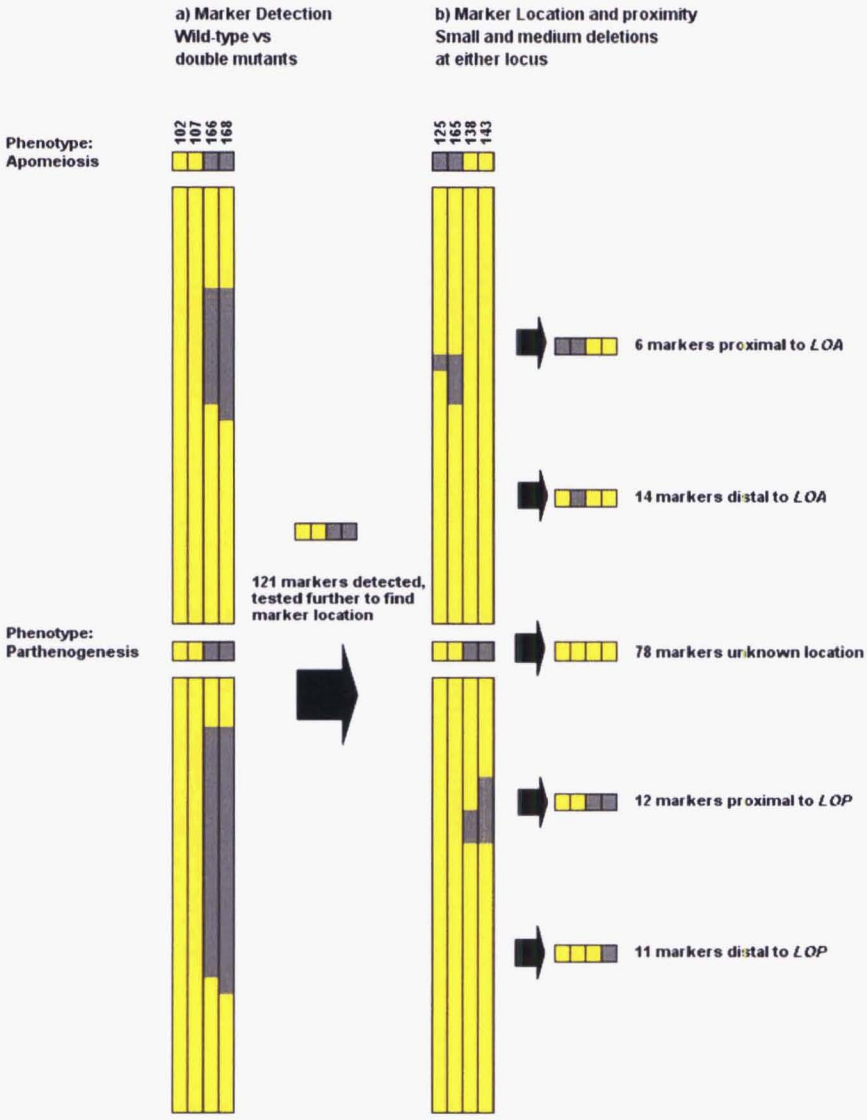


Fig. 5.3 The two stage "sieve and bin" strategy for high-throughput screening of mutants with further SDAFLP primer combinations to detect further markers located at *LOP* and *LOA*. Each column represents the phenotypes and the deletions at *LOA* and *LOP* of a plant. Groups of four squares represent presence and absence patterns of putative markers. a) A mini-panel of two wild-type plants (C4Dγ102 and C4D107) and two Loa-/Lop- mutants (C4Dγ166 and C4Dγ168) with relatively large deletions at both loci were screened with 228 primer combinations. A total of 121 potential markers were detected by their presence in both wild type and their absence in both Loa-/Lop- mutants. b) The primer combinations that detected the 121 potential markers were then used to screen a second mini-panel of two Loa- mutants with small and medium deletions (C4Dγ125 and C4Dγ165 respectively) and two Lop- mutants with small and medium deletions (C4Dγ138 and C4Dγ143 respectively). The presence and absence patterns of each marker in this mini-panel enabled assignment of markers to a locus and an approximate distance from the locus centre, proximal or distal. Markers absent from either both Loa-, or both Lop- mutants of this mini-panel are expected to be more proximal to determinants than markers lost only in one mutant. Markers that are lost in no mutants of this mini-panel are at unknown locations.

pattern, 43 of the 121 potential markers were assigned to a locus (either *LOA* or *LOP*) and given a relative position (proximal or distal). The other 78 markers showed no absence in the marker-location mini-panel, indicating that they were positioned beyond the deletions of the mini-panel members.

5.5.2 The bias of C4Dγ138 and C4Dγ143; further screening with C4Dγ116

The screening of the marker location mini-panel produced 12 further markers proximal to *LOP* (absent in both Lop- mutants) and 11 markers distal to *LOP* (absent in only C4Dγ143). Eight and one of the proximal and distal markers respectively, were mapped. Mapping of the markers indicated that the deletions of the Lop- mutants C4Dγ138 and C4Dγ143, lay with a bias to one side of the locus. The biased deletions of these mutants therefore caused a bias of the location of identified markers to that side of the locus centre (putatively located near the marker lop110). The deletions of mutants C4Dγ138 and C4Dγ143 and how they lie relative to the most central marker of *LOP* detected at this point of the SDAFLP screen, are illustrated in Fig. 5.4.

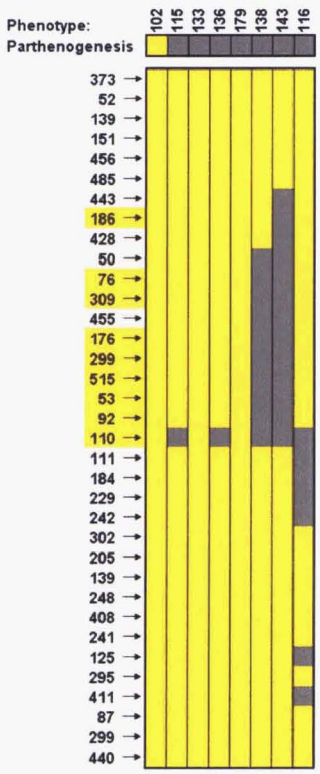


Fig. 5.4 The bias of new markers (highlighted in yellow) towards one side of *LOP*. Putatively the most central marker is lop110, which was the marker most adjacent to the breakpoints of the deletions of mutants C4Dγ138 and C4Dγ143.

To raise the density of markers located at the opposite side of the centre of *LOP*, mutant C4Dγ116, with a deletion biased to that side (see Fig. 5.4), was screened for the absence of

the 78 markers of unknown location, and the 11 markers scored as distal to *LOP* (Fig. 5.5). Of those markers, a total of 19 were found to be absent in C4Dγ116 and therefore likely to be positioned within its deletion at *LOP*.

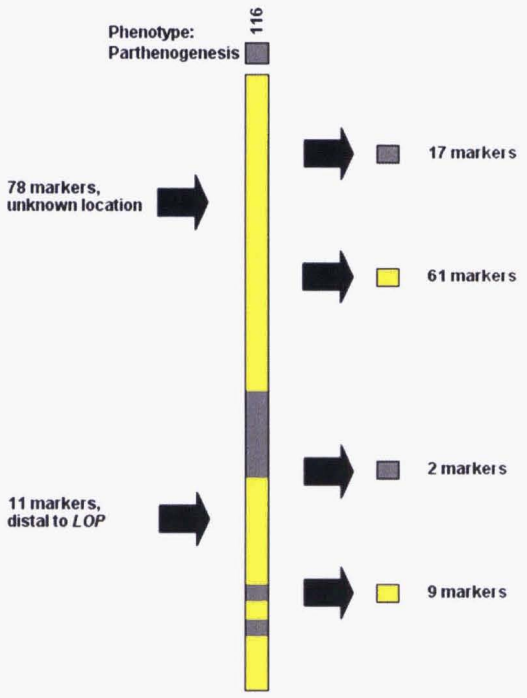


Fig. 5.5 The screen C4Dγ116 with markers of unknown location, and scored as "distal to *LOP*", for their presence or absence in the mutant C4Dγ116. Of the 78 markers of unknown location, and the 11 markers distal to *LOP*, 17 and two markers respectively were found to be absent in C4Dγ116, and were putatively located in the region of the mutant's deletion.

5.5.3 Mapping of identified markers

The mapping of the identified markers was deduced by testing each marker for its presence or absence in 20 additional mutants with informative breakpoints. Testing was restricted to markers that were proximal to putative loci centres, and were able to be scored with confidence. Table 5.1 summarises the mapping of markers that were placed in categories according to their approximate locations based on the results of the "sieve and bin" screening. The final mutant-by-marker matrices are illustrated in Fig. 5.6. Some primer combinations were tested against only a subset of the 20 additional mutants if the data points could not assist with mapping (noted as not determined). The markers most frequently absent in association with loss of the traits, loa223 and loa300 of *LOA* and lop110 of *LOP*, are most likely to be most proximal to determinants.

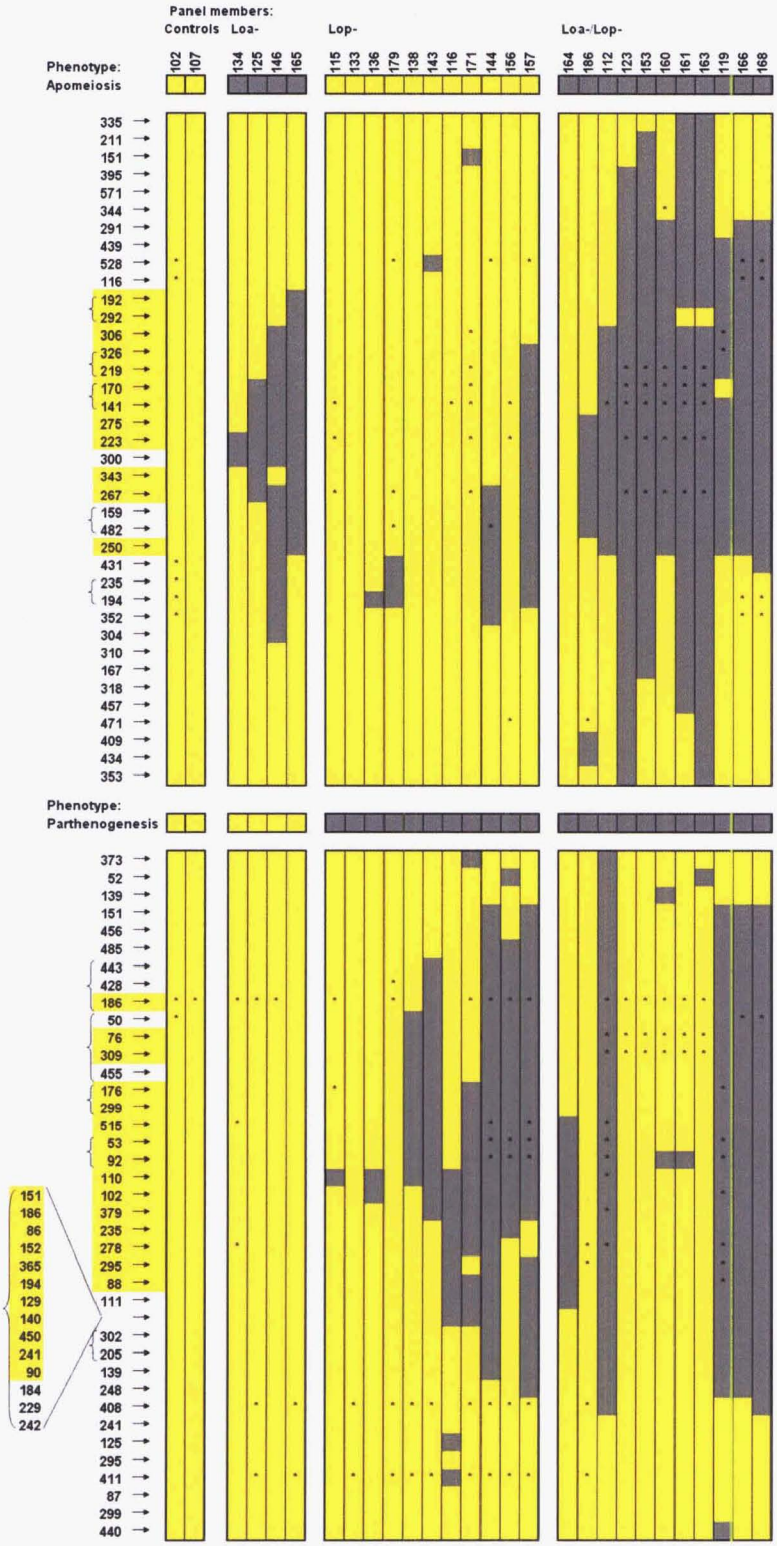


Fig. 5.6 Mutant-by-marker matrices generated by the mapping of markers detected by the "sieve and bin" strategy, at *LOA* and *LOP*, based on their presence and absence in 26 key mutants. Newly discovered markers are highlighted in yellow. Asterisks indicate markers whose presence or absence was not determined. A significant block of markers, lop151 through to lop242, could not be ordered with respect to each other. Other blocks of markers that could not be ordered with respect to each other are in brackets.

All Loa- mutants and all except one Loa-/Lop- mutant showed corresponding deletions at *LOA*. Conversely, however, the Lop- mutant C4Dγ157 carries a deletion at *LOA* that mapping indicates is likely to include a putative determinant, yet assessment of nuclear DNA contents of its progeny indicates the production of 2n+n hybrids from unreduced embryo sacs. The cause of this outlier is unknown. Fertilisation of reduced eggs by unreduced pollen, which produces progeny whose nuclear DNA contents resemble those derived from unreduced embryo sacs, is unlikely, as unreduced pollen from *H. aurantiacum* (A4) is rare. Although possible, it is also unlikely that a determinant for apomeiosis has been retained despite a significant deletion that includes flanking markers. This would require the deletion to be non-contiguous sparing the determinant. Verification of the phenotype and genotype of this mutant is therefore required, by reanalysis of the nuclear DNA contents of its progeny and verification of the source of material for DNA analysis.

Table 5.1 A summary of the mapping of markers identified from screening using the "sieve and bin" strategy. Identified markers that were readily scorable went on to be mapped by screening for their presence or absence in 26 key members of the mutant panel (*denotes markers that were later found to be absent in C4Dγ116).

Marker Category	Markers identified	Markers mapped
Proximal to *LOA*	6	5
Distal to *LOA*	14	6
Proximal to *LOP*	12	8
Distal to *LOP*	11	1 (+2*)
Additional *LOP* markers absent in C4Dγ116	19	17
Unknown	61	0

While the two central *LOA* markers, loa223 and loa300, are frequently absent in association with loss of apomeiosis, the central *LOP* marker, lop110, is more commonly retained despite loss of parthenogenesis. Two Lop- mutants, C4Dγ133 and C4Dγ179, have no deletions at *LOP* detected. The phenotypes of both mutants were distinctively loss-of-parthenogenesis; no alternative gametic or zygotic lethality was evident as both produced substantial quantities of germinable n+n and 2n+n hybrid seed on fertilisation (data not shown). The apparently intact copies of *LOP* in these mutants when parthenogenesis is lost raises the probability that mutations at other loci might be involved. Data from the G5 mutant panel and the PG segregating population indicate that deletions at any of three loci are able to cause significant reductions in parthenogenetic seed set, with

one locus acting as a key determinant and two additional loci acting as modifiers. Modifiers are similarly likely to play a role in parthenogenesis in C4D, and it is possible that C4Dγ133 and C4Dγ179 carry deletions of modifying loci. It remains possible, however, that mutation at *LOP* has occurred in these mutants. The two mutants may still possess deletions that are too small for resolution by the current screen. The deletions of C4Dγ115 and C4Dγ136, defined by one and two markers respectively, were resolved with the most recently identified markers of this screen, and further resolution of undetected deletions at *LOP* may be possible with further primer combinations. Alternatively it is possible that the γ-irradiation has caused chromosomal inversions or translocations that have resulted in a loss of function of the genetic determinant(s) but no accompanying marker loss. Similarly, if mutations in genes of *trans*-acting factors involved in epigenetic changes, resulted in the loss of apomixis, an accompanied common marker loss detectable by this screen may not occur. A previous marker screen, undertaken by others of the Crop & Food Research Apomixis Group, using methylation sensitive AFLP, indicated that *Hieracium* mutants with loss of apomixis had genomes with variable methylation states.

It is interesting to note that the Loa-/Lop- mutants C4Dγ160 and C4Dγ161 both show deletion of one of the most recently identified markers lop92, where deletions were not previously detected. Deletions at *LOP* cannot easily be fully attributed as the causes of loss of parthenogenesis in Loa-/Lop- mutants as loss of the trait may conceivably be a pleiotropic effect of the deletions of *LOA*. In the cases of C4Dγ160 and C4Dγ161, there is no additional data to verify whether the deletions of lop92 caused loss of parthenogenesis. However, they do provide a tentative indication that a determinant of parthenogenesis lies between lop110 and lop92, rather than between lop110 and lop102 (see Fig. 5.6).

A total of 256 primer combinations were used, and there were approximately 170 scorable bands generated from each primer combination. Therefore, a total of approximately 43500 candidate markers were assessed over the course of the screen. Given an approximate genome size of C4D of 7.2×10^9 bp, the average distance between each marker may be estimated at 165 kb. It should be noted, however, that this may be an under-estimation of the average inter-marker distance. Bands of repetitive sequences are not necessarily scorable and therefore cannot be fairly considered as candidate markers. Accurate estimations of physical distances between markers will be made on gaining BAC tiling paths that contain multiple markers.

The mapping validated the sieve and bin approach that was taken: first identifying markers with a few mutants with key deletions, then mapping of the identified markers with further mutants with informative breakpoints. The Loa- mutants C4Dγ125 and C4Dγ165 have deletions that span the putative central markers of *LOA*, loa223 and loa300, with little biases towards either side of the locus. The deletions of the Lop- mutants C4Dγ138 and C4Dγ143, however, showed significant biases towards one side of *LOP*, but these were adequately compensated for by screening for absence of identified markers in C4Dγ116. Markers identified due to their absence in this mutant included the centrally located markers lop102 and lop379, and these markers provided resolution between the proximal breakpoints of C4Dγ138 and C4Dγ143, which were previously unresolved.

5.6 Marker isolation

The effort towards the isolation of markers for their use as probes and SCAR markers for BAC library screening was focussed towards markers proximal to determinants at *LOA* and *LOP*. Other markers that shared primer combinations with key markers were able to be isolated for sequencing and characterisation with little additional effort.

5.6.1 Mini-sequencing of markers

Mini-sequencing was limited to markers over 100 bp in length. For mini-sequencing, each sample requires the analysis of a series of 12 mini-sequence profiles. For accurate identification of the peaks, a control series of profiles was required in which the peaks for mini-sequencing were absent. The mini-sequencing was therefore performed on the Loa- mutant C4Dγ140 and the Lop- mutant C4Dγ129 (see Fig. 5.1). Each mutant was able to serve as a control for the other, as they each possessed large deletions at either *LOA* or *LOP*. This enabled markers from either or both loci to be mini-sequenced from the same mini-sequencing profiles. Using the mini-sequencing profiles of these two mutants, a total of 45 markers were subject to mini-sequencing of which mini-sequence of between one and three bases was generated from 25 *LOA* and 18 *LOP* markers (Table 5.2 columns 1-3). Some mini-sequences gave more than one base at one or more of the three mini-sequenced bases, a characteristic common with some markers of the G5 mutant panel (see Table 3.1 of Chapter 3). This is believed to be due similarly to the presence of SNPs within the mini-sequenced bases at linked DNA duplications.

5.6.2 Design of *Mse*I primers with additional selective bases

In an effort to reduce the complexity of electrophoresed SDAFLP profiles for band isolation while increasing the intensity of marker bands, 14 extended *Mse*I selective primers were designed using the mini-sequence data as two or three additional selective bases. Each primer was tested as the *Mse*I selective primer for SDAFLP using templates from the wild type control C4D107 and the Loa-/Lop- mutant C4Dγ166. Ten markers were successfully amplified from the wild type template while showing absence of amplification from the mutant template, thereby confirming the utility of each primer for band isolation, and the mini-sequence data from which they were designed (Table 5.2 columns 4 and 5).

5.6.3 Marker band isolation

Band isolation of a total of 15 and 11 proximal markers at *LOA* and *LOP* respectively was attempted from profiles generated from *Pst*I primers combined with conventional and extended *Mse*I primers. Initial attempts at isolation of the 26 markers were made from conventional SDAFLP profiles of two wild type samples (C4Dγ102 and C4D107) electrophoresed alongside profiles from two Loa-/Lop- mutants (C4Dγ166 and C4Dγ168) as negative controls. From these standard profiles, five central markers were successfully isolated. Three additional markers distal to *LOP* (lop428, lop450 and lop302) were isolated due to their virtue of being amplified by primer combinations in common with those of proximal markers. All markers that were not successfully isolated and for which extended *Mse*I+5N/6N primers had been designed were then amplified using their *Pst*I primers combined with their *Mse*I+5N/6N primers and electrophoresed enabling isolation of a further nine marker bands (Table 5.3).

Amplification of markers using their *Mse*I+5N/6N primers generally proved successful in reducing the complexity of the SDAFLP banding profiles and lifting the intensities of the bands. In some cases the use of *Mse*I+5N/6N primers was necessary for band visibility. There was also evidence that band intensity was enhanced by increasing the concentration of the primers. Most marker bands, including some that amplified only faintly, were successfully isolated directly from silver-stained gels. Fig. 5.7 illustrates partial SDAFLP profiles containing four markers that were isolated. The use of radio-labelled SDAFLP profiles, which are generally more intense than silver-stained profiles, was considered for the isolation of fainter bands but it was decided not to proceed. Radio-labelled profiles depend on indirect band isolation from gel locations deduced from autoradiographs, thereby raising the risk of incorrect band isolation.

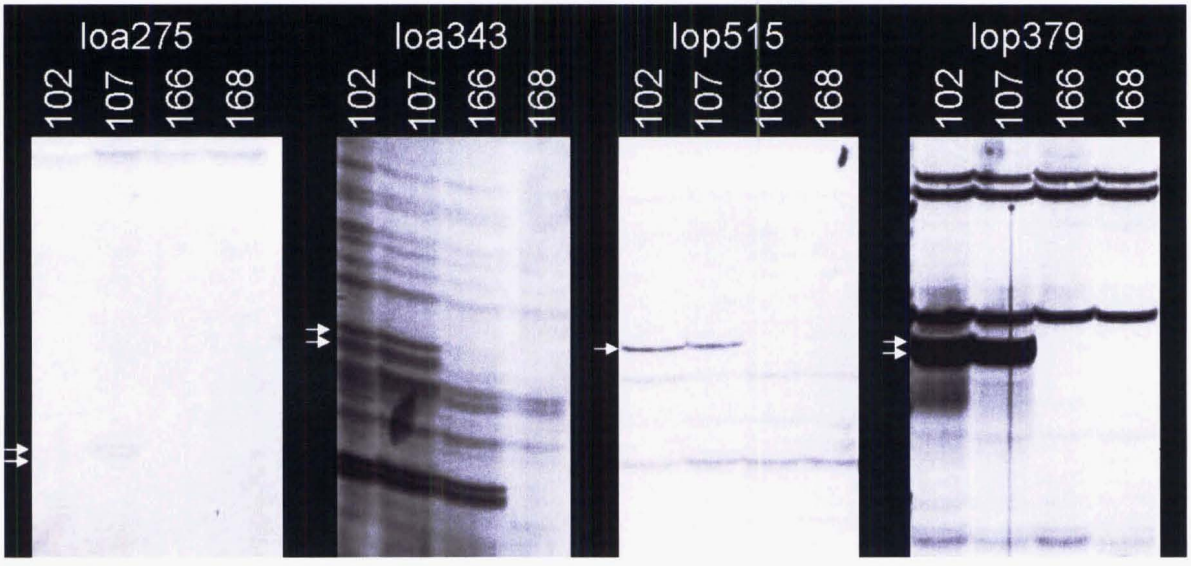


Fig. 5.7 Examples of markers within gel sections of profiles amplified using *Mse*I+5N/6N primers. Profiles of two wild type samples (C4Dγ102 and C4D107) and two Loa-/Lop- samples (C4Dγ166 and C4Dγ168) were electrophoresed and silver-stained and bands were excised from each wild type sample. The markers loa275, loa343 and lop379 have amplified as doublets that are believed to be due to variable A tailing.

Some markers were unexpectedly apparent as doublets or even triplets (see Fig 5.7), while sequences of each band of a doublet or triplet were identical or showed close to 100% similarity. Microsatellites within linked duplications of marker sequences might be expected to cause a pattern of multiple banding but no similar evidence of microsatellites was evident in standard SDAFLP profiles. The multiple banding may be due to A tailing of the products throughout amplification giving rise to different species of the markers with variable lengths. This may only have occurred in profiles for band isolation due to their increased primer concentrations, a step that was taken to raise the intensity of bands.

In the cases of four markers, initial attempts at isolation resulted in the final sequences being shorter than expected. This suggested that alternative bands were being isolated. The profiles from which these bands were isolated were consistent with this possibility. Very strongly amplified bands that preceded the bands of interest in the lanes on the gels often ran on the gel with a trailing streak, which possibly contaminated the gel slice containing the band of interest. This was a significant issue; due to their lower molecular weights and the efficiency at which they were amplified by PCR, these contaminating bands were preferentially re-amplified and later cloned during the isolation process. For loa223, a band of the expected length could not be isolated despite repeated attempts. This was the result of persistent contamination by a lower molecular-weight insert.

Table 5.2 Mini-sequence data of selected markers with confidence scores assigned on the basis of the clarity of each mini-sequence peak. Confidence scores range from 1 to 5, with 5 representing the highest confidence, for each base-position of the mini-sequence. *Mse*I+5N/6N primers for some central markers with mini-sequences of high confidence were designed. The primers were tested for successful amplification of their respective SDAFLP markers.

Marker	Mini-sequence	Confidence (bases 1,2 and 3	MseI+5N/6N primers	Amplification using MseI+5N/6N primers
loa395	CAG	555		
loa571	TTC	544		
loa344	A/TAA	444		
loa291	CAA	544		
loa439	GTC	433		
loa116	GGG	444		
loa292	?CA	-44		
loa326	CTT	255		
loa219	CGG	444	Y	Y
loa170	CCA	233		
loa141	GCC	555	Y	
loa275	TAT	555	Y	Y
loa223	CTT	444	Y	
loa300	GAA	545	Y	Y
loa343	CAT	553	Y	Y
loa267	GTC	554	Y	Y
loa159	ATA	555	Y	Y
loa250	ACC	235		
loa235	GG/TC	333		
loa194	AGT	433		
loa352	GCG	455		
loa304	TCC	444		
loa310	GAT	554		
loa318	ACC	555		
loa409	CCT	555		
lop50	AG	44		
lop76	ATC/G/T	433		
lop309	A/CCT	444		
lop455	GG	53	Y	
lop515	CCC	555	Y	Y
lop110	CCA	555	Y	Y
lop379	GTG	553	Y	Y
lop235	AAC	545	Y	Y
lop295	GGA	425		
lop111	AA/TA	553	Y	
lop365	??T	--4		
lop129	?T?	-5-		
lop450	G	5		
lop184	TGG	544		
lop151	G?G	3?5		
lop248	GTT	543		
lop125	GTG	555		
lop299	GAA	542		

Table 5.3 Isolation of markers at *LOA* and *LOP*, from SDAFLP profiles that were generated using either standard or extended *Mse*I+5N/6N primers and electrophoresed.

Marker	Isolated from MseI+3N	Isolated from MseI+5N/6N
loa192		
loa292		
loa306		
loa326		
loa219		Y
loa170		
loa141		
loa275		Y
loa223		
loa300		Y
loa343		Y
loa267		Y
loa159		Y
loa482	Y	
loa250		
lop428	Y	
lop309		
lop455	Y	
lop299	Y	
lop515		Y
lop110		Y
lop102	Y	
lop379		Y
lop235		Y
lop278	Y	
lop295		
lop111		
lop450	Y	
lop302	Y	

5.6.3.1 Amplification and isolation of additional markers

The *Mse*I+5N/6N primers were designed specifically for individual markers, resulting in a high degree of specificity. Surprisingly, three additional markers were amplified from wild type samples using *Mse*I+5N/6N primers, while showing distinct absence in the Loa-/Lop- mutants C4Dγ166 and C4Dγ168. None of the three markers showed any amplification using the less specific *Mse*I+3N primers. For example, a 418 bp product that was strongly amplified using *Mse*I+CCAATA showed no amplification using *Mse*I+CCA, which intuitively would be expected to have occurred. It is possible that the three additional markers were amplified in standard SDAFLP profiles using *Mse*I+3N primers,

but only at levels that were not detectable. If this were the case, it highlights the competitive nature of AFLP and SDAFLP where the absence of amplification of some bands results in the increased amplification of others.

5.7 Marker sequencing and characterisation

In general, for each marker, three or more sequences were generated from two isolated bands, to help verify that the correct bands were isolated, and to gain a consensus sequence. The sequences included a direct sequence from the band excised from the lane of sample C4Dγ102 and two individual colonies with inserts of cloned fragments of C4D107. Finished sequence was derived whenever possible from consensus sequences derived from isolated bands of C4D107, as this plant was not subject to any mutation-causing γ-irradiation. In the case of lop278, the fragment proved difficult to clone and therefore the direct sequence from C4Dγ102 was used. Based on the consensus between sequences and sequence length, sequenced markers were attributed confidence scores on being correct bands and being correct sequence (Appendix 7.2). Some variation between the lengths of the markers as predicted by the genetic analyser and the lengths of the corresponding marker sequences were observed. In most cases this was attributable to the different primer positions of the *Mse*I+3N and the *Mse*+5N/6N primers.

5.7.1 Bioinformatic analysis of marker sequences

Significant ORFs predicted by the ORF finder and exons predicted by GENSCAN in the 15 central markers with finished sequences are listed in Table 5.4. Predictions of exons showed general consistency with predicted ORFs. As expected, the exon prediction of GENSCAN appeared to be more conservative; only three ORFs, the major ORFs of loa159, loa482 and lop379, were predicted as exons by GENSCAN and only the ORF of loa482 was predicted to be an exon in its entirety. All of the predicted exons corresponded with the largest ORF of each marker. However, this does not eliminate the possibility of partial exons at either boundary of some markers.

While many of the marker sequences contain extended potential ORFs characteristic of coding sequences, only two showed similarity to nucleotide sequences of the nr database of GenBank. No markers showed significant similarity to known sequences at the nucleotide level. However, at the peptide level (using the tblastx algorithm), markers lop299 and lop379 showed significant similarity (Expect ≤ 0.0001) to known sequences. Lop299 contains two extensive potential ORFs, one spanning almost the entire marker sequence.

While the sequence shows no significant similarity at the nucleotide level to known sequences, part of the ORF shows low-level similarity at the peptide level to genomic sequence of *Lactuca sativa* (lettuce) and of *Stevia rebaudiana*, with Expect values of 4×10^{-5} and 0.002 respectively. Both species, like *Hieracium*, are from the Asteraceae family, and, as described in Chapter 3, apomixis has been reported in *Stevia rebaudiana* (de Oliveira et al., 2004). Conservation at the peptide level, when divergence has occurred at the nucleotide level, implies escape from mutational decay, and therefore implies a possible function. Attribution of function cannot be made as the similarity extends only over approximately 60bp. It is likely, however, that *Hieracium*, *Lactuca* and *Stevia* share descendents of ancestral transposable elements. The similarity that lop299 has with genomic sequence of *Lactuca* and *Stevia* may reflect the marker's derivation from a transposable element, whose functionality if lost, has only been lost recently in evolutionary time.

Of more interest were the similarities that marker lop379 showed with plant protein kinases. At the nucleotide level, these similarities were restricted to two short regions of lop379. The sequence of the marker between bases 211 and 280 showed similarity to a kinase gene of *Arabidopsis* with an Expect value of 8×10^{-5}. A second region between bases 251 and 275 showed similarity to sequences from a diverse range of genomes. This region, including four repeats of a CAC trinucleotide microsatellite, is a sequence of low complexity, and local similarity with sequences that contain simple repeats occur readily by chance.

While blastn searches for similarities between the query sequences and the non-redundant (nr) nucleotide database of GenBank, tblastx and blastx translate the query into all 6 possible peptide sequences prior to conducting similarity searches of translated nucleotide and peptide databases. The translations result in more sensitive searches as they allow for the redundancy of codon triplets that causes nucleotide sequences to diverge sufficiently to limit the detection of similarity by blastn. Similarity searches conducted using tblastx and blastx to search the nr nucleotide and peptide databases gave much more significant results. Similarities at the peptide level were found that spanned the entire length of lop379 with Expect values as low as 6×10^{-38}. The proteins that showed similarity to lop379 were predominantly with receptor protein kinases or (Pto-like) serine/threonine kinases from a wide range of plant species including *Arabidopsis* and rice. Interestingly, the four repeats of the trinucleotide CAC that are present within the microsatellite described above predict

the presence of four histidine residues, which were not common in any similar sequence. These extra residues may be due to slippage at the DNA replication fork that occurs at microsatellites, or they may represent a unique functional feature of the gene sequence of lop379.

Table 5.4 Summary of open reading frames, predicted by the ORF Finder, and exons, predicted by the GENSCAN, of sequences of markers at *LOA* and *LOP*.

		Output from ORF Finder			Output from GENSCAN	
Marker	**Genomic Sequence Length**	**ORF predictions (frames)**	**ORF length**	**ORF features**	**Exon prediction (strand)**	**Exon features and probability**
loa219	194	3-164 (-1) 38-190 (+2) 2-133 (-2)	162 153 131	 Stop at 190	None	
loa275	252	2-199 (+2) 60-251 (+3) 54-194 (-2)	198 193 141	Stop at 199	None	
loa300	281	1-108 (-3) 3-77 (+3) 1-66 (+1) 3-59 (-1) 219-272 (-1)	108 75 66 57 54	 Stop at 77 Stop at 66 Stop at 219	None	
loa343	319	80-318 (-1) 37-195 (-2) 213-317 (-3)	240 159 105	Stop at 80 Stop at 37 Stop at 213	None	
loa267	243	1-197 (-2) 104-242 (+2) 18-155 (+3)	197 140 138	 Stop at 155	None	
loa159	136	26-121 (+2) 1-68 (-3) 2-58 (-1)	96 68 57	Start at 26, Stop at 121	26-102 (+) 26-92 (+)	Initiation, 0.96 Initiation, 0.016
loa482	462	56-442 (-3) 26-316 (+2) 309-395 (+3) 401-461 (+2) 406-461 (-1)	387 291 87 62 57	Stop at 56 Stop at 316 Stop at 395 Stop at 406	442-56 (-) 409-56 (-)	ATG-Stop, 0.918
lop455	436	294-435 (+3)	143	No more found	None	
lop299	275	3-263 (-1) 15-245 (+3)	261 231	 Stop at 245	None	
lop515	496	213-494 (-3) 1-179 (-3) 1-111 (+1)	282 179 111	Stop at 213 Stop at 111	None	
lop110	88	2-87 (-1) 1-72 (-2) 3-59 (+3)	87 72 57	 Stop at 59	None	
lop102	74	1-54 (-3)	54	No more found	None	
lop379	357	1-327 (-1) 210-356 (+3)	327 148		324-48 (-) 324-30 (-) 324-39 (-) 327-39 (-) 327-48 (-)	Initiation, 0.3 Initiation, 0.011 Initiation, 0.298 Initiation, 0.188 Initiation, 0.188
lop235	205	28-204 (+1) 2-163 (+2) 1-125 (-3)	177 162 125	 Stop at 163		
lop278	255	28-117 (+1) 11-94 (+2) 1-82 (-3)	90 84 82	Stop at 117 Stop at 94		

Despite the uncommon histidine residues and the low levels of similarity that lop379 has with known gene sequences at the nucleotide level, it is highly likely that the marker codes for an actively expressed protein kinase, and is not part of a pseudogene. A domain search for conserved peptide domains against the CDD database of NCBI (Marchler-Bauer and Bryant, 2004) indicated that the sequence of lop379 codes for part of a catalytic domain with similarity to those of Tyrosine kinases and Serine/Threonine protein kinases. Furthermore, lop379 contains all invariant residues of the protein kinase catalytic domain, highlighting the conservation of function in the sequence that has occurred (Stone and Walker, 1995).

5.7.2 Potential role in parthenogenesis of the gene at lop379

Protein kinases catalyse the transfer of the γ-phosphate from ATP to amino acid side chains of proteins, thereby reversibly changing the conformation of the downstream protein. The regulation of protein kinases is mediated via a variety of signals, including second messengers such as cyclic nucleotides and calcium, or by phosphorylation by protein kinases upstream in a phosphorylation cascade. Some protein kinases possess transmembrane domains and are activated by extracellular signals. Protein kinases feature in mediating signal transduction for many processes in response to both environmental stimuli and endogenous signals and may often play essential roles in conferring traits (Stone and Walker, 1995).

The functional roles of protein kinases are diverse; therefore no function can be attributed solely on the basis of nucleotide sequence. However, the marker's proximity to the central region of *LOP* attracts further investigation into its potential role in parthenogenesis. To change the state of an embryo sac from quiescence to the activation of the cell division of embryogenesis in the absence of fertilisation requires signal transduction that is likely to involve one or more protein kinases. Pivotal roles of protein kinases in aspects of plant reproduction are well documented, including ovule development (Benjamins et al., 2001; Chevalier et al., 2005) and megagametogenesis (Pischke et al., 2002). Most interestingly, the genomic sequence of lop379 showed significant similarity at the peptide level to SERK and the *SERK*-like family of genes of a number of species ranging from *Arabidopsis* to maize. As outlined in Chapter 1, *SERK* was discovered as a marker gene expressed in carrot competent and embryogenic cells (Schmidt et al., 1997). In the context of parthenogenesis, *SERK* has attracted a great deal of interest. Its role in parthenogenetic cell types, however, resembles that in sexual reproduction (Tucker et al., 2003). Its expression

is therefore likely to be in response to upstream signals that have unique roles and unique expression patterns associated with either sexuality or parthenogenesis.

While it is possible that the gene at lop379 plays a role in parthenogenesis, the deletion mutant panel has five Lop- mutants that possess lop379, and are therefore likely to possess the full gene sequence. This implies that there are additional requirements for parthenogenesis to be expressed. An epistatic system whereby a number of genes act, perhaps as an operon of a high number of single dose alleles at a single locus, has been proposed for apospory in *Pennisetum* (Ozias-Akins et al., 1998), and it is possible that an operon exists at *LOP*. Whether an operon is present at *LOP*, and whether lop379 is part of such an operon, will be investigated further by the Apomixis Programme at Crop & Food Research.

5.8 SCAR marker design and amplification

Sequence analyses of isolated fragments of genomic DNA, during the present study and during previous investigations, indicate that genomes of *Hieracium* species are rich in active and remnant transposable elements, resulting in genomes that are composed largely of moderately to highly replicated DNA. Given a highly repetitive genome, the use of isolated SDAFLP markers as probes for BAC clone filters is expected to result in hybridisation to a large number of non-specific clones that possess broadly similar sequences of repetitive DNA. SCAR marker detection within BAC clone pools is potentially a more discerning technique than hybridisation of probes to BAC colony filters, as the tolerance of base-to-base mismatching during primer annealing is much less than base-to-base mismatching during probe hybridisation.

Primers were designed based on the sequences of the SDAFLP markers and were tested for amplification of single-locus SCAR markers. At least one primer pair based on each of the 15 central sequenced markers was tested. Primer pairs were initially tested as SCAR markers on the wild type C4D107 and the Loa-/Lop- C4Dγ166 which carries large deletions at both *LOP* and *LOA*. A total of six SCAR markers (loa275, loa300, loa267, lop379, lop235 and lop278) showed no amplification or differential amplification in C4Dγ166, providing initial indications that they amplified from the genomic locations of their SDAFLP counterparts. Interestingly, the SCAR of loa300 showed amplification in both C4D107 and C4Dγ166. An additional band, however, of approximately 550bp was

amplified in the wild type but not in the mutant (data not shown). Fig. 5.8 shows a representative gel image of SCAR marker PCR testing.

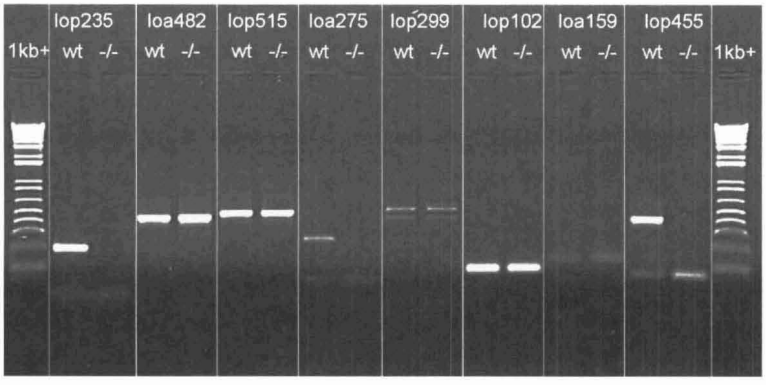


Fig. 5.8 Representative PCR tests of DNA from the wild type C4D107 (wt) and the Loa-/Lop- mutant C4Dγ166 (-/-), using SCAR marker primers. Of the SCAR markers illustrated, lop235, loa275 and lop455 showed discernment between the wild type and the mutant, with their respective presence and absence.

The six discerning SCAR marker primer combinations were then used for amplification of 16 samples including one wild type control, seven Loa- mutants, seven Lop- mutants, and one Loa-/Lop- mutant. The results of the testing of four markers are illustrated in Fig. 5.9. The six SCAR markers amplified in correspondence with the SDAFLP data in most samples. Exceptions included amplification of SCARs loa235, loa267 and loa275 from mutants C4Dγ156, C4Dγ134 and C4Dγ125 respectively, which showed loss of the corresponding SDAFLP markers. Two additional exceptions included amplification of SCAR markers lop379 from C4Dγ143 and loa267 from C4Dγ144 at much reduced efficiencies. Apart from amplification of loa275 from C4Dγ125, all exceptions were markers that were predicted to be located at the margins of deletions. The additional 550bp product amplified with loa300 was tested for its presence in a similar group of mutants by Takashi Okada (CSIRO Plant Industry, Adelaide), and was found to amplify in mutants in which the SDAFLP marker was found to be absent. This marker, while linked to *LOA*, is therefore not likely to correspond directly to the SDAFLP marker from which it was derived.

It is possible that the inconsistent presence and absence patterns of the two marker types are due to extraneous SCAR amplifications at linked duplications that were discernable by SDAFLP but not by SCAR amplification. This explanation, however, requires expansion to be sufficient. With the exception of loa267, the SCAR marker primers contained the same restriction sites and selective bases that served as polymorphisms for the generation of the SDAFLP markers. It is therefore reasonable to believe that the two marker types should amplify at the same genomic locations. However, it is possible that mismatching

between the 5' ends of the SCAR primers and the genomic sequence is tolerated at highly similar sequence duplications enabling the extraneous amplification to occur. The sequences of the 5' ends of the SCAR primers serve as restriction sites for SDAFLP markers which is likely to be a more reliable form of polymorphism detection.

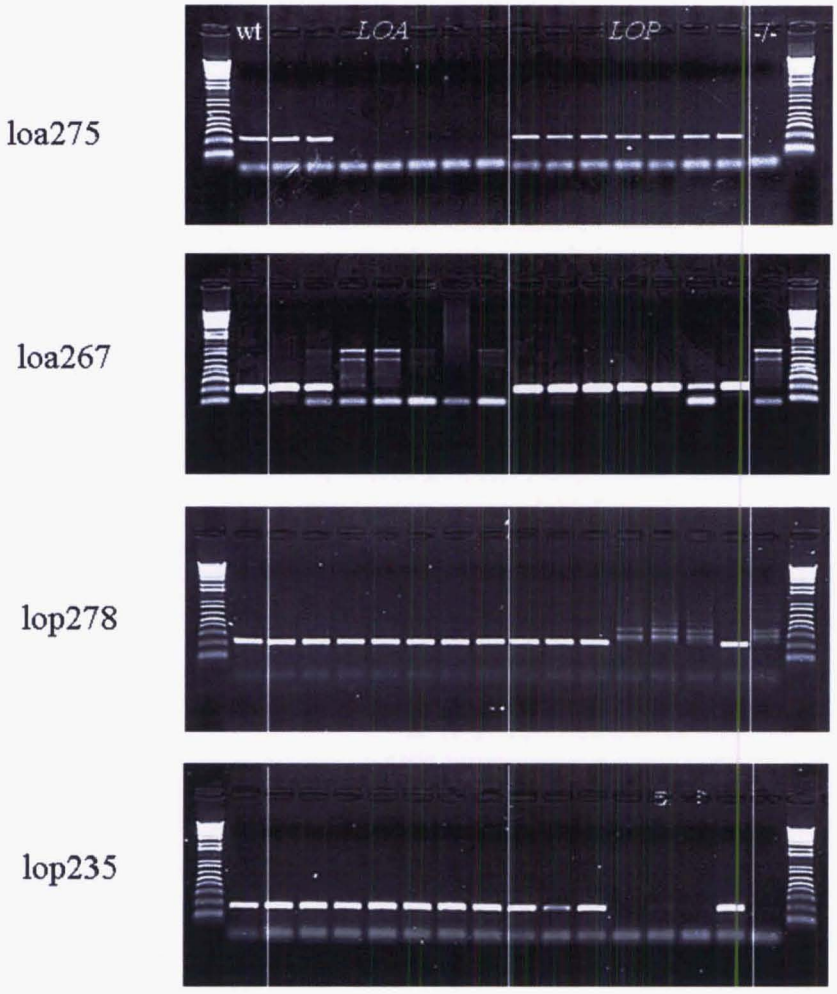


Fig. 5.9 Amplification of two *LOA* and two *LOP* SCAR markers to test for their correspondence with their SDAFLP marker counterparts. Samples amplified were lane 1, C4D108; lanes 2-8, seven Loa- mutants (C4Dγ134, C4Dγ125, C4Dγ165, C4Dγ152, C4Dγ146, C4Dγ135, C4Dγ132); lanes 9-15, seven Lop- mutants (C4Dγ179, C4Dγ138, C4Dγ143, C4Dγ116, C4Dγ171, C4Dγ144, C4Dγ156) and lane 16, Loa-/Lop- mutant, C4Dγ168.

An alternative explanation for the inconsistencies between the marker types is that the proposed linked duplications that are amplified by SCAR primers possess small insertions or deletions (in/dels). These would result in length polymorphisms within the SCAR products that are not resolvable on an agarose gel. If this were the case, SDAFLP profiles would be expected to contain markers of different lengths derived from linked duplications that are deleted together. This sometimes occurred; the SDAFLP marker loa267 amplified as a doublet and was deleted as a doublet, except in the case of C4Dγ134, which retained one of the doublet peaks, and which showed SCAR amplification. An extreme case of an insertion is evident from the additional 550bp product amplified by the loa300 SCAR

primers, which was found to contain an insertion of 255bp (Takashi Okada, pers. comm.). More subtle length polymorphisms between duplications may only be resolved using PAGE or capillary based DNA analysis. Using more discerning methods of analysis for resolution of length polymorphisms may be worthwhile for later utility of the SCAR markers for BAC clone isolation.

5.8.1 Genomic representation of marker sequences

The quality of the 15 markers for use as probes for BAC colony hybridisation was assessed by hybridising the probes to Southern blots of *H. caespitosum* genomic DNA. This was undertaken by Takashi Okada. Table 5.5 compares the results of the Southern analysis of the probes with the use of the derived SCAR markers. Four out of the five successful SCAR markers are based on probes that hybridise as medium to high copy markers. This indicates that the marker is hybridising to duplications that are discernable by the SCAR primers. Surprisingly, those markers that hybridise to a single band have shown a low rate of successful conversion to SCAR markers. It is likely then that these sequences are represented more than once in the genome, possibly as alleles, or alternative duplications, that are not discernable as RFLPs. The exception is the marker lop379, which hybridised as a single copy probe and was successfully converted into a SCAR marker. This suggests that this marker may be hemizygous; however, evidence of a hemizygous state of lop379 requires its use as a probe against DNA of a segregating population, in order to demonstrate segregation in linkage with the trait and with the SDAFLP marker derivative.

In *Pennisetum*, 11 of 15 sequenced RAPD markers were successfully converted into SCAR markers (Ozias-Akins et al., 1998), a much higher success rate than the six out of 15 SDAFLP markers from *LOA* and *LOP* that were successfully converted. It is possible that the higher success rate of conversion of the RAPD markers of *Pennisetum* is due to the proposed hemizygous nature of the ASGR (Ozias-Akins et al., 1998). An additional reason might be that SCAR markers, derived from RAPD markers, might be expected to be more stringent than those derived from AFLP markers, when sequences that include the original polymorphisms are incorporated into the primers. RAPD primers are decamers therefore, assuming the primers anneal with no mismatches, the markers reflect 20 bp of unique nucleotide sequence. SDAFLP markers reflect 14 bp of unique nucleotide sequence composed of two restriction sites (one 6 bp and the other 4 bp) and 4 selective bases.

Table 5.5 Ten of the 15 SDAFLP markers used as probes against *H. caespitosum* DNA to test for approximate copy number. The behaviour of each probe is compared with its behaviour as a SCAR. *Loa300 gives a SCAR of approximately 550bp.

Marker	Southern hybridisation pattern	Single-locus SCAR
loa219	Single band	N
loa275	Smears	Y
loa300	Faint hybridisation	Y*
loa343	Faint smear and 2-4 distinct bands	N
loa267	Intense smear	Y
loa159		N
loa482	Intense smear	N
lop455		N
lop299		N
lop515		N
lop110	Single band	N
lop102		N
lop379	Single band	Y
lop235	Smear	Y
lop278	Smear	Y

5.9 Phenotype segregation and marker association in a segregating population

To test segregation ratios of apomeiosis and parthenogenesis, a population of 101 *H. caespitosum* X *H. pilosella* segregants was selected for phenotype characterisation. Four markers from each locus, *LOA* and *LOP*, were scored for their presence or absence in a random selection of 44 segregants to test for cosegregation of each locus with apomeiosis and parthenogenesis, and recombination at the loci.

5.9.1 Characterising phenotypes of the segregating population

Scores for dark seed production and parthenogenesis of the 101 segregants are given in Appendix 7.3, and for apomeiosis are given in Appendix 7.4. For the scoring of dark seed and parthenogenesis, seed was assessed from both decapitated floral heads and from non-decapitated heads. Only one incidence of putative pollination due to lack of decapitation was found; PC28 yielded two dark seeds from 17 non-decapitated buds of which one

germinated. This is most likely to have been a n+n self or hybrid as the result of inadvertent pollination, as the very low rate of dark seed production (0.12/head) indicates that parthenogenesis is absent. Furthermore, three decapitated buds yielded only one dark seed which did not germinate. Some segregants gave unexpectedly lower means of numbers of dark seed and germination from decapitated heads than from non-decapitated heads. This may be due to damage to reproductive structures during the decapitation. However, noting the exception of PC28, the respective data from decapitated and nondecapitated buds provided the same scores for parthenogenesis for all other PC segregant. The data from decapitated and nondecapitated buds were therefore compiled for the purposes of this analysis.

The ratios of the four classes of segregants based on the detection (or lack of detection) of apomeiosis and parthenogenesis are given in Table 5.6. Production of endosperm, as dark seed, was also assessed. Many Lop+ segregants produced small numbers of seed from cut heads that germinated only to form very small masses of callus, some with a single stunted root. The amounts of tissue available were often too small to test for nuclear DNA contents; however those that were tested were found to be derived from either meiotic or apomeiotic embryo sacs.

Table 5.6 Rates of transmission of apomeiosis and parthenogenesis amongst 101 *H. pilosella* X *H. caespitosum* segregants. Numbers in parenthesis are proportions of segregants that fall into each phenotypic class.

	Apomeiosis detected	**Apomeiosis not detected**	**Total**
Parthenogenesis detected	15 (Loa+/Lop+)	55 (Loa-/Lop+)	70 (0.69)
Parthenogenesis not detected	10 (Loa+/Lop-)	21 (Loa-/Lop-)	31 (0.31)
Total	25 (0.25)	76 (0.75)	101

5.9.2 Segregation distortion of *LOA* and *LOP*

Segregation distortion against *LOA* and apomeiosis was evident in the PC segregants with inheritance by only 25% of the progeny. Mapping via segregation assumes that all mega- and micro-gametophytes have equal chances of survival, and, following fusion, that all zygotes are equally likely to survive. In tetraploids, an allele in simplex is expected to segregate 1:1, similarly to the heterozygous condition in diploids. Distortion from the expected 1:1 ratio implies either that simple Mendelian inheritance does not apply to the trait (due to a duplex condition or to additional genes operating) or that certain gametic or zygotic genetic combinations have reduced chances of survival.

Segregation distortion of apomixis is often noted in the literature, and linked gametic lethality is commonly proposed as a cause. Linked gametic lethality may be caused by the apomixis allele itself or by a linked mutant allele of a housekeeping gene necessary for gamete (either egg or pollen or both) development. Segregation of a gametic lethal factor to haploid gametes precludes their development. When gametes are diploid, segregation distortion may become apparent if gene dosage is important, or when the apomixis allele is in combination with other alleles that lower gamete fitness. A linked gametic lethal factor was proposed to cause transmission of the ASGR in *Pennisetum* to F1 hybrids through the male germ-line at rates less than 1:1 (Ozias-Akins et al., 1998). A similar linked factor is proposed to cause segregation distortion of apomixis in maize-*Tripsacum* hybrids. The distortion in this case was not seen to occur in the F1 progeny. Instead, a very profound segregation distortion of sexuality to apomixis of 16:1 was observed from a subsequent backcross between maize-*Tripsacum* hybrids with maize. The female hybrid parents of this cross possessed only a diploid complement of *Tripsacum* chromosomes; therefore the distortion occurred on attempting to transmit the apomixis factor in the haploid condition (Grimanelli et al., 1998a). The authors have suggested that rare crossovers between the apomixis factor and the lethal factor enable infrequent "escapes" of the apomixis factor in the simplex condition. Of additional interest in this case was that the distortion of the apomixis factor was observed on testing transmission through the female germ-line. Most studies test for segregation of apomixis factors through the male germ-line and are likely to reflect lethality towards male gametes. In *Ranunculus* segregation distortion was not reported, but the apospory factor was found to be lethal to microgametes in the homozygous condition (Nogler, 1984). In *Taraxacum*, *DIPLOSPORY* in simplex undergoes Mendelian transmission through diploid pollen, (van Dijk and Bakx-Schotman, 2004), whereas transmission by haploid pollen shows severe segregation distortion (van Dijk, 2003).

In the present study of *Hieracium* segregants, the low inheritance of apomeiosis indicates gametic lethality of a linked factor. The rate of inheritance may reflect a low frequency of crossover between *LOA* and a linked gametic lethality factor, and thus the genetic distance between the two. However, as in *Pennisetum*, the distortion occurred when the gametes were diploid. Recessive lethality may affect diploid gametes less than haploid gametes, except when gene dosage plays a role. An alternative cause of the distortion may be more

simply the detrimental expression of gene products of apomixis/apomeiosis factors in the context of a reduced gamete, causing some gametes to be lost.

The transmission frequency of apomeiosis of 0.25 is half the 1:1 ratio expected and suggests the involvement of an additional factor. However, the strong association between apomeiosis and *LOA* demonstrates that *LOA* is sufficient for expression of the trait (see section 5.10). It may be speculated instead that an additional factor is required for the survival of gametes carrying *LOA*. Hypothetically, this may be either one of the three alternative *loa* alleles, or an alternative gene in simplex, that acts as a "balancer" to counteract gametic lethality of *LOA* (Fig. 5.10). Such a mechanism would make itself apparent in the form of pseudo-linkage between *LOA* and the hypothetical balancer locus and could be tested with a more directed marker screen of the PC population.

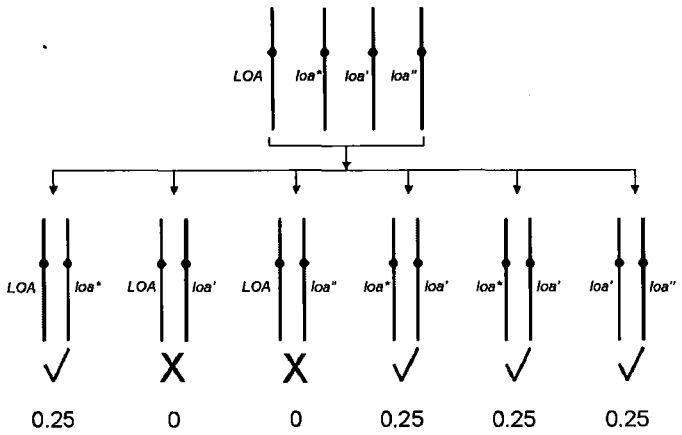


Fig. 5.10 Hypothetical segregation of *LOA* and the wild alleles *loa, *loa'* and *loa''* in male gametes that give rise to the PC segregants. The allele *loa** acts as a balancer for gametic lethality of *LOA*, which is not fulfilled by the other two wild alleles *loa'* and *loa''*. A single allele at an alternative locus that acts as a balancer would induce the same segregation ratio of *LOA*.**

The common observation of segregation distortion against apomixis (or apomeiosis) that is apparent in the literature suggests that the phenomenon is somehow related to apomixis *per se*. However, the distortion is often attributed to linked gametic lethality. A widespread presence of a factor that is linked to a variety of modes of apomixis functioning in disparate species suggests a selective advantage. Selection of a linked mechanism for gametic lethality may ensure the sustained survival of the agamic complex. Grimanelli et al. (1998b) noted that should apomicts and sexuals of an agamic complex be of the same ploidy, then apomixis would predominate, sexuality would disappear, and a "blind alley of evolution" would eventuate with no source of new genetic variation to be drawn from. A linked gametic lethality whose effect is almost certain in haploid gametes may serve to protect diploid sexuals from invasion of apomixis loci, thereby sustaining a source of genetic variability, and might explain the exclusive domain of polyploids for apomixis.

While segregation distortion against apomeiosis was observed in the PC segregants, distortion in favour of parthenogenesis was evident, with inheritance by 68% of the progeny. A similar segregation distortion towards parthenogenesis from a cross between diploid sexual *H. pilosella* and triploid *H. piloselloides* was reported previously (Bicknell et al., 2000). Complicating factors associated with the triploid nature of the male parent might have influenced these data, although transmission of the factor for parthenogenesis through both diploid and haploid pollen was observed. Segregation distortion towards a locus indicates that meiotic drive may be acting whereby small regions of the genome preferentially segregate under influence of a trans-acting driver locus (Mroczek et al., 2006). Alternatively, parthenogenesis may be in linkage to a factor that confers gametic advantage to the pollen for functions such as pollen germination or pollen tube growth. It is also possible that one of the alternative alleles of *LOP* is gametic or embryonic lethal. If no other factors influence segregation of *LOP*, lethality of an alternative allele would theoretically result in transmission of *LOP* to approximately 66% of segregants (Fig. 5.11).

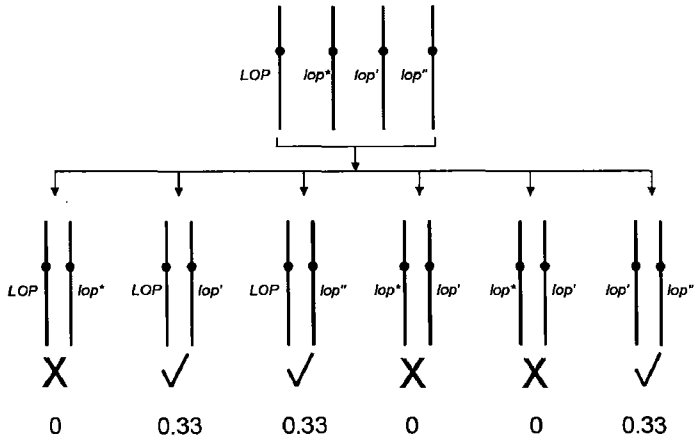


Fig. 5.11 Hypothetical segregation of *LOP* and the wild alleles *lop, *lop'* and *lop''* in male gametes that give rise to the PC segregants. The allele *lop** confers either gametic or embryonic lethality, resulting in segregation of *LOP* to approximately 66% of the progeny.**

Both hypotheses given for the segregation distortion of *LOA* and *LOP* may be tested with the use of allele specific markers such as microsatellites.

5.10 Marker testing of segregants

Nine and eight markers of *LOA* and *LOP* respectively were tested in 44 of the characterised segregants. Segregant-by-marker matrices representing segregation of these markers and the phenotypes is illustrated in Fig. 5.12. The most central markers tested (loa300 and lop102) segregated with the respective traits in all except two segregants, which possess the markers but no corresponding phenotypes were observed. PC3, a Lop- segregant, carries *LOA*, yet on fertilisation, only n+n hybrids were found, indicating the utilisation of solely meiotic embryo sacs. It is possible, however, that *LOA* has very low

penetrance in this segregant and further crossings would yield 2n+n progeny. The second non-corresponding segregant, PC107, has *LOP* but was characterised as typically sexual; few dark seed and no germinable seed formed on uncut heads while abundant germinable seed was produced when floral heads were pollinated with A4 pollen. The reason for the outlying non-correspondence between genotype and phenotype of this segregant is unclear and closer investigation is required to discount a labelling error or greenhouse pot-to-pot contamination. Despite the outliers, the data offer strong validation of the deletion mutagenesis approach towards identifying markers in linkage with apomeiosis and parthenogenesis, and indicate that no other major loci are necessary for their expression.

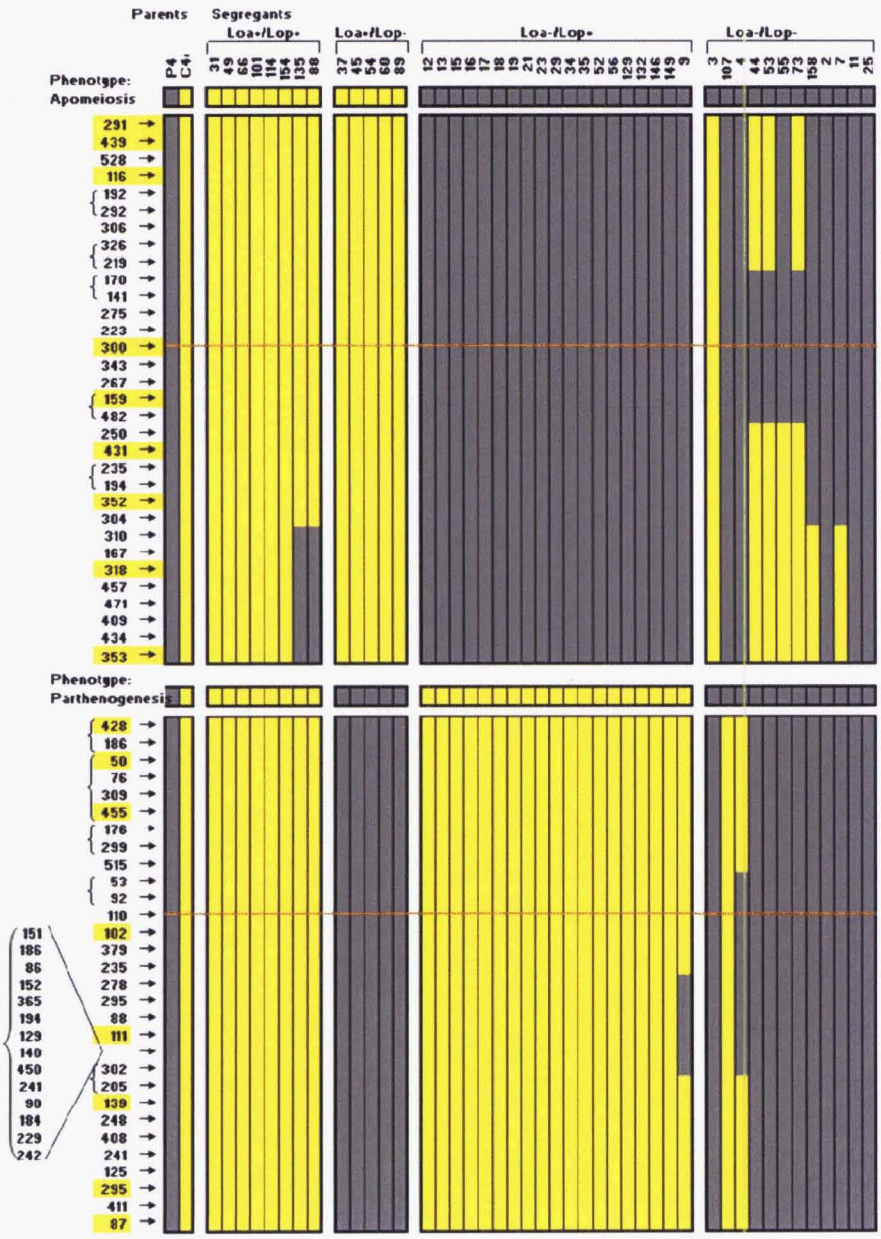


Fig. 5.12 Segregant-by-marker matrices illustrating segregation of nine and eight markers of *LOA* and *LOP* respectively, in 44 segregants characterised into four classes, from the fully apomictic (Loa+/Lop+) to the fully sexual (Loa-/Lop-), with the two intermediate classes, Loa+/Lop- and Loa-/Lop+. Markers highlighted in yellow were tested while the scoring of all other markers was inferred from the data and is indicative only. Orange horizontal lines indicate the most central markers.

5.10.1 Apomeiosis and parthenogenesis and their associated loci, *LOA* and *LOP*, segregate independently

Apomixis until recently was widely proposed to be conferred by a single dominant locus. Monogenic inheritance of apospory was reported in *Ranunculus* (Nogler, 1984), *Pennisetum* (Ozias-Akins et al., 1998) and *Tripsacum* (Grimanelli et al., 1998b). The genetics of parthenogenesis as a distinct component of apomixis has received less direct investigation. Some authors have hypothesised parthenogenesis to be a pleiotropic consequence of apomeiosis due to the shortening of megasporogenesis associated with apomeiosis (Grimanelli et al., 1998b; Grimanelli et al., 2001). Parthenogenesis in *H. aurantiacum* and *H. piloselloides* was reported as a monogenic dominant trait (Bicknell et al., 2000), but no measure of meiosis was taken at that time.

More recent evidence suggests that monogenic inheritance of apomixis is over-simplified and that each component may be conferred independently by dominant genes. Recombination between apomeiosis and parthenogenesis has been reported in *Taraxacum* (van Dijk et al., 1999) and in *Poa* (Albertini et al., 2001; Matzk et al., 2005). In the present study, no evidence of linkage between *LOA* and *LOP* is apparent in the segregation data of the PC population. There is also no evidence of linkage in repulsion between the two loci, therefore it is assumed that *LOA* and *LOP* are independent. Furthermore, there is no compelling evidence of either apomeiosis or parthenogenesis having any contingency on the other.

The segregation between apomeiosis and parthenogenesis gives rise to genotypes that are evolutionary dead ends, either through repeated polyploidisation or haploidisation. It may be speculated then that natural selection will select in favour of occasional combinations (e.g. through genomic translocations) of apomeiosis and parthenogenesis that are in linkage, implying that the evolution of apomixis in C4D is recent. While linkage between apomeiosis and parthenogenesis may be favoured over time, apomixis itself offers close "linkage" between all genes of a genotype, which may serve to neutralise the advantage of linkage thereby causing sluggish selection of the linked state.

5.10.2 Meiotic recombination within *LOA* and *LOP*

Previous studies of model apomicts have indicated that loci that confer apomixis and particularly apomeiosis are often located at large non-recombinant hemizygous regions of chromosomal DNA (Ozias-Akins et al., 1998; Noyes and Rieseberg, 2000). Traits appear to cosegregate with large marker blocks in most segregants. However, by contrast to the

ASGR in *Pennisetum*, recombination at *LOA* and *LOP* does not appear to be suppressed. These data provide evidence of viable meiotic recombination occurring between markers of *LOA* and *LOP* in eight and two of the 44 segregants, respectively. The highest level of recombination at *LOA*, however, were blocks of no fewer than four and perhaps as many as 15 markers. Likewise, neither of the two examples of recombination at *LOP* is likely to be a block of a small numbers of markers. Although only 44 segregants were assessed for recombination, it appears from the current data that a large number of meioses would need to be screened to gain the marker resolution that was gained using deletion mutagenesis.

Severe restriction of recombination caused some degree of sequence divergence at the ASGR locus in *Pennisetum* from the rest of the genome which has resulted in a large partially hemizygous region of chromosomal DNA. Of 12 markers linked to the ASGR, six markers hybridised to genomic DNA as low copy number sequences. In the present study, three out of 15 markers at *LOA* and *LOP* hybridised as low copy number sequences, and all others hybridised as moderately to highly represented sequences (see Table 5.5). There is therefore little evidence that *LOA* and *LOP* are regions that have undergone sequence divergence that might have resulted from suppressed recombination.

Asexual species of older lineages of the genus *Hypericum* possess significantly larger genomes than sexual species, probably due to an increased propensity to accumulate retrotransposons that are not subjected to elimination via unequal homologous recombination at meiosis (Matzk et al., 2003). Logically, the accumulation of retrotransposons in asexual species may be expected to be compounded at loci that confer apomeiosis due to their strong association with suppressed meiotic recombination. The expression of apomeiosis itself suppresses recombination of the locus that confers it in the female germline, and in many species investigated, recombination has become suppressed in the male germline as well. Sequence divergence, however, due to the lack of recombination, may be countered by inward migration of retrotransposons, and the associated genome shuffling and proliferation caused by retrotransposon activity.

5.10.3 Association of autonomous endospermy with parthenogenesis

In addition to the lack of germinable seed, only seven (10%) Lop- segregants produced any dark seed on either cut or uncut heads. Dark seed in *H. piloselloides* and *H. aurantiacum* is associated with successful development of endosperm, which acts as the primary nutritional source of parthenogenetic embryos (Koltunow et al., 1998). The rate of dark

seed production of Lop- segregants was always very low, ranging from 0.1 to 0.4 seeds per head, which corresponds with the significant reduction in dark seed observed in Lop- deletion mutants. Therefore, the association of autonomous endospermy with parthenogenesis in C4D is very tight. A similar association was also observed in G5 (see Chapter 3). Genetic linkage between parthenogenesis and autonomous endospermy may confer a selective advantage, as seed requires the presence of functional endosperm to germinate. Production of endosperm that provides no benefit to a functional embryo is likely to be costly to the plant and therefore be selected against. Alternatively, parthenogenesis and autonomous endospermy may be pleiotropic effects of *LOP*, perhaps resulting from the two processes being interrelated and occurring in positive response to the same mechanism. Autonomous endospermy at very low rates is apparently still possible in some Lop- segregants. This suggests that *LOP* is not required for the capacity of autonomous endospermy, but may instead provide a molecular trigger of a downstream event, which may infrequently occur spontaneously.

Fig. 5.13 shows a semi-log scatter plot of mean dark seed versus mean germination per seed head of Lop+ segregants. Dark seed is an indicator of successful endosperm development in *H. piloselloides* and *H. aurantiacum*. However, an embryo is not always present alongside successful endosperm development (Koltunow et al., 1998). Although not tested directly, non-germinable dark seeds of the Lop+ PC segregants are also likely to lack embryos. While only a proportion of dark seed are germinable, the logarithmic relationship between dark seed and germination implies that the greater the numbers of dark seed per head, the greater the chance of each seed being occupied by an embryo. If *LOP* carries a determinant that is a trigger simultaneously for parthenogenesis and for autonomous endospermy, the threshold of expression of the determinant may be lower for expression of autonomous endospermy than for expression of parthenogenesis. Under this hypothesis once the level of expression of the determinant at *LOP* is high (reflected by higher quantities of dark seed), embryogenesis is promoted. Conversely, those segregants whose ratios of germination to dark seed show deviation from the logarithmic relationship may possess modifiers that act on either trait individually.

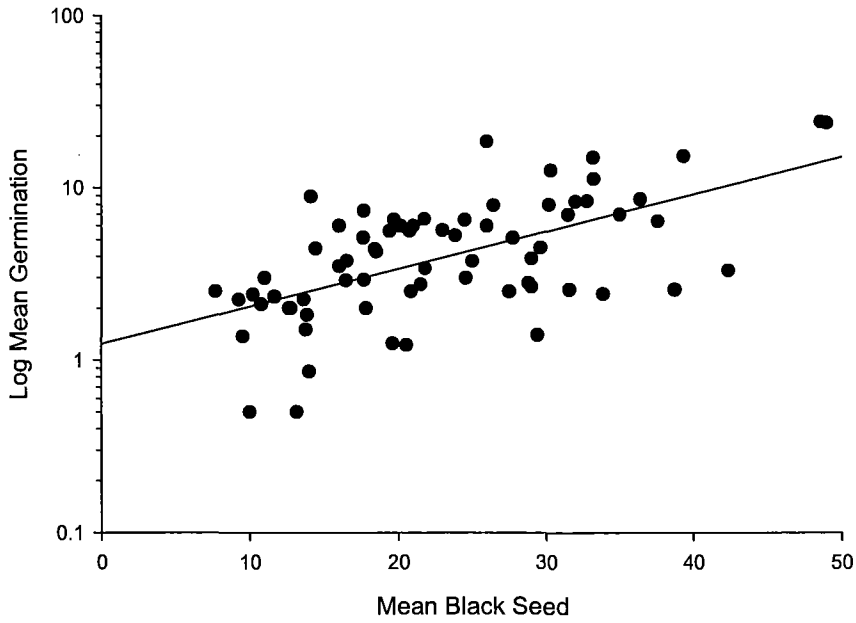


Fig. 5.13 Semi-log scatter plot showing the logarithmic relationship between mean dark seed production mean and germination from each of the PC segregants.

5.10.4 Is apomeiosis a modifier of parthenogenesis? Parthenogenesis is a gametophytic gene

The mean dark seed and germination per head of Loa+/Lop+ are compared with those of Loa-/Lop+ segregants in Table 5.7. While *LOA* and *LOP* can act independently and their expressed traits have no contingency on each other, *LOA* appears to have an effect on the per-head rates of dark seed set and germination. This effect enabled *LOA* to be discovered as part of the deletion mutant screen as Loa- mutants showed a significant reduction in seed set. Of Lop+ segregants, dark seed set and germination per head is on average higher in Loa+ segregants than in Loa- segregants. In addition, the average ratio of germination per dark seed is higher in Loa+ segregants than in Loa- segregants; any dark seed that is produced by a Loa+ segregant has a 35% chance of germinating while a dark seed of a Loa- segregant has a 19% chance of germinating (see Table 5.7). Fig 5.14 illustrates parthenogenetic PC segregants placed in frequency ranges of germinable seed produced per head. None of 55 Loa-/Lop+ segregants produced on average more than 9 germinable seed per head whereas approximately half of the 15 Loa+/Lop+ segregants produced on average more than 9 germinable seed per head. Two of the 15 Loa+/Lop+ segregants produced 24 or more germinable seed per head, although it should be noted that for PC221 only one head was assessed. It is also notable that all six *H. pilosella* X *H. glaciale* (PG) segregants that gave good apomictic seed set were apomeiotic. However, the establishment

of a similar modification of parthenogenesis by the presence of apomeiosis in that population was not possible due to the low population size and due to the high inheritance of apomeiosis among parthenogenetic and non-parthenogenetic segregants alike (see Chapter 3).

While the present data show an apparent modification of parthenogenesis by *LOA*, the increase in seed set associated with apomeiosis in the PC segregants is better explained by *LOP* being a gametophytic gene. The requisite inheritance of *LOP* for parthenogenetic development was inferred from a population of n+0 polyhaploids that were shown to consistently possess *LOP* (Ross Bicknell and Sylvia Erasmuson, Crop & Food Research, pers. comm.). Given that *LOP* carries a gametophytic gene, the locus is inherited more frequently by gametes of apomeiotic PC segregants than by those of meiotic segregants, and seed set would correspondingly occur at a higher frequency, and the data suggests that this is the case.

Table 5.7 Average rates of dark seed set, germination, and ratio of germination per dark seed produced by Loa+ and Loa- parthenogenetic (Lop+) PC segregants.

	Loa+ (n=15)		Loa- (n=55)		
	Mean	**SD**	**Mean**	**SD**	***Ho*: Loa+ = Loa-**
Dark seed/head	35.6	10.38	20.8	8.02	$t(68) = 4.33$, $P < 0.001$
Germination	11.1	7.13	3.8	2.14	$t(14.7) = 3.91$, $P = 0.001$
Germination/dark seed	0.35	0.19	0.19	0.09	$t((14) = 5.93$, $P < 0.001$

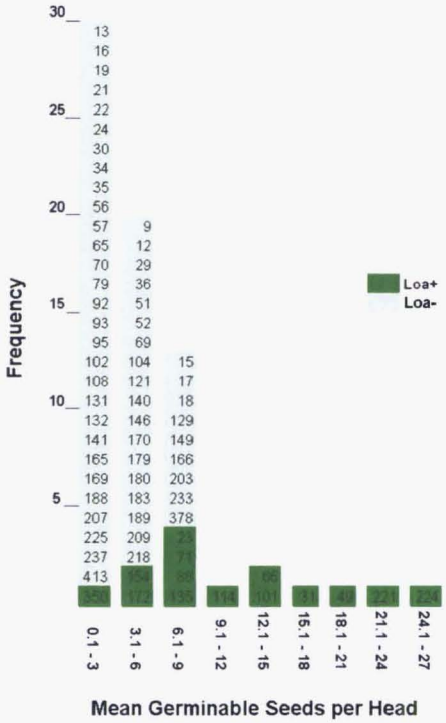


Fig. 5.14 Parthenogenetic PC segregants placed in frequency ranges according to their mean germinable seed per head. Those segregants highlighted in dark green are Loa+/Lop+ while those in light green are Loa-/Lop+.

It is interesting to note that apomixis showed some features of control by a gametophytic gene in maize-*Tripsacum* hybrids (Grimanelli et al., 1998a). Sexually derived backcross derivatives generated by pollinating a maize-*Tripsacum* hybrid with maize (n+n) were exclusively sexual, while apomictic development from the same hybrid (n+0) resulted in some apomictic polyhaploid plants, and some sterile plants. Therefore, only those progeny that were parthenogenetically produced were found to be capable of parthenogenesis. This observation is consistent with a gametophytic status of genes for parthenogenesis in *Tripsacum*, which may be more widespread among apomictic genera.

5.11 Concluding remarks

It is likely that the successful transfer of apomixis to economically important crops may only be undertaken once the genetic determinants of the trait and their co-ordinated actions are understood. Investigations into native apomicts have often aimed to isolate proximal markers as a step towards the isolation of the genetic determinants themselves, but suppressed recombination at apomixis loci has presented considerable challenges for the ordering and utility of cosegregating markers. By using a marker screen of the *H. caespitosum* deletion mutant panel, these challenges have been overcome. This research has demonstrated that all determinants that are required for apomixis are present at two key loci: *LOA* that enables apomeiosis, and *LOP* that enables parthenogenesis coupled with autonomous endosperm development. The generated markers at *LOA* and *LOP*, together with the BAC library of the *H. caespitosum* genome, now provide a real opportunity to identify genetic determinants at *LOA* and *LOP*, and elucidate the molecular mechanisms of apomixis. With that knowledge we may progress towards our goals of fixed heterosis, and large yield gains for global agriculture.

Chapter 6 Future prospects

6.1 Variations of apomixis in *Hieracium*: an evolutionary perspective

The presence of apomixis in a wide variety of forms in disparate and related species indicates that it has evolved several times independently utilising pre-existing mechanisms of sexual reproduction. Independent evolution of apomixis is likely with the utility of apospory and diplospory in the related subgenera *Pilosella* and *Hieracium* respectively. However, it remains possible that a recent secondary adaptation of an ancestral form of apomixis may have generated the variations of apomixis that are visible in the two subgenera today. Similarly, the utilisation of apospory and diplospory within the genus *Poa* (Grun, 1955) may reflect two independent evolutionary events or alternatively, more recent occurrences of secondary evolution. In *Hypericum*, apomixis appears in two sections, and is suggested to have evolved in one secondarily from a sexual intermediate species (Matzk et al., 2003). By contrast, conservation of the ASGR is evidence of radiation of the locus within *Pennisetum* and its relatives.

There are features of apomixis in *Hieracium* subgenus *Pilosella* that indicate that the trait evolved once followed by radiation during speciation. Apomixis in the subgenus *Pilosella* is exclusively apospory, and determinants of parthenogenesis in the apomicts *H. aurantiacum* and *H. piloselloides* are likely to be allelic, or at least at linked locations (Bicknell et al., 2000). The forms of apospory between species, however, are quite variable (Koltunow et al., 2000), and the present study suggests variable genetic mechanisms for both apomeiosis and parthenogenesis in G5 and C4D. It is possible therefore that apospory evolved in *Hieracium* more than once, each time utilising a predisposition to apospory based on genetic and mechanistic features of sexual forms.

How apomixis evolved in *Hieracium*, and why apospory evolved as the mode of apomixis, are questions that may require a multifaceted approach to answer. A preliminary investigation into sequence similarity between the determining loci of G5 and C4D may be made by assessing whether the species share any cosegregating markers. Tests may also be made for allelism by generating G5 X C4D hybrids that carry markers of determining loci from both parents, and testing those markers for their segregation among test-cross progeny. Allelism indicates radiation of apomixis after its evolution, but it does not exclude the possibility that apomixis evolved more than once from similar pre-existing

genetic conditions of sexual plants. Full characterisation of key determinants at *LOA* and *LOP*, and their mechanism of signal transduction, will elucidate genetic differences and similarities of apomixis between G5 and C4D. Comparisons of the genetic mechanisms of apomixis may be extended to other species as well. The key genetic features that distinguish tetraploid apomictic and sexual genotypes of the species in which both occur such as *H. pilosella* may also enable insight into the likely mechanism of evolution of apomixis in *Hieracium*.

6.2 Utility of markers for the isolation of BAC clones

The successful sequencing of *LOA* and *LOP* and the characterisation of the genetic determinants that they contain requires the construction of BAC contigs that span the two loci. The efficiency of this task will in part depend upon the accuracy of the marker data and the density of the markers at the proximal regions of the two loci. It will also depend upon the genomic representation of the DNA sequences of the most proximal markers. The representation of the markers will in turn influence their utility for the accurate identification, with few false positives, of the BAC clones that they represent. High densities of markers at *LOA* and *LOP* should reduce the numbers of BAC clones between markers and that are required to span the genomic regions that contain the determinants. In addition, higher marker density raises the chances that two or more markers are located on the same BAC clones, providing immediate verification of the marker orders generated by deletion mapping.

The estimation of the average physical distance between markers is 165 kb. Takashi Okada has recently constructed BAC contigs using the SCAR markers loa300, loa275 and loa267 of 300 kb, 150 kb and 240 kb respectively. None of the contigs contain more than one of these three markers, which was not an unexpected result as they are not neighbouring markers. However, the contig constructed using loa267 was found to contain the neighbouring SCAR markers loa159 and loa343. This suggests that the physical distance between these markers may be between 100 kb and 200 kb, close to the estimation of the average distance of 165 kb between markers. The level of saturation of markers at *LOA* and *LOP* achieved using 256 primer combinations appears to be sufficient to expedite the assembly of BAC contigs, with few extensive gaps between markers to be filled.

6.2.1 Further strategies towards clone isolation

The genome of *H. caespitosum* is moderately large, and being polyploid, there is a high quantity of duplicated nuclear DNA. Furthermore, preliminary data indicates that *Hieracium* genomes have accumulated a large amount of repetitive DNA composed largely of retrotransposons. It is possible that the utilisation of apomixis as a primary form of reproduction, with little recombination due to meiosis, has provided ideal conditions for the accumulation of repetitive DNA. Furthermore, if *LOA* and *LOP* are regions of reduced recombination, the local accumulation of retrotransposons may be compounded. A high density of markers allows some attrition of markers composed of highly repetitive sequences that are difficult to use as hybridisation probes, but recent experience shows that moderately repetitive sequences can also hybridise to a large number of extraneous clones. More discerning methods of BAC clone isolation will almost certainly need to be used. Unexpectedly, SCAR markers that were based on the sequenced SDAFLP markers, and that included the *Pst*I and *Mse*I restriction sites in their primers, did not always offer the same resolution as their corresponding SDAFLP markers. This implies that the annealing of the SCAR primers, probably at their 5' ends which anneal to the restriction sites, is not as stringent as the recognition of the sites by the restriction enzymes themselves.

More discerning analyses of SCAR marker amplification may detect polymorphisms that enable the distinction between BACs that carry the genuine SDAFLP marker and those that carry duplicated sequences that cause falsely positive amplification. Examples include single strand conformation polymorphisms (SSCP), cleaved amplified polymorphic sequences (CAPS) and short sequence repeats (SSRs). All of these techniques require preliminary analysis of each marker to establish any discerning polymorphisms, which may be difficult to find within markers that are composed of highly repetitive sequence.

A recent attempt to improve the discernment of BAC clone screening, undertaken by Ross Bicknell, Sylvia Erasmuson and Saira Wilson (Crop & Food Research, Lincoln), was the use of SDAFLP to screen BAC clone pools for the presence of the markers detected by the screen of the mutant panel. This technique shows some promise. However, the efficiency of SDAFLP amplification of a marker of interest depends not only on its own representation in the sample, but also on the representation of other competing sequences. The sequence most highly represented in BAC pools is bacterial sequence; each pool contains 384 different BAC clones therefore the representation of bacterial DNA is potentially higher than that of insert DNA.

Modifications that raise the specificity of SDAFLP screening of BAC pools are therefore proposed. It is clear that the higher specificity of *Mse*I+5N/6N primers resulted in SDAFLP profiles with reduced complexity and raised the relative intensities of the desired bands (see Chapter 5, section 5.6.3). The similar use of *Mse*I+5N/6N primers for SDAFLP BAC pool screening may offer similar improvements to those profiles. This principle of increasing the specificity of the selective primers for SDAFLP selective amplification may be taken to the degree that the primers are specific to the sequence of the marker of interest. By using internal primers as selective SDAFLP primers, the specificity of the preamplification process is combined with the specificity of internal SCAR primers. Verification of BAC pools and clones detected in this way may be gained by repeating with a different set of internal marker primers. It is hoped that this form of SDAFLP screening will result in the highly specific detection of BAC clone pools.

6.3 Does *H. caespitosum* carry additional genes associated with apomixis?

While it appears that *LOA* and *LOP* together fulfil the genetic requirements for apomixis to occur in *Hieracium*, two PC segregants carry all markers tested at either *LOA* or *LOP* with no expression of the corresponding traits. Further investigation is required to establish the reasons for their non-expression, but low levels of expression may be detected with further testing. It remains possible, however, that these plants do not carry critical modifiers necessary for effective expression of their traits. It should also be noted that whether *LOA* and *LOP* are single gene loci or complex loci will only be revealed once they are sequenced and extensively characterised.

Wild type apomictic *Hieracium* species are able to produce functional meiotic embryo sacs in addition to those that are apomeiotic. Meiotic embryo sacs may undergo parthenogenetic development to form n+0 progeny, or undergo fertilisation to form n+n hybrids. This facultative mode of apomeiosis was largely conserved during transmission of *LOA* into segregating progeny. The occurrence of reduced meiotic embryo sacs in the presence of *LOA* was very common; exclusive utilisation of apomeiotic embryo sacs was observed in only two of the 17 Loa+/Lop+ segregants and one of the 11 Loa+/Lop- segregants. While complete penetrance of apomeiosis in these segregants may only be ascertained by testing the nuclear DNA contents of large numbers of their progeny, this result does suggest a variable penetrance of apomeiosis.

The screen of the C4D deletion mutants did not yield any additional modifying loci. It is likely, however, that modifiers are involved in determining the expressivity of both apomeiosis and parthenogenesis. The mutant screen of G5 led to one principle locus and two modifying loci that together, strongly promote parthenogenesis. It is possible that the detection of three loci in that genotype reflects an alternative genetic mechanism for apomixis. Alternatively, the mutant screen of C4D might have been more stringent resulting in a focus towards principle loci. Extensive quantitative scoring of apomeiosis and parthenogenesis and molecular mapping using an extended segregating population may offer a means to locate any quantitative trait loci (QTL) associated with apomeiosis and parthenogenesis. The current BAC library will enable BAC clone isolation and sequencing of the QTL. An alternative approach may lie in the analysis of transduction of the signal yielded by the key determinants carried by *LOA* and *LOP*. Of additional interest is the observation of dominant alleles for genes that prevent apomeiosis and parthenogenesis carried by sexual genotypes of *Poa pratensis* (Matzk et al., 2005). The potential presence of genes that prevent apomixis in the sexual P4 may also be investigated in this population, or, alternatively, with enhancer/suppressor screens of apomictic hybrids.

6.4 Downstream analysis: signal transduction

The sequencing of BAC contigs spanning *LOA* and *LOP* will provide a large amount of data that will need to be screened for candidate genes. This may initially take a form of *in silico* screening and annotation of gene sequences within the sequence data, using a programme such as GENSCAN to screen for open reading frames and splice sites, and then comparing the putative gene sequences with unigene sets using Blast for assignment of putative gene function. Candidate genes may include regulatory genes that code for molecules such as transcription factors or protein kinases.

There are a number of avenues available for the analysis of signal transduction. Recent efforts have been made towards the transformation of deletion mutants with binary vectors that carry inserts as large as 100 kb. If this technique is successful it may then be possible to transfer BAC inserts in an attempt to restore function in deletion mutants. Alternatively, candidate genes may be tested for function using RNA interference (RNAi) to see if a "knockdown" of apomeiosis or parthenogenesis is obtained. Successful gain and/or loss of function tests will be followed up by full characterisation of expression patterns using northern and *in situ* hybridisation, and promoter/reporter gene fusions.

The protein kinase that is coded by lop379 is a candidate gene for a role in parthenogenesis. However, Lop- deletion mutants in which lop379 remains intact indicate that alternative genes are likely to be primary determinants of parthenogenesis. This protein kinase, however, may play a role in signal transduction that is downstream from any key determinant. For this reason, analysis of this protein kinase will not proceed until *LOP* is more fully characterised. Analysis of signal transduction will best proceed with knowledge of all candidates, beginning with the analysis of those most likely to be candidate primary determinants.

6.5 The potential role of epigenetic regulation

Prior to the sequencing of *LOA* and *LOP* and the testing of candidate genes, the types of genes that control apomixis may only be subjects of speculation. However, apomixis in *Hieracium* appears to be a process that harnesses the mechanisms of sexual reproduction, but with altered gene regulation to enable the avoidance of meiosis and fertilisation (Tucker et al., 2001; Tucker et al., 2003). It may be expected then that genes controlling apomixis in some way regulate gene expression in the aposporous initial cell and the parthenogenetic egg cell, to take identities and the competencies normally reserved for the megaspore and the zygote respectively. It has been proposed that this altered gene regulation occurs by means of epigenetic control, to explain how apomeiosis, parthenogenesis and autonomous endospermy evolved simultaneously when any component in isolation is disadvantageous (Koltunow and Grossniklaus, 2003). Epigenetic control of apomixis may occur in *Hieracium*. However, the multi-locus nature of apomixis in the present study suggests that any epigenetic control of apomixis is not mediated by a single locus in *Hieracium*. Apomeiosis and parthenogenesis can occur independently. Therefore global epigenetic control of apomixis in *Hieracium* is unlikely. Epigenetic control, if it is involved, is likely to instead be mediated downstream of the loci that confer each trait.

In mammals, fertilisation is necessary for embryogenesis in part because of critical gender-specific epigenetic modification that occurs via the imprinting of each genome during gametogenesis. It was found that successful parthenogenesis of a reconstructed mouse oocyte with two haploid maternal genomes could occur providing there was an increased expression of the gene for the growth factor IGFII (*Igf2*) and mono-allelic expression of the non-coding RNA *H19*. These modifications together led to appropriate

expression of a wide range of genes so that development could proceed to term (Kono et al., 2004). *H19* and *Igf2* are normally imprinted reciprocally in the egg and the sperm to achieve differential maternal expression of *H19* and paternal expression of *Igf2* (Ferguson-Smith and Surani, 2001).

It is now well established that gender-specific imprinting is reflected in the 2m:1p "balance" of maternal to paternal genome contributions to the endosperm, that is obtained on fertilisation of the 2n central cell of the embryo sac (Grant-Downton and Dickinson, 2005). In most angiosperm species, the 2m:1p ratio is critical to endosperm development; greater maternal or paternal genome contributions lead to abnormal development of the endosperm, and often abortion of the seed (Lin, 1984; Spielman et al., 2001). It appears, however, that similar gender-specific epigenetic information is not essential for plant embryo development. While paternal expression of many genes in early plant embryos is lower than maternal expression (Vielle-Calzada et al., 2000; Weijers et al., 2001), any complete dependence of plant embryogenesis on gender-specific imprinting is yet to be seen. Furthermore, it is argued that the multiple ways that an asexual embryo can form suggest that gender-specific epigenetic information from either parent is non-essential for embryo development (Gehring et al., 2004). Successful rescue of embryos from their deficient endosperms following interspecific or intergeneric crosses adds support to this view.

Embryonic differential imprinting may not hold the key to initiating parthenogenesis in plants in the way that it apparently does in the mouse. However, it is conceivable that components of apomixis may operate via epigenetic mechanisms, and non-coding RNA molecules have been implicated. A notable mammalian example of epigenetic regulation is X chromosome inactivation. The random inactivation of one X chromosome in every cell requires the X inactive specific transcript gene (*XIST*) that codes for a 15 to 17 kb RNA molecule that coats and triggers cytosine methylation and heterochromatin assembly (Morey and Avner, 2004). RNA-directed DNA methylation is well characterised in plants for silencing of viruses and transposons (Mathieu and Bender, 2004) and the roles of short interfering RNA in aspects of plant development, including floral development, are well documented (Achard et al., 2004; Grant-Downton and Dickinson, 2005). Bioinformatic screens for candidate determinants therefore cannot be limited to those that are dependent on the presence of features of structural genes such as open reading frames and intron

splice sites. Additional bioinformatic screens for any non-coding RNAs (Weinberg and Ruzzo, 2006) present at *LOA* and *LOP* will also be conducted.

6.6 The mechanism of autonomous endosperm development

Autonomous endosperm development is prevalent in apomictic Asteraceae, and is consistently the case in the well described family members *Hieracium* (Koltunow et al., 1998), *Taraxacum* (Richards, 1970) and *Erigeron* (Noyes, 2000). This contrasts with the apomictic grasses, which are predominantly pseudogamous requiring fertilisation of the central cell to initiate endosperm development. Some pseudogamous apomicts have employed mechanisms to maintain the 2m:1p parental genome ratio in the endosperm including the use of only one unreduced polar nucleus as the central cell, or utilising both pollen for fertilisation, resulting in a 4m:2p ratio. Others have relaxed the requirements of the genome ratio, but still need at least some paternal contribution (Spielman et al., 2003).

Successful endosperm development is considered to be a critical hurdle in the engineering of apomixis – one that may be the last to be surmounted. Almost all autonomous apomicts are found in the Asteraceae. How apomictic genera of the Asteraceae are able to undergo autonomous endosperm development is therefore an important question. If there is no requirement of paternal genes for endosperm development, the maternal imprints of paternal genes may be absent. It has therefore been proposed that genomic imprinting may have become attenutated in the family (Vinkenoog and Scott, 2001). This hypothesis holds up well given that interploidy crosses (which generally result in abortive seed in species that require the 2m:1p genome balance) readily occur in *Hieracium* (Bicknell et al., 2000), *Taraxacum* (Tas and van Dijk, 1999; van Dijk and Bakx-Schotman, 2004) and in *Erigeron* (Noyes, 2000). The lack of pseudogamous mutants in the mutant panels is consistent with this hypothesis, and indicates that endosperm development does not depend on additional genetic information to be autonomous. Autonomous endospermy, given the presence of *LOP*, appears to instead be the default mechanism. The relationships between autonomous endospermy, the relaxation of parental genome contribution requirements, and attenuation of imprinting or its effects, all deserve focus in the future.

As discussed in Chapter 5, the phenotypic data of the PC segregants suggest that parthenogenesis and autonomous endospermy are either closely linked traits, or that they are pleiotropic effects of *LOP*, perhaps with modifiers that affect each trait. The production

of dark seed (which is correlated with the presence of endosperm) is generally restricted to Lop+ segregants. Autonomous endospermy therefore does not occur needlessly in a Lop- background. However, in a Lop+ background, only a fraction of the dark seeds germinate. The production of non-germinable dark seed suggests that endosperm may develop in the absence of an embryo, indicating some independence of the regulation of autonomous endospermy, from that of parthenogenesis. This question will almost certainly be answered while exploring signal transduction of determinants at *LOP*. Preliminary investigations however, may be made into the prevalence of embryos within dark seed. It is possible that a compromised embryo may serve as a signal for autonomous endospermy, but may fail to develop sufficiently for the seed to be germinable. If this is the case then an embryo-endosperm interaction may operate.

6.7 The engineering of apomixis in sexual species

Attempts at the engineering of apomixis in sexual species have until now focussed on introgression of the trait by wide-crossing, or on deregulation of sexual processes via mutation. Both of these approaches are yet to prove successful. It now remains to be seen if a detailed knowledge of the genetic and mechanistic processes, followed by genetic engineering of the process into economically important species will fare better.

What form would an engineered apomict take? This may well depend on native predispositions of the target species, implying that a full understanding of the pre-existing sexual processes may be an additional pre-requisite. The first engineered apomict may therefore be derived from a sexual species that is well described rather than one that is economically important. Intuitively, it may be surmised that diplospory, rather than apospory, may be more readily engineered. Diplospory demonstrates the utility of pre-existing meiotic components. By contrast, apospory depends on the production of new cell types, and may be more likely to lead to polyembryony. A number of *Arabidopsis* and maize mutants that show hallmarks of non-reduction or nuclear restitution of megaspores indicate that it may be possible to induce diplospory via mutation. These mutations, however, result in high levels of sterility. If additional regulation by dominant genes is required - which appears to be the case for native diplosporous apomicts such as *Taraxacum* (van Dijk and Bakx-Schotman, 2004) - the apparent advantages over apospory may not be so clear. Whether diplospory or apospory is utilised, and in which form, may be more dependent on the amenability of the crop to either system.

The ideal engineered system will be inducible to allow incorporation of the engineered apomict into breeding programmes, and will be highly penetrant once induced. Like apomeiosis, parthenogenesis also needs to be highly penetrant for efficient setting of quality seed. The trigger for parthenogenesis may be a more universal process shared by different native apomicts, although future analyses of native apomicts will establish if this is indeed the case. By contrast, endospermy is likely to take a range of forms, and may be difficult to engineer in some systems. Some crops may be amenable to the engineering of autonomous endospermy but in others a pseudogamous mechanism may be required. It is very likely that in many systems, obstacles concerning gender-specific imprinting may need to be surmounted. In others, the imprinting requirements for endosperm may be able to be relaxed. The ability of *Arabidopsis* to form differentiated endosperm under the combined loss of FIE function and maternal hypomethylation (Vinkenoog et al., 2000) suggests how relaxation of imprinting requirements may be implemented.

The mechanism of apomixis will one day be fully elucidated. It is possible that this will be achieved in one species, or different components will be elucidated from different species. Comparative descriptions of similar and more distinctive mechanisms in other species will quickly follow, which will provide a mechanistic "palette" from which components of the process may be taken for the successful transfer of apomixis into species of agronomic importance.

Chapter 7 Appendices

7.1 Glossary and abbreviations

- **A3.4:** an aneuploid (triploid plus 4 chromosomes) apomictic accession of *Hieracium aurantiacum.*
- **A4:** a tetraploid apomictic accession of *Hieracium aurantiacum.*
- **AFLP:** amplified fragment length polymorphism, a PCR based technique of generation of multiple dominant markers.
- **Apomeiosis:** avoidance of meiosis resulting in an unreduced embryo sac.
- **Apomixis:** asexual reproduction through seed.
- **Apospory:** apomeiotic embryo sac development from cells of nucellar origin.
- **ASGR:** apospory-specific genomic region.
- **BAC:** bacterial artificial chromosome. A BAC vector contains an insert of exogenous genomic DNA of approximately 100kb.
- **BAC contig:** a series of contiguous overlapping BAC clones.
- **Band:** a fragment of DNA electrophoresed on a gel from which markers are identified.
- **blastn:** an algorithm/programme that searches for similarities between a query nucleotide sequence and a database of nucleotide sequences.
- **blastx:** an algorithm/programme that searches for similarities between a query nucleotide sequence that is translated into all six possible translation frames and a database of peptide sequences.
- **bp:** base pairs.
- **C4D:** a tetraploid apomictic accession of *Hieracium caespitosum.*
- **CAPS:** cleaved amplified polymorphic sequence, a polymorphism detectable by the presence of an internal restriction site.
- **cDNA-AFLP:** AFLP that generates profiles of gene expression utilising cDNA as template.
- **Diplospory:** apomeiotic embryo sac development from a megaspore mother cell.
- **Embryo sac:** the female gametophyte which contains the egg cell and the central cell.

- **Expect value:** a level of significance attributed to a similarity hit of a blast search based on the number of hits with the same level of similarity that are expected to occur by chance.
- **G5:** a pentaploid apomictic accession of *Hieracium glaciale.*
- **Gametophyte:** the phase of a plant that produces gametes.
- **γ:** gamma.
- ***In silico*:** by computer, e.g. bioinformatics.
- **kb(p):** kilobase (pairs)
- ***LOA* or *loa*:** loss of apomeiosis.
- ***LOP* or *lop*:** loss of parthenogenesis.
- **Marker:** a band or peak that may be attributed a genomic location.
- **Mbp:** mega-base pairs
- **Megaspore:** the selected spore that, following meiosis, undergoes mitotic divisions to develop into an embryo sac.
- **Megaspore mother cell:** a cell within the ovule that undergoes differentiation and meiosis to become a haploid megaspore and three minor spores.
- **Microcolinearity or microsynteny:** conservation between species at a level of DNA sequence.
- **Microgamete:** male gamete as a pollen grain and the pollen tube that grows from it.
- **nr:** non redundant, refers to the comprehensive database of DNA sequence at GenBank.
- **Nucellus:** the central tissue of an ovule that surrounds the embryo sac.
- **ORF:** open reading frame.
- **P4:** a tetraploid sexual accession of *Hieracium pilosella.*
- **PAGE:** polyacrylamide gel electrophoresis.
- **Parthenogenesis:** autonomous development of the embryo without fertilisation.
- **PCR:** polymerase chain reaction.
- **Polyhaploid:** a plant derived from parthenogenetic development of a meiotic embryo sac.

- **Pseudogamous:** refers to an apomictic plant that requires fertilisation of the central cell for endosperm development.
- **QTL:** quantitative trait loci, loci detected by their quantitative effects on a trait.
- **RAPD:** randomly amplified polymorphic DNA, PCR generation of multiple dominant markers from 10 base primers that anneal throughout the genome.
- **RFLP:** restriction fragment length polymorphism, detected by hybridising a probe to digested and electrophoresed genomic DNA.
- **RNAi:** RNA interference, a technique of post transcriptional gene silencing mediated by short double stranded RNA molecules with complementary sequence.
- **SDAFLP:** secondary digest AFLP, a methylation insensitive form of AFLP.
- **SCAR:** sequence characterised amplified region, a dominant marker that is detectable as a PCR product.
- **SNP:** single nucleotide polymorphism, based on a change of a single base.
- **Sporophyte:** the phase of the plant that produces spores.
- **SSCP:** single stranded conformation polymorphism, a technique of resolving discrete polymorphisms that alter conformations of single stranded DNA.
- **SSR:** short sequence repeat, a repeat of between approximately two and five bases at which slippage occurs at the DNA replication fork generating a polymorphism.
- **TAIL-PCR:** thermally asymmetric interlaced PCR, enables the amplification of unknown sequence that borders known sequence.
- **tblastx:** an algorithm/programme that searches for similarities between query and database nucleotide sequences, each translated into all six possible translation frames.
- **Tm:** melting temperature.

7.2 Band and sequence confidence scores of markers at *LOA* and *LOP*

In general, a direct sequence was obtained from C4Dγ102 (irradiated wild type) and two cloned sequences were obtained from C4D107 (non-irradiated wild type). Confidence scores were given for the bands and sequences of markers. Band confidence scores (1-5) were based on: the intensity of the bands on the gel, the ease with which they were excised, the consensus between sequences from independent isolations (from C4Dγ102 and C4D107) and from independent colonies, and from consensus between the sequence length and the marker length predicted by the genetic analyser. Sequence confidence scores were based on sequence consensus between samples and clones, and sequence quality.

Marker	Isolated from mini-sequence	Match betw. C4D102 and C4D107	Match betw. C4D107 clones	Band confidence	Sequence confidence	Notes
loa219	Y	With one C4D107 clone	Match (except SNP)	5	5	Sequence of clone with consensus with direct sequence
loa275	Y	With one C4D107 clone	No match	4	5	functioned as a SCAR
loa300	Y	Match	Match	5	5	
loa343	Y	Match	Match but with SNPs	5	3	Sequence contains SNPs
loa267	Y	Match	Match	5	5	Functioned as a SCAR
loa159	Y	No match	No match	3	5	Band homology with band of previous isolation attempt
loa482	N	Match	Match	5	4	Region of dubious sequence due to short sequence runs
lop455	N	Match	Match	4	4	
lop299	N	Match	No match	3	5	Verification based only on size
lop515	Y	Match	Match	5	4	Lower case denotes areas of dubious sequence
lop110	Y	Match	Match, except SNP	5	5	
lop102	N	With one C4D107 clone	No match	4	5	Sequence of clone with consensus with direct sequence
lop379	Y	Some matches	Match	5	5	Functioned as a SCAR
lop235	Y	Match	Match	5	5	
lop278	Y	n/a	n/a	4	4	Sequence only obtained from direct sequence (however functioned as a SCAR).

7.3 Germination rates of seed of PC segregants

For phenotypes (Loa+, Lop+), 2 is positive and 0 is negative. Scores were taken for decapitated (yellow) and non-decapitated (blue) heads and were compiled (green).

Plant			Decapitated floral heads					Non-decapitated floral heads					Total deacapitated and non-decapitated					
PC#	Lop+	Loa+	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Germ. / dark seeds
23	2	2	0	0	0	0.0	0.0	2	49	13	24.5	6.5	2	49	13	24.50	6.50	0.27
31	2	2	6	236	92	39.3	15.3	0	0	0	0.0	0.0	6	236	92	39.33	15.33	0.39
49	2	2	1	27	20	27.0	20.0	3	77	54	44.5	32.0	4	104	74	26.00	18.50	0.71
66	2	2	3	91	38	30.3	12.7	0	0	0	0.0	0.0	3	91	38	30.33	12.67	0.42
71	2	2	0	0	0	0.0	0.0	8	113	71	30.1	19.4	8	113	71	14.13	8.88	0.63
88	2	2	0	0	0	0.0	0.0	5	182	43	74.2	17.2	5	182	43	36.40	8.60	0.24
101	2	2	0	0	0	0.0	0.0	5	166	75	33.2	15.0	5	166	75	33.20	15.00	0.45
114	2	2	0	0	0	0.0	0.0	8	266	90	33.3	11.3	8	266	90	33.25	11.25	0.34
135	2	2	1	23	13	23.0	13.0	2	30	9	15.0	4.5	3	53	22	17.67	7.33	0.42
154	2	2	1	23	4	23.0	4.0	7	143	41	20.4	5.9	8	166	45	20.75	5.63	0.27
172	2	2	4	98	12	24.5	3.0	5	163	23	32.6	4.6	9	261	35	29.00	3.89	0.13
218	2	2	0	0	0	0.0	0.0	6	254	20	42.3	3.3	6	254	20	42.33	3.33	0.08
221	2	2	1	49	24	49.0	24.0	0	0	0	0.0	0.0	1	49	24	49.00	24.00	0.49
224	2	2	3	148	70	49.3	23.3	2	95	51	47.5	25.5	5	243	121	48.60	24.20	0.50
350	2	2	5	147	7	29.4	1.4	0	0	0	0.0	0.0	5	147	7	29.40	1.40	0.05
9	2	0	1	7	6	7.0	6.0	6	94	25	31.0	8.0	7	101	31	14.43	4.43	0.31
12	2	0	3	64	16	21.3	5.3	3	32	5	16.0	2.5	6	96	21	16.00	3.50	0.22
13	2	0	0	0	0	0.0	0.0	4	51	8	25.5	4.0	4	51	8	12.75	2.00	0.16
15	2	0	5	123	37	24.6	7.4	6	168	50	58.4	14.0	11	291	87	26.45	7.91	0.30
16	2	0	1	4	0	4.0	0.0	5	79	11	15.8	2.2	6	83	11	13.83	1.83	0.13
17	2	0	0	0	0	0.0	0.0	5	164	42	62.7	16.0	5	164	42	32.80	8.40	0.26
18	2	0	0	0	0	0.0	0.0	3	96	25	61.0	15.5	3	96	25	32.00	8.33	0.26
19	2	0	1	10	3	10.0	3.0	3	34	9	11.3	3.0	4	44	12	11.00	3.00	0.27
21	2	0	0	0	0	0.0	0.0	7	98	6	14.0	0.9	7	98	6	14.00	0.86	0.06
22	2	0	4	72	9	18.0	2.3	1	17	1	17.0	1.0	5	89	10	17.80	2.00	0.11
24	2	0	4	92	11	23.0	2.8	2	33	4	16.5	2.0	6	125	15	20.83	2.50	0.12
29	2	0	1	31	6	31.0	6.0	1	21	6	21.0	6.0	2	52	12	26.00	6.00	0.23
30	2	0	3	24	4	8.0	1.3	6	73	15	24.3	5.0	9	97	19	10.78	2.11	0.20
34	2	0	1	33	3	33.0	3.0	1	22	2	22.0	2.0	2	55	5	27.50	2.50	0.09
35	2	0	0	0	0	0.0	0.0	7	271	18	74.4	5.3	7	271	18	38.71	2.57	0.07
36	2	0	3	48	18	16.0	6.0	0	0	0	0.0	0.0	3	48	18	16.00	6.00	0.38
51	2	0	0	0	0	0.0	0.0	4	100	15	25.0	3.8	4	100	15	25.00	3.75	0.15
52	2	0	6	138	34	23.0	5.7	0	0	0	0.0	0.0	6	138	34	23.00	5.67	0.25
56	2	0	4	55	6	13.8	1.5	0	0	0	0.0	0.0	4	55	6	13.75	1.50	0.11
57	2	0	4	49	8	12.3	2.0	2	21	6	10.5	3.0	6	70	14	11.67	2.33	0.20

Plant			Decapitated floral heads					Non-decapitated floral heads					Total deacapitated and non-decapitated					
PC#	Lop+	Loa+	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Germ. / dark seeds
65	2	0	3	29	7	9.7	2.3	1	8	2	8.0	2.0	4	37	9	9.25	2.25	0.24
69	2	0	5	88	26	17.6	5.2	3	53	15	17.7	5.0	8	141	41	17.63	5.13	0.29
70	2	0	0	0	0	0.0	0.0	9	148	26	16.4	2.9	9	148	26	16.44	2.89	0.18
79	2	0	0	0	0	0.0	0.0	8	109	18	13.6	2.3	8	109	18	13.63	2.25	0.17
92	2	0	0	0	0	0.0	0.0	12	212	35	17.7	2.9	12	212	35	17.67	2.92	0.17
93	2	0	3	65	6	21.7	2.0	1	21	5	21.0	5.0	4	86	11	21.50	2.75	0.13
95	2	0	3	33	12	11.0	4.0	2	18	0	9.0	0.0	5	51	12	10.20	2.40	0.24
102	2	0	0	0	0	0.0	0.0	7	221	18	31.6	2.6	7	221	18	31.57	2.57	0.08
104	2	0	6	121	36	20.2	6.0	0	0	0	0.0	0.0	6	121	36	20.17	6.00	0.30
108	2	0	0	0	0	0.0	0.0	4	40	2	20.7	0.7	4	40	2	10.00	0.50	0.05
121	2	0	6	162	30	27.0	5.0	2	60	11	30.0	5.5	8	222	41	27.75	5.13	0.18
129	2	0	0	0	0	0.0	0.0	5	188	32	69.5	10.3	5	188	32	37.60	6.40	0.17
131	2	0	5	118	0	23.6	0.0	3	39	10	13.0	3.3	8	157	10	19.63	1.25	0.06
132	2	0	0	0	0	0.0	0.0	8	76	11	9.5	1.4	8	76	11	9.50	1.38	0.14
140	2	0	3	63	18	21.0	6.0	0	0	0	0.0	0.0	3	63	18	21.00	6.00	0.29
141	2	0	6	202	15	33.7	2.5	1	35	2	35.0	2.0	7	237	17	33.86	2.43	0.07
146	2	0	3	57	2	19.0	0.7	5	180	34	63.8	10.0	8	237	36	29.63	4.50	0.15
149	2	0	1	35	7	35.0	7.0	0	0	0	0.0	0.0	1	35	7	35.00	7.00	0.20
165	2	0	0	0	0	0.0	0.0	5	144	14	28.8	2.8	5	144	14	28.80	2.80	0.10
166	2	0	10	215	67	21.5	6.7	2	46	12	23.0	6.0	12	261	79	21.75	6.58	0.30
169	2	0	7	140	9	20.0	1.3	2	45	2	22.5	1.0	9	185	11	20.56	1.22	0.06
170	2	0	3	55	15	18.3	5.0	1	19	2	19.0	2.0	4	74	17	18.50	4.25	0.23
179	2	0	0	0	0	0.0	0.0	5	97	28	19.4	5.6	5	97	28	19.40	5.60	0.29
180	2	0	4	66	15	16.5	3.8	0	0	0	0.0	0.0	4	66	15	16.50	3.75	0.23
183	2	0	0	0	0	0.0	0.0	7	167	37	23.9	5.3	7	167	37	23.86	5.29	0.22
188	2	0	0	0	0	0.0	0.0	6	79	3	13.2	0.5	6	79	3	13.17	0.50	0.04
189	2	0	4	78	21	19.5	5.3	1	14	1	14.0	1.0	5	92	22	18.40	4.40	0.24
203	2	0	0	0	0	0.0	0.0	2	63	14	31.5	7.0	2	63	14	31.50	7.00	0.22
207	2	0	6	46	15	7.7	2.5	0	0	0	0.0	0.0	6	46	15	7.67	2.50	0.33
209	2	0	4	86	15	21.5	3.8	1	23	2	23.0	2.0	5	109	17	21.80	3.40	0.16
225	2	0	0	0	0	0.0	0.0	3	87	8	29.0	2.7	3	87	8	29.00	2.67	0.09
233	2	0	0	0	0	0.0	0.0	5	151	40	30.2	8.0	5	151	40	30.20	8.00	0.26
237	2	0	5	120	12	24.0	2.4	2	52	9	26.0	4.5	7	172	21	24.57	3.00	0.12
378	2	0	0	0	0	0.0	0.0	10	197	65	19.7	6.5	10	197	65	19.70	6.50	0.33
413	2	0	3	16	2	5.3	0.7	5	85	14	34.7	6.2	8	101	16	12.63	2.00	0.16

Plant			Decapitated floral heads					Non-decapitated floral heads					Total deacapitated and non-decapitated					
PC#	Lop+	Loa+	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Heads	Dark seed	Germ.	Mean dark seed	Mean germ.	Germ. / dark seeds
37	0	2	2	0	0	0.0	0.0	4	1	0	0.3	0.0	6	1	0	0.17	0.00	
45	0	2	0	0	0	0.0	0.0	10	0	0	0.0	0.0	10	0	0	0.00	0.00	
54	0	2	0	0	0	0.0	0.0	4	1	0	0.3	0.0	4	1	0	0.25	0.00	
60	0	2	0	0	0	0.0	0.0	11	0	0	0.0	0.0	11	0	0	0.00	0.00	
89	0	2	1	0	0	0.0	0.0	7	0	0	0.0	0.0	8	0	0	0.00	0.00	
94	0	2	6	0	0	0.0	0.0	2	0	0	0.0	0.0	8	0	0	0.00	0.00	
96	0	2	4	0	0	0.0	0.0	2	0	0	0.0	0.0	6	0	0	0.00	0.00	
151	0	2	3	0	0	0.0	0.0	0	0	0	0.0	0.0	3	0	0	0.00	0.00	
168	0	2	5	0	0	0.0	0.0	3	0	0	0.0	0.0	8	0	0	0.00	0.00	
175	0	2	12	0	0	0.0	0.0	0	0	0	0.0	0.0	12	0	0	0.00	0.00	
2	0	0	5	0	0	0.0	0.0	3	0	0	0.0	0.0	8	0	0	0.00	0.00	
3	0	0	0	0	0	0.0	0.0	5	0	0	0.0	0.0	5	0	0	0.00	0.00	
4	0	0	0	0	0	0.0	0.0	6	0	0	0.0	0.0	6	0	0	0.00	0.00	
11	0	0	2	0	0	0.0	0.0	3	0	0	0.0	0.0	5	0	0	0.00	0.00	
25	0	0	1	0	0	0.0	0.0	5	0	0	0.0	0.0	6	0	0	0.00	0.00	
28	0	0	3	1	0	0.3	0.0	14	1	1	0.1	0.1	17	2	1	0.12	0.06	
40	0	0	0	0	0	0.0	0.0	8	0	0	0.0	0.0	8	0	0	0.00	0.00	
44	0	0	0	0	0	0.0	0.0	5	0	0	0.0	0.0	5	0	0	0.00	0.00	
53	0	0	6	0	0	0.0	0.0	4	5	0	1.3	0.0	10	5	0	0.50	0.00	
55	0	0	3	0	0	0.0	0.0	0	0	0	0.0	0.0	3	0	0	0.00	0.00	
73	0	0	1	0	0	0.0	0.0	4	0	0	0.0	0.0	5	0	0	0.00	0.00	
80	0	0	4	0	0	0.0	0.0	2	1	0	0.5	0.0	6	1	0	0.17	0.00	
91	0	0	1	0	0	0.0	0.0	5	0	0	0.0	0.0	6	0	0	0.00	0.00	
97	0	0	0	0	0	0.0	0.0	18	2	0	0.2	0.0	18	2	0	0.11	0.00	
107	0	0	4	0	0	0.0	0.0	3	1	0	0.3	0.0	7	1	0	0.14	0.00	
117	0	0	2	0	0	0.0	0.0	1	0	0	0.0	0.0	3	0	0	0.00	0.00	
128	0	0	2	0	0	0.0	0.0	2	0	0	0.0	0.0	4	0	0	0.00	0.00	
148	0	0	8	0	0	0.0	0.0	3	0	0	0.0	0.0	11	0	0	0.00	0.00	
158	0	0	0	0	0	0.0	0.0	4	0	0	0.0	0.0	4	0	0	0.00	0.00	
161	0	0	0	0	0	0.0	0.0	5	2	0	1.0	0.0	5	2	0	0.40	0.00	
193	0	0	0	0	0	0.0	0.0	5	2	0	0.4	0.0	5	2	0	0.40	0.00	

7.4 Apomeiosis scores for PC segregants

Nuclear DNA contents of seedlings derived from decapitated heads of parthenogenetic segregants and from crossed heads of non-parthenogenetic segregants were measured by flow cytometry. Relative DNA contents indicate whether seedlings are derived from meiotic or apomeiotic embryo sacs. The formation of parthenogenetic seedlings that are 2n, or hybrid seedlings that are 3n, indicate that the parental plant is capable of apomeiosis.

Plant			Heads assessed		Flow cytometry	
PC#	Lop+	Loa+	Cut	Crossed	Seedlings	Relative nuclear DNA contents
23	2	2	2	0	6	n, 2n
31	2	2	6	0	12	n, 2n
49	2	2	4	0	18	2n
66	2	2	3	0	6	n, 2n
71	2	2	8	0	10	n, 2n
88	2	2	5	0	12	n, 2n
101	2	2	5	0	6	2n
114	2	2	8	0	6	2n
135	2	2	3	0	4	n, 2n
154	2	2	8	0	19	n, 2n
172	2	2	9	0	20	n, 2n
218	2	2	6	0	7	n, 2n
221	2	2	1	0	6	n, 2n
224	2	2	5	0	18	2n
350	2	2	5	0	6	n, 2n
9	2	0	7	0	24	n
12	2	0	6	0	11	n
13	2	0	4	0	4	n
15	2	0	11	0	30	n
16	2	0	6	0	6	n
17	2	0	5	0	12	n
18	2	0	3	0	8	n
19	2	0	4	0	7	n
21	2	0	7	0	4	n
22	2	0	5	0	7	n, 2n*
24	2	0	6	0	10	n
29	2	0	2	0	10	n
30	2	0	9	0	12	n
34	2	0	2	0	5	n
35	2	0	7	0	10	n
36	2	0	3	0	6	n
51	2	0	4	0	10	n
52	2	0	6	0	16	n
56	2	0	4	0	5	n
57	2	0	6	0	9	n
65	2	0	4	0	8	n
69	2	0	8	0	18	n
70	2	0	9	0	4	n
79	2	0	8	0	6	n
92	2	0	12	0	12	n
93	2	0	4	0	10	n
95	2	0	5	0	3	n
102	2	0	7	0	3	n
104	2	0	6	0	6	n
108	2	0	4	0	2	n

*possibly one 2n seedling found

Plant			Heads assessed		Flow cytometry	
PC#	Lop+	Loa+	Cut	Crossed	Seedlings	Relative nuclear DNA contents
121	2	0	8	0	16	n
129	2	0	5	0	9	n
131	2	0	8	0	13	n
132	2	0	8	0	4	n
140	2	0	3	0	6	n
141	2	0	7	0	12	n
146	2	0	8	0	10	n
149	2	0	1	0	6	n
165	2	0	5	0	7	n
166	2	0	12	0	18	n
169	2	0	9	0	9	n
170	2	0	4	0	8	n
179	2	0	5	0	7	n
180	2	0	4	0	12	n
183	2	0	7	0	4	n
188	2	0	6	0	2	n
189	2	0	5	0	7	n
203	2	0	2	0	7	n
207	2	0	6	0	7	n
209	2	0	5	0	8	n
225	2	0	3	0	4	n
233	2	0	5	0	6	n
237	2	0	7	0	10	n
378	2	0	10	0	7	n
413	2	0	8	0	7	n
37	0	2	0	3	6	2n,3n
45	0	2	0	5	12	2n,3n
54	0	2	0	1	6	2n,3n
60	0	2	0	3	6	2n,3n
89	0	2	0	5	12	2n,3n
94	0	2	0	1	6	2n,3n
96	0	2	0	5	12	2n,3n
151	0	2	0	1	6	3n
168	0	2	0	2	6	2n,3n
175	0	2	0	4	6	2n,3n
2	0	0	0	4	18	2n
3	0	0	0	5	6	2n
4	0	0	0	1	6	2n
11	0	0	0	4	12	2n
25	0	0	0	4	12	2n
28	0	0	0	2	6	2n
40	0	0	0	6	19	2n
44	0	0	0	2	6	2n
53	0	0	0	2	6	2n
55	0	0	0	3	6	2n
73	0	0	0	2	6	2n
80	0	0	0	6	13	2n
91	0	0	0	7	12	2n
97	0	0	0	5	12	2n
107	0	0	0	5	12	2n
117	0	0	0	1	6	2n
128	0	0	0	9	13	2n
148	0	0	0	2	6	2n
158	0	0	0	6	7	2n
161	0	0	0	3	6	2n
193	0	0	0	3	6	2n

7.5 References

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7.6 Publications

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Catanach, A. S., Bicknell, R. A., Erasmuson, S. K., Fletcher, P. J. (2005). Identification of key loci associated with apomixis in *Hieracium*. Poster presentation, Queenstown Molecular Biology Meeting 2005.

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Catanach, A. S., Bicknell, R.A. Erasmuson, S. K., Fletcher, P. J. (2006). Use of deletion mutagenesis to isolate apomixis genes in *Hieracium*. Poster and oral presentations, 8th International Congress of Plant Molecular Biology.

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CROP & FOOD RESEARCH

Mana Kai Rangahau

Use of deletion mutagenesis to isolate apomixis genes in *Hieracium*

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The ultimate goal – clonal propagation through seed

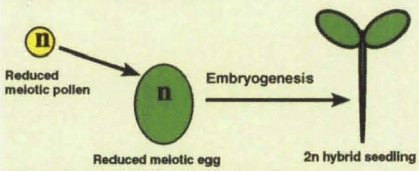
If wheat and rice (and other inbreeding crop species) produced clonal seed faithfully through successive generations, it would be economic to produce hybrid seed of these major crop species, which could be grown with the substantial yield advantages of hybrid vigour.

The biology – apomixis in *Hieracium*

Some plants naturally produce clonal seed with apomixis. A classical example of an apomict is the dandelion. We are working with a number of apomictic species of the fellow composite *Hieracium*, to isolate genetic determinants of apomixis and elucidate the mechanism.

Comparison of Sex and Apomixis

Figure 1 Sex = meiosis + fertilisation

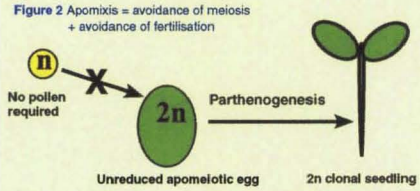


Sexual reproduction has two essential components:

1. **Meiosis:** To produce reduced 1n gametes (egg and pollen), with half of the genome of the parents.
2. **Fertilisation:** Union of a 1n egg with a 1n pollen which triggers embryogenesis.

The seedling is a 2n hybrid.

Figure 2 Apomixis = avoidance of meiosis + avoidance of fertilisation



Apomictic *Hieracium* avoid each component of sexual reproduction with two over-riding processes:

1. **Apomeiosis:** To produce an unreduced 2n egg.
2. **Parthenogenesis:** Spontaneous embryogenesis of a 2n egg with no fertilisation.

The seedling is 2n and clonal.

Mapping with a deletion mutant panel – two key loci found

In a deletion mutant panel, the more a marker is absent with the absence of a trait, the closer the marker is likely to be to the gene that confers the trait. We phenotyped 79 loss-of-apomixis mutants into three classes: loss-of-apomeiosis; loss-of-parthenogenesis; and loss of both traits. In an initial screen using eight AFLP primer combinations we identified two loci in which markers are commonly lost with the corresponding loss of either component of apomixis (**Figure 3**). We have termed these loci *LOSS OF APOMEIOSIS (LOA)* and *LOSS OF PARTHENOGENESIS (LOP)*. We then focussed on increasing marker density around the central regions at each locus.

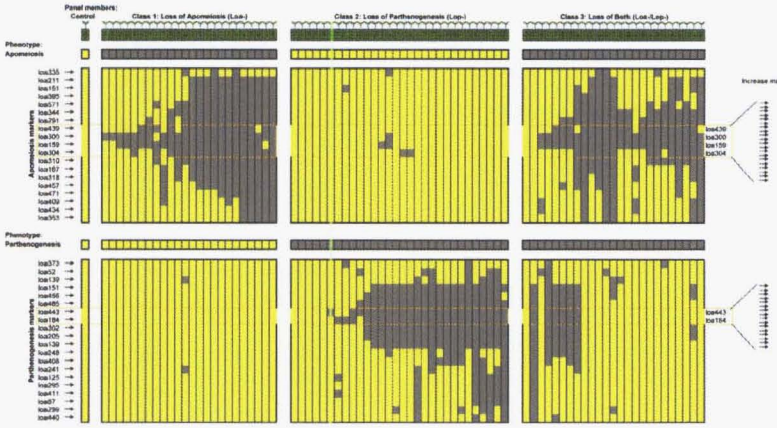


Figure 3 Graphical representation of marker loss in three classes of *H. caespitosum* mutants. Columns represent plants and rows represent marker loci. The gold boundaries indicate regions of most common marker loss which were focussed on for increased marker density (see **Figure 4**).

Increasing marker density, sequencing and SCARs

Increasing marker density at *LOA* and *LOP* should correspondingly increase marker proximity to key determinants. Another 248 more AFLP primer combinations were used to screen subsets of the panel composed of mutants with centrally located deletion breakpoints. This enabled many new markers to be placed at the centres of *LOA* and *LOP* (**Figure 4**). We have sequenced 15 of the most central markers from both loci, and one sequence shows similarity to known genes. It is likely, however, that other genes are involved, as some Lop- mutants have retained this marker. Five markers were successfully converted into SCARs that show amplification in close correspondence with their source AFLP markers.

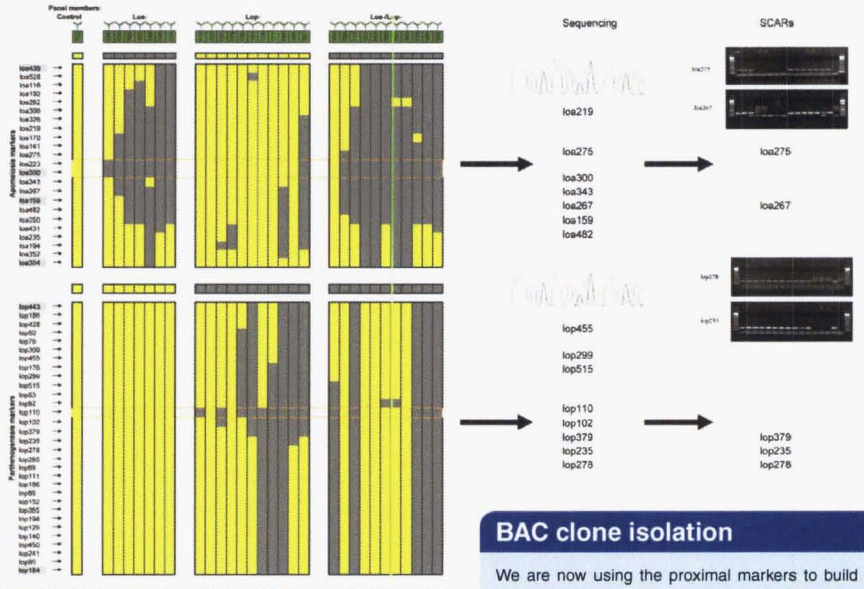


Figure 4 Graphical representation of *LOA* and *LOP* after screening with a total of 256 AFLP primer combinations. Markers that are in grey were identified during the initial screen. The gold boundaries indicate central regions where key determinants are likely to lie. Fifteen markers were sequenced and of those five were successfully converted into SCARs.

BAC clone isolation

We are now using the proximal markers to build BAC contigs which will be screened *in silico* for candidate genes. We intend to test candidate sequences via transformation of the mutants and assessing for complementation.

Research undertaken in partnership with LINCOLN UNIVERSITY

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Identification of key loci associated with apomixis in Hieracium

Andrew Catanach, Ross Bicknell, Sylvia Erasmuson and Pam Fletcher.

The ultimate goal

If wheat and rice (and other inbreeding crop species) produced clonal seed faithfully through successive generations, it would be economic to produce hybrid seed of these major crop species, which could be grown with the substantial yield advantages of hybrid vigour.

Apomixis = clonal propagation through seed

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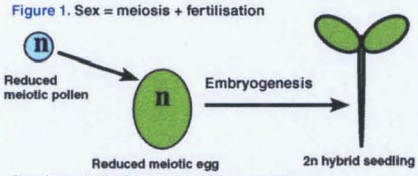
Comparison of Sex and Apomixis

Figure 1. Sex = meiosis + fertilisation

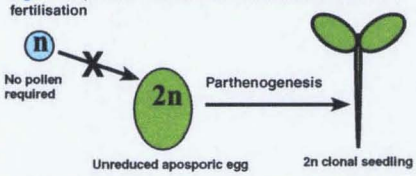


Sexual reproduction has two essential components:

1. **Meiosis:** To produce reduced 1n gametes (egg and pollen), with half of the genome of the parents.
2. **Fertilisation:** Union of a 1n egg with a 1n pollen which triggers embryogenesis.

The seedling is a 2n hybrid.

Figure 2. Apomixis = avoidance of meiosis + avoidance of fertilisation



Apomictic Hieracium avoid each component of sexual reproduction with two over-riding processes:

1. **Apospory:** To produce an unreduced 2n egg.
2. **Parthenogenesis:** Spontaneous embryogenesis of a 2n egg with no fertilisation.

The seedling is 2n and clonal.

Mapping with a deletion mutant panel

In a segregating population, the more a molecular marker is present with the presence of a trait, the closer that marker is likely to be to the gene that confers that trait. Similarly, in a deletion mutant panel, the more a molecular marker is absent with the absence of a trait, the closer that marker is to the gene that confers that trait. We have characterised chromosomal deletions of 30 loss-of-parthenogenesis and 24 loss-of-apospory Hieracium caespitosum mutants, in terms of commonly lost AFLP markers. (Fig. 3). The most useful mutants are those that have deletion breakpoints close to determinants. To date we have screened either the whole panel, or subsets of the panel, with 256 AFLP primer combinations and have identified 62 markers.

Figure 3a Mutants with loss of parthenogenesis

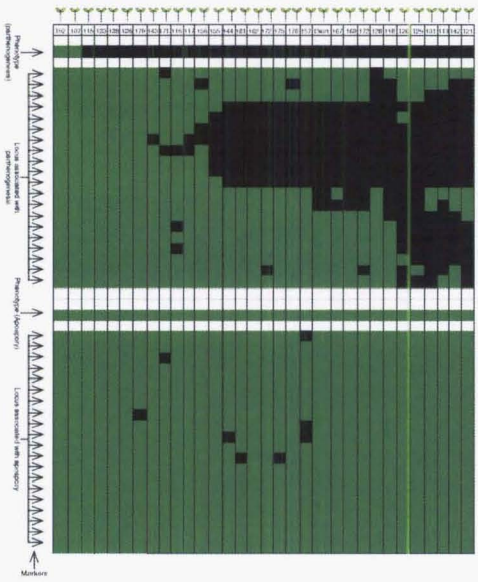
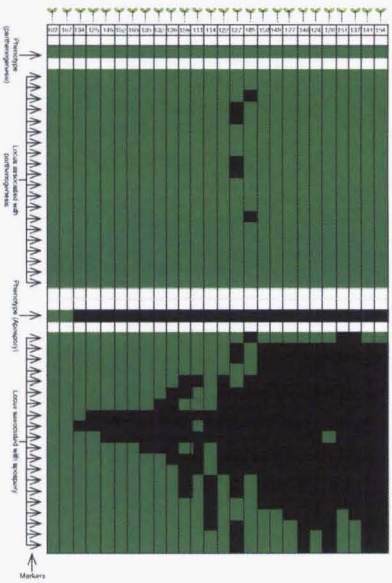


Figure 3b Mutants with loss of apospory



KEY
presence
absence

Figure 3. Graphical representation of marker loss in loss-of-parthenogenesis (a) and loss-of-apospory (b) mutant panels. Plant phenotypes are represented in the header rows. Each column represents a control or mutant plant and each row represents a marker. Plants 102 and 107 are wild-type controls.

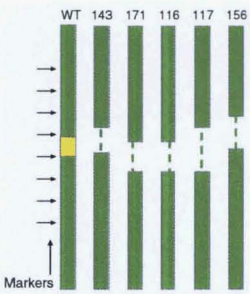


Figure 4a Deletions that result in loss of parthenogenesis

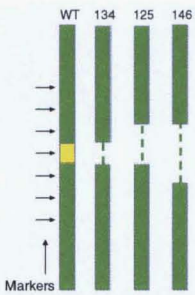


Figure 4b Deletions that result in loss of apospory

Figure 4. Graphical representation of genomic deletions with breakpoints close to determinants for a) parthenogenesis and b) apospory. The areas coloured yellow span regions where putative determinants are expected to lie.

Isolation of AFLP markers and Probing of a BAC library

We have generated a BAC library with 5X coverage of the tetraploid Hieracium caespitosum genome. The next step is the isolation and sequencing of the most proximal AFLP markers for use as probes for the isolation of clones from the BAC library. The clones will then be sequenced and assessed for candidate genes.

Research undertaken in partnership with LINCOLN UNIVERSITY
Te Whare Wānaka o Aoraki

production and design by
illustration department C&FR 20665 August 200

A chromosomal region associated with parthenogenesis in *Hieracium* is deleted in a T-DNA mutant

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INTRODUCTION

While most angiosperms utilise sex for production of seed, some reproduce asexually, utilising apomixis to produce seed that is genetically identical to the parental plant. The process of gametophytic apomixis deviates from sexual reproduction at two key developmental stages. First, maternal meiosis is avoided resulting in an unreduced embryo sac. This may be achieved either through arrest of meiosis of the megaspore mother cell (diplospory), or by replacement of the megaspore with an unreduced embryo sac derived from nucellar tissue (apospory). The second deviation is avoidance of fertilisation, with the embryo sac undergoing parthenogenetic development of the embryo directly.

HIERACIUM GLACIALE AS A WILD-TYPE APOMICT

Hieracium glaciale is a non-obligate apomict that utilises aposporv. Most seed of the wild-type plant is derived from parthenogenetic development of unreduced aposporic embryo sacs. Some seed, however, may be derived from fertilisation of either unreduced aposporous, or reduced meiotic embryo sacs, giving rise to 3n or 2n hybrid plants (Fig 1).

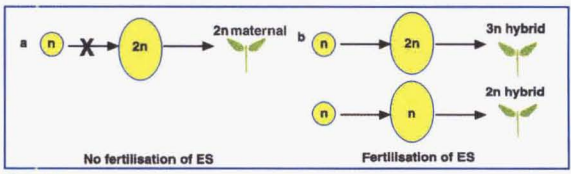

Figure 1.
Most seed of wild-type *H. glaciale* is derived from parthenogenetic development of 2n aposporous embryo sacs (ES), resulting in 2n maternal plants (a). Some seed is derived from fertilisation of either aposporous or meiotic embryo sacs, resulting in 3n or 2n hybrids (b).

A *LOSS OF APOMIXIS* MUTANT, MUTANT 110

We have isolated a mutant of *H. glaciale* following *Agrobacterium* - mediated transformation, which we named mutant 110. The mutant shows no ability of parthenogenetic reproduction. Instead, the mutant demonstrates increased potential for sexual reproduction via fertilisation of unreduced aposporous or reduced meiotic embryo sacs (Fig 2).

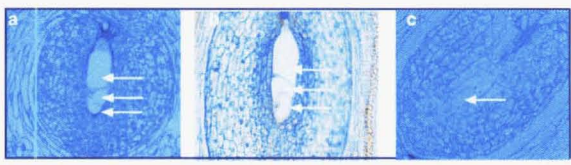

Figure 2.
a) Three developing aposporous initial cells (arrowed) within an ovule of wild-type *H. glaciale*. Aposporous embryo sacs normally undergo parthenogenetic development into 2n maternal seedlings.
b) Aposporous initials (arrowed) within an ovule of mutant 110, demonstrating similar development to those of the wild-type. No parthenogenetic development of aposporous embryo sacs of mutant 110 is observed but they may be fertilised to form 3n hybrid seedlings.
c) Meiotic tetrad within an ovule of mutant 110. The megaspore is arrowed. The descendent embryo sac, when fertilised, gives rise to a 2n hybrid seedling.

T-DNA INSERTS OF MUTANT 110

Genetic analyses of segregants of crosses with mutant 110 as the pistillate parent indicate the mutant has three independently segregating T-DNA inserts. We developed PCR assays from sequenced T-DNA vectors and TAIL-PCR products of neighbouring genomic DNA, which enabled each insert to be detected independently (Fig 3).

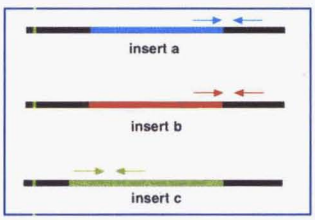

Figure 3.
Three T-DNA inserts detected in mutant 110 (inserts a-c). Inserts a and b have truncated left borders and are detectable by PCR from the right borders to neighbouring genomic DNA. Insert c is detectable by using a left border specific PCR assay.

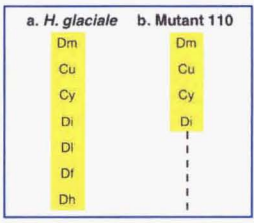

Figure 4.
The genomic region associated with parthenogenesis in wild-type *H. glaciale* defined by markers Dm through to Dh (a). This region is extensively deleted in mutant 110 (b).

DELETION OF A GENOMIC REGION ASSOCIATED WITH PARTHENOGENESIS

Mutant 110 was included in an AFLP screen of a panel of 73 *H. glaciale* radiation deletion mutants. Surprisingly, the mutant was found to carry a deletion of a region of genomic DNA associated with parthenogenesis (Fig 4). This region, defined by three AFLP markers, is deleted in 17 (23%) of the 73 radiation deletion mutants and is linked to parthenogenesis in an F1 population segregating for the trait (more information will be given in an oral presentation by Ross Bicknell in session 8).

WAS T-DNA INSERTION THE CAUSE OF DELETION?

Deletion of genomic DNA associated with T-DNA insertion has been reported previously in *Arabidopsis* (Kaya et al., 2000, Plant Cell Physiol. 41(9), 1055-1066). As all three T-DNA inserts can be detected independently in progeny, we assessed the possibility of T-DNA insertion as a cause of deletion. If T-DNA insertion was the cause of the deletion, a T-DNA insert may co-segregate with markers linked to the deletion. We conducted interspecific crosses with mutant 110 as the maternal parent. Hybrid progeny originating from fertilisation of reduced meiotic embryo sacs were scored for each T-DNA insert, and for two AFLP markers, Di and Cy, that are linked to the deleted region (Fig 5).

	Plant	Insert a	Insert b	Insert c	Di	Cy
Parents:	♀Mutant 110					
	♂A3					
F1s:	110XA3#8					
	110XA3#12					
	110XA3#15					
	110XA3#17					
	110XA3#18					
	110XA3#20					
	110XA3#21					
	110XA3#30					
	110XA3#32					
	110XA3#33					
	110XA3#37					
	110XA3#39					
	110XA3#40					
	110XA3#45					
	110XA3#48					
	110XA3#50					
	110XA3#54					
	110XA3#62					
	110XA3#65					
	110XA3#66					
	110XA3#67					
	110XA3#69					

Figure 5.
Results of genetic analysis to determine if any of T-DNA inserts a, b or c, is associated with the deletion of mutant 110. F1 segregants from crosses with mutant 110 as the pistillate parent and *H. aurantiacum* (A3) as the pollen parent were assessed. Coloured cells indicate positive detection of each T-DNA insert (a, b and c) and AFLP markers Di and Cy.

CONCLUSION

While mutant 110 is a T-DNA transformant that carries a genomic deletion associated with parthenogenesis, significant association between any of the three T-DNA inserts and the deletion was not established. It is possible a deletion-causing T-DNA insert might have been translocated, or significantly truncated and remains undetected. Alternatively, the deletion has been caused by unknown factors, perhaps associated with *Agrobacterium* transformation.

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Deletion mapping of genetic regions associated with apomixis in *Hieracium*

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Although apomixis has been quoted as a technology with the potential to deliver benefits similar in scale to those achieved with the Green Revolution, very little is currently known of the genetic mechanisms that control this trait in plants. To address this issue, we developed *Hieracium*, a genus of daisies native to Eurasia and North America, as a genetic model to study apomixis. In a molecular mapping study, we defined the number of genetic loci involved in apomixis, and we explored dominance and linkage relationships between these loci. To avoid difficulties often encountered with inheritance studies of apomicts, we based our mapping effort on the use of deletion mutagenesis, coupled with amplified fragment length polymorphism (AFLP) as a genomic fingerprinting tool. The results indicate that apomixis in *Hieracium caespitosum* is controlled at two principal loci, one of which regulates events associated with the avoidance of meiosis (apomeiosis) and the other, an unlinked locus that controls events associated with the avoidance of fertilization (parthenogenesis). AFLP bands identified as central to both loci were isolated, sequenced, and used to develop sequence-characterized amplified region (SCAR) markers. The validity of the AFLP markers was verified by using a segregating population generated by hybridization. The validity of the SCAR markers was verified by their pattern of presence/absence in specific mutants. The mutants, markers, and genetic data derived from this work are now being used to isolate genes controlling apomixis in this system.

amplified fragment length polymorphism (AFLP) | meiosis | parthenogenesis

Apomixis is the asexual formation of seed. It is a process that results in the formation of genetically uniform populations (1, 2) and also in unique patterns of speciation (3, 4). Approximately 400 flowering plant taxa are recorded as apomictic, including members of 35 diverse plant families (5, 6). Very few crop species, however, are known to be apomictic. Among those that are, most are tropical tree species, such as citrus and mango, or tropical forage grasses, such as *Brachiaria* and *Paspalum*.

It is widely reported that apomixis holds the promise of providing significant benefits to agriculture and to overall global welfare if it could be installed into seed-propagated crops in an inducible format (7–11). For rice alone, an economic analysis conducted on the scenario of free access and relatively modest adoption rates of apomixis predicted an improvement in welfare in excess of 4 billion U.S. dollars per annum (12). Despite this recognized potential, very little is known about the genetic and developmental processes that underlie the expression of apomixis, in part because of the absence of apomixis in classic model species. Some aspects of apomixis are under study in *Arabidopsis*, using mutagenesis to explore possible mechanisms for converting this obligate sexual species into an apomict (13). Most notably, this work has highlighted the critical role played by chromatin-remodeling factors and other epigenetic factors in the specification of early embryo and endosperm development (14–19). Other researchers are developing apomictic species into model systems to study the trait in its native form. Several models are emerging, including *Panicum* (20, 21), *Pennisetum* (22), *Paspalum* (23), *Tripsacum* (24–26), *Brachiaria* (27), *Poa* (28, 29), *Taraxacum* (30, 31), *Hypericum* (32), and *Erigeron* (33).

One of the best characterized systems is *Hieracium*, a genus of daisies native to Eurasia and North America (34). In *Hieracium*, apomixis occurs by apospory, a developmental process characterized by three distinct deviations from sexual reproduction (Fig. 1). In the first instance, a cell type develops within the ovule that initiates embryo sac formation without first proceeding through meiosis. This process is known as apomeiosis, and the cell type is called an aposporous initial. Aposporous initials typically develop near the time of meiosis at sites adjacent to the meiotic apparatus. They then divide and enlarge in apparent competition with meiotic products during early ovule development (35). Ultimately, their development results in the formation of one or more unreduced (2n) embryo sacs (35). Tucker *et al.* (36) monitored gene expression in the unreduced embryo sacs of an apomictic accession of *Hieracium* and in the reduced embryo sacs of a sexual accession. After initiation, the two were seen to be very similar. This finding is in agreement with the generally held belief that apomixis represents a modified form of sexual reproduction (13), as illustrated in Fig. 1. The second major deviation from sexual reproduction occurs at the level of egg-cell fate. Within each unreduced embryo sac, an egg cell develops. In common with meiotically derived egg cells, they may be fertilized by a suitable sperm cell, resulting in the formation of a zygote. Most commonly, however, the unreduced eggs of apomicts divide spontaneously, directly initiating the processes of embryogenesis. The spontaneous formation of an embryo is common to all apomictic systems, and it is also recorded in many animal systems (37). In both plants and animals, this process is known as parthenogenesis. Finally, the endosperm of *Hieracium* develops spontaneously without requiring the fertilization of the polar nuclei, a phenomenon referred to as autonomous endospermy.

Apomixis in *Hieracium* is reported to be genetically controlled (38–40). Intriguingly, Mendel studied inheritance in this genus (41), but apomixis would remain undescribed in these plants until the observations of Ostenfeld (42–44) and Rosenberg (45, 46; see also ref. 47). Several authors have reported that apomixis in *Hieracium* is conferred by the inheritance of dominant genetic elements (38–40), which is also widely reported for other

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Abbreviations: AFLP, amplified fragment length polymorphism; *LOA* or *loa*, loss of apomeiosis; *LOP* or *lop*, loss of parthenogenesis; SCAR, sequence-characterized amplified region; SDAFLP, secondary digest-amplified fragment length polymorphism.

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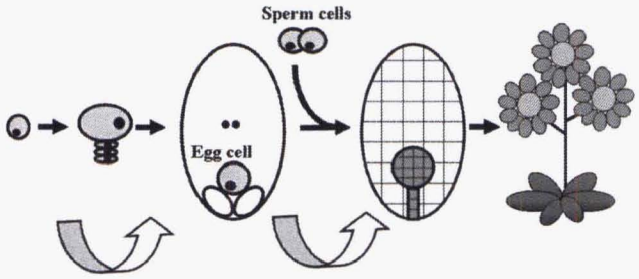

Fig. 1. Apomixis in *Hieracium* follows the developmental mechanism of apospory. Three critical deviations from sexual reproduction are apparent: an avoidance of meiosis (apomeiosis), an avoidance of fertilization before embryo formation (parthenogenesis), and an avoidance of fertilization before endosperm formation (autonomous endospermy).

apomicts as well (for reviews, see refs. 1 and 9). Ozias-Akins and colleagues (48–53) noted that the molecular mapping of apomixis loci in the grass genus *Pennisetum* was frustrated by an apparent repression of meiotic recombination around the site of an apospory-specific genomic region (ASGR). The size of this region of repressed recombination remains unclear, but it is estimated to be in excess of 50 megabases (52). Intriguingly, this region appears to be hemizygous in the apomicts studied because no similar region was found in sexual relatives (50). Furthermore, a very similar ASGR has also been described in the related apomict *Cenchrus ciliaris* (22, 51). Repressed recombination in association with elements of apomixis has also recorded for *Paspalum* (54, 55).

The observation that recombination is frequently repressed around loci associated with apomixis, together with the dominant inheritance of this trait in *Hieracium*, prompted the choice of deletion mutagenesis as a mechanism for mapping these elements in this system. This approach has several advantages over mapping that uses a segregating population: it utilizes near-isogenic mutants to define map positions; it is independent of difficulties associated with the suppression of cross-over at meiosis; and it simplifies marker validation because the mutants represent genomic subsets of the wild-type plant. *Hieracium* is also well suited for this approach because it can be regenerated easily in culture from very small tissue segments. This feature made it possible to break tissue chimerism and therefore to base the screen at the M_1 level.

Results and Discussion

The Mutant Screen. From an initial sample of ≈5,000 germinable seeds, ≈2,400 plants reached maturity after irradiation. The initial screen of chimeric M_1 plants yielded 220 with potentially valuable mutant sectors, each of which was introduced into tissue culture for regeneration. Ninety regenerants were found to demonstrate deficiencies in apomeiosis and/or parthenogenesis, and 79 of these regenerants were identified as being sufficiently viable to be used for further study. Table 1 lists the mutant classes and the phenotypic characteristics of each. Mutants with a specific loss of the apomeiosis component of apomixis used reduced (meiotic) eggs to form progeny. Because these plants were tetraploids and they retained parthenogenesis, diploid seedlings were frequently recovered after the suppression of pollination (Table 1). In the apomixis literature, progeny of this type are called polyhaploids, and they are given the descriptive nomenclature n+0 to represent the formation of a seedling from an unreduced, unfertilized (1n) gamete (56). Following agreed conventions regarding the naming of loci identified by mutation in *Arabidopsis*, we suggest the designation loss of apomeiosis (*LOA*) for this locus in *Hieracium*. In the wild type, the dominant allele confers apomeiosis (*LOA*), whereas the recessive phenotype is reductional meiosis.

Mutants unable to perform the parthenogenesis component of apomixis did not form seed when pollination was prevented. However, they readily formed hybrid seed after pollination with the tetraploid tester plant A4Z (Table 1). Because apomeiosis remained intact in these plants, unreduced eggs were typically fertilized with diploid A4Z sperm cells, resulting in hexaploid-addition hybrids. In the apomixis literature, progeny of this type are given the descriptive nomenclature 2n+n to represent the fertilization of a 2n gamete with a reduced male gamete (56). We suggest the designation loss of parthenogenesis (*LOP*) for this locus in *Hieracium*. In the wild type, the dominant allele confers parthenogenesis (*LOP*), whereas the recessive phenotype is syngamy.

Another class of mutants was identified with deficiencies in both apomeiosis and parthenogenesis, which re-creates the sexual phenotype that is described by the nomenclature n+n (56). In many cases, these plants were weak, difficult to maintain, and displayed large deletions, making them of limited value for mapping. We also suspect that many of these plants represented mutants with embryo-lethal mutations that could be recovered by hybridization. Intriguingly, this type of mutation appears to have been identified principally in plants also demonstrating a loss-of-apomeiosis (*loa*) phenotype and not in plants that had only lost parthenogenesis (see Fig. 2). In *loa* mutants, the egg cell is reduced because of the reductional division of meiosis. Recessive, deleterious alleles causing embryo lethality are therefore more likely to be expressed in this background than in *LOA* plants that form unreduced, tetraploid egg cells. Mutants with the *loa/LOP* genotype form reduced eggs that can initiate parthenogenetic development (Table 2) to produce a polyhaploid (n+0) seedling. If they also carried a recessive embryo-lethal factor, however, they would fail to complete seed formation unless hybridization succeeded in complementing the lethality factor. At the level of detection for the mutant screen, therefore, these plants appeared initially to have the genotype *loa/lop*

Table 1. Mutant classes

Mutant class	Progeny type(s) after bud decapitation*	Progeny type(s) after pollination	Locus	No. of mutants
Wild-type apomict	Unreduced maternal seedlings (2n+0)	Maternal seedlings (2n+0)	WT	NA
Loss of apomeiosis	Reduced polyhaploid seedlings (n+0)	Polyhaploids (n+0)	*LOA*	24
Loss of parthenogenesis	No seed forms	Unreduced hybrids (2n+n)	*LOP*	30
Combined class*	No seed forms	Reduced hybrids (n+n)	*LOA + LOP*	25
Loss of autonomous endospermy†	No seed forms	Maternal seedlings (2n+0)	Unidentified	0

Nomenclature in parentheses follows the convention of Harlan and de Wet (56) in which the nuclear state of the egg is represented on the left of the addition sign and the nuclear state of the sperm is on the right. WT, wild-type; NA, not applicable.

*Many of the mutants in the combined class were severely compromised and/or had very large deletions that could not be used for mapping.

†Predicted mutant class that was not observed.

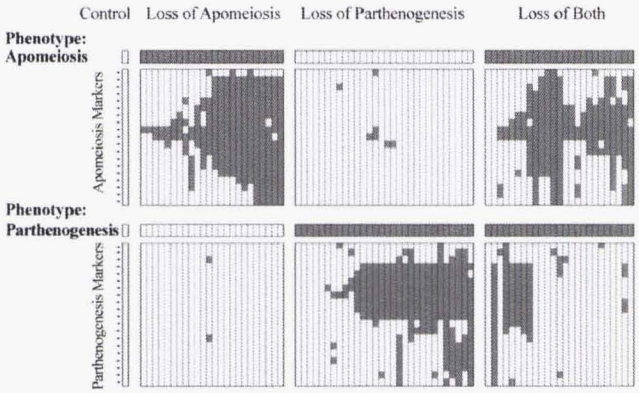

Fig. 2. AFLP markers in a selection of 81 mutants in which apomeiosis and/or parthenogenesis was dysfunctional. Vertical columns represent mutants, horizontal rows represent markers, a light square represents a marker that is present, and a dark square represents a marker that is absent. Phenotypic data are shown in the bar at the top of each group. The controls were subjected to irradiation and regeneration, but they did not express a mutant phenotype with respect to apomixis.

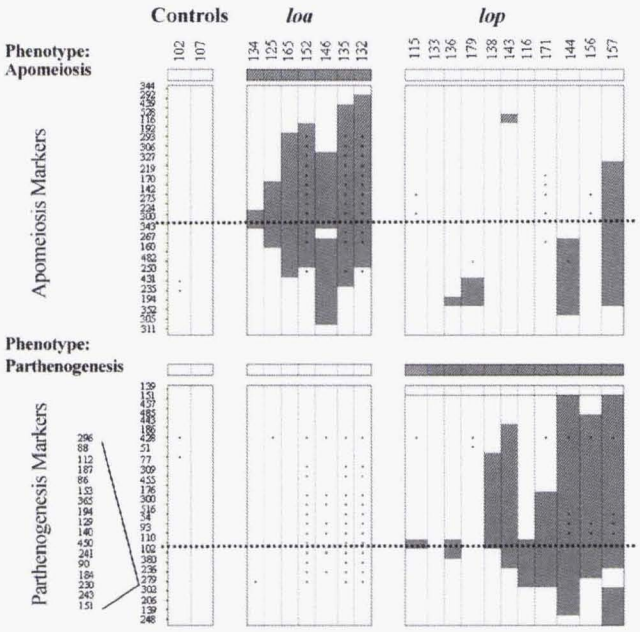

Fig. 3. AFLP markers in an advanced selection of mutants displaying small deletions in either the *LOA* or *LOP* loci. Vertical columns represent mutants, horizontal rows represent markers, a light square represents a marker that is present, and a dark square represents a marker that is absent. Phenotypic data are shown in the bar at the top of each group. The control was subjected to irradiation and regeneration, but it did not express a mutant phenotype with respect to apomixis. Dotted lines indicate apparent midpoints for the consensus deletions, and therefore they are the most probable sites of critical loci. An asterisk represents a data point that was inferred from a previous experiment but not directly tested. A group of 17 *lop* markers was mapped to a site between markers lop 279 and lop 302. No further ordering is possible for this group because discriminating breakpoints were not available.

because reduced eggs formed and fertilization was required for seed development. We noticed, however, that they differed critically from this genotype in the ultimate quantity of seed that developed. Because *loa/LOP* plants are capable of parthenogenesis, hybridization is a rare event in this genotype. If these plants also carried an embryo-lethal factor requiring recovery through complementation, seed set after pollination would also be expected to be poor, which was observed (data not shown). In contrast, true *loa/lop* plants were seen to produce abundant seed after hybridization.

The mutant screen was also designed to detect a final expected mutant class (Table 1). In apomictic forms of *Hieracium*, the endosperm tissue forms spontaneously. In sexual forms, however, it will only form after fertilization (35). We had anticipated a mutant class in which the embryos arose asexually but the endosperm would need to be the product of fertilization. Many native apomicts utilize this mechanism (known as pseudogamy); however, for reasons that are unclear, it was not seen among the mutants recovered. Preliminary histological results indicate that *lop* mutants consistently demonstrated an inability to form either an embryo or an endosperm without fertilization (data not presented), indicating that the *LOP* locus may influence the formation of both tissues in this system.

Mapping. Figs. 2 and 3 illustrate our current model for marker order in the *LOA* and *LOP* loci. The two genomic regions identified align well with phenotypic data from the mutants (Figs. 2 and 3), indicating that both *LOA* and *LOP* have been correctly identified from regions of common marker loss. One mutant, γ-induced mutant 164 (γ164), demonstrated a combined loss-of-apomeiosis-and-parthenogenesis phenotype, yet it retained the most centrally located markers at the *LOA* locus (loa 300 and loa 343). Similarly, two mutants with a *lop* phenotype (γ133 and γ179) retained the most centrally positioned marker identified for this locus. We interpret these data as resulting from mutations that are either too small to be identified by the markers known to date or possibly because of positional changes, such as translocations and/or inversions that are not discernable by amplified fragment length polymorphisms (AFLP).

Seven AFLP bands were successfully converted into polymorphic sequence-characterized amplified region (SCAR) markers, including four markers for the *LOA* locus and three markers for the *LOP* locus. Fig. 4 illustrates the results for four of these markers, tested against a panel of seven *loa* mutants, seven *lop* mutants, one mutant demonstrating a loss of both characters, and a wild-type plant. In all but three cases, the SCAR marker results reflected those from the AFLP analysis. The exceptions

Table 2. Segregating population: P4 (sexual) × C4D (apomict)

Segregant class	Progeny type(s) after bud decapitation	Progeny type(s) after pollination	Proposed genotype	No. of segregants
Apomict: apomeiotic, not requiring fertilization	Unreduced maternal seedlings (2n+0)	Maternal seedlings (2n+0)	*AaaaPppp*	13
Meiotic, not requiring fertilization	Reduced polyhaploid seedlings (n+0)	Polyhaploids (n+0)	*aaaaPppp*	48
Apomeiotic, requiring fertilization	No seed forms	Unreduced hybrids (2n+n)	*Aaaapppp*	9
Sexual: meiotic, requiring fertilization	No seed forms	Reduced hybrids (n+n)	*aaaapppp*	22

Nomenclature in parentheses follows the convention of Harlan and de Wet (56) in which the nuclear state of the egg is represented on the left of the addition sign and the nuclear state of the sperm is on the right.

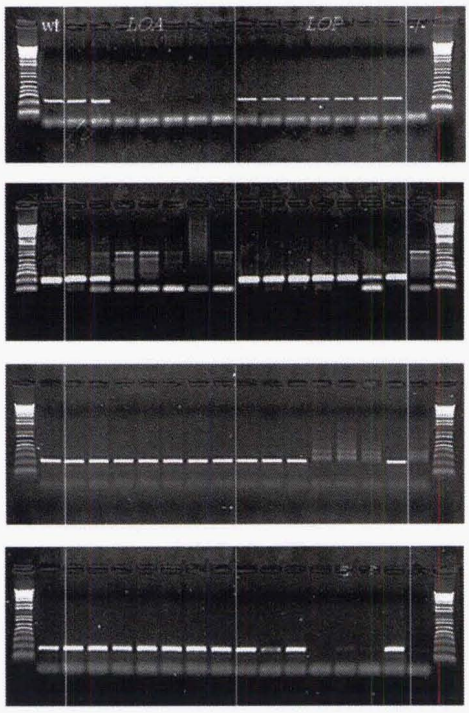

Fig. 4. Demonstration of SCAR marker use against the panel of mutants. In each gel lane 1 is a wild-type plant, lanes 2–8 are seven mutants in LOA (134, 125, 165, 152, 146, 135, and 132), lanes 9–15 are seven mutants in LOP (179, 138, 143, 116, 171, 144, and 156), and lane 16 is the dual mutant 168. The markers used are listed to the left of each gel.

were positive results for the SCARs loa 275 and loa 267 against mutant γ125, and lop 235 against mutant γ156 (Fig. 4). In all cases, the AFLP analysis had indicted a loss of the marker in the given mutant. For markers loa 267 and lop 235, AFLP predicted locations as just within the deletion regions of mutants γ125 and γ156, respectively. For loa 275, the predicted location was more central in mutant γ125. We suspect that some level of adjacent sequence duplication occurs at these sites, enabling the SCAR primers to amplify fragments, whereas the loss of critical restriction sites led to the loss of the AFLP markers.

The Segregating Population. Plant phenotyping for this population also indicated the action of two loci, one associated with the inheritance of apomeiosis and the other with the inheritance of parthenogenesis, which is in agreement with reported observations in two other daisy genera, *Taraxacum* (30, 31) and *Erigeron* (33, 57). In a previous publication (38), we concluded that apomixis was inherited as a monogenic trait in *Hieracium*. It is now clear that this finding is not the case and that the earlier incorrect conclusion resulted from the screen used to measure apomixis at that time. That screen represented a score of parthenogenesis rather than of apomixis as a whole. For both the *LOA* and *LOP* loci, segregation distortion appeared to have acted during the formation of the hybrid population. For *LOP*, the dominant allele was inherited by 67% of the progeny, whereas the dominant allele for *LOA* was only inherited by 24% of the progeny. Roche *et al.* (51) noted a similar imbalance in the inheritance of apomixis in *Pennisetum*, and Grimanelli *et al.* (25) also reported it in *Tripsacum*. Nogler (58) noted that a dominant allele for apomixis in *Ranunculus auricomus* appeared to be gamete-lethal in homozygous form. In a previous study (38), we observed a similar effect in *Hieracium*. In this case, however, the effect appeared to result from a zygote-lethal mechanism. In either case, the effect need not be the direct result of apomixis genes but rather the product of linkage drag, where deleterious lethal alleles arise within regions of reduced recombination associated with the components of apomixis (1, 59). Matzk *et al.* (32) have proposed that this effect may be associated with the accumulation of transposable elements in these regions. Factors for apomeiosis (*LOA*) and parthenogenesis (*LOP*) segregated independently in this population. No significant linkage association was apparent among the segregant classes (Table 2).

The segregation patterns of two AFLP markers, one linked to the *LOA* locus (loa 300) and one linked to the *LOP* locus (lop 102), were determined for 37 members of the P4 × C4D hybrid population.

For parthenogenesis, the marker and trait demonstrated absolute cosegregation across all 37 of the plants tested. For apomeiosis, all 11 of the plants that scored positive for apomeiosis had inherited the loa 300 marker. Of the plants that scored negative for apomeiosis, 24 of 26 lacked the loa 300 marker, indicating that the loa 300 marker lies ≈5 cm from the *LOA* locus. We conclude that the markers used are closely linked to the *LOA* and *LOP* loci in *Hieracium*. Furthermore, no evidence was seen to indicate a role for any other major loci in the inheritance of apomixis in these plants, although modifier loci almost certainly influenced trait expression as previously suggested (60).

In summary, two major genomic regions in *Hieracium caespitosum* have been identified that collectively control apomixis at the level of the avoidance of meiosis and the avoidance of fertilization, respectively. The mutants and markers described in this work have become the basis of a gene-isolation strategy in *Hieracium*. A BAC library for C4D has been made, and it is now being used to isolate sequences corresponding to the *LOA* and *LOP* loci. Ultimately, it is our hope that this information will be used to install apomixis into target species and therefore to advance our goal of using this technology for the improvement of crop species to benefit global welfare.

Materials and Methods

Plant Material and Tissue Culture. A tetraploid, apomictic accession of the species *H. caespitosum* (designated C4D), obtained from Dijon, France, was used for the mutagenesis and mapping studies reported in this work. Preliminary results indicated that this plant was simplex for dominant alleles associated with apomixis at both the *LOA* and *LOP* loci (Tables 1 and 2 and data not shown). Two other plant accessions were used as pollination partners: a tetraploid, apomictic accession of *Hieracium aurantiacum*, from Zurich, Switzerland (A4Z); and a tetraploid, sexual accession of *Hieracium pilosella*, from Caen, France (P4). All stock plants and mutants were maintained vegetatively in tissue culture and within a glasshouse. Flowering during winter months was encouraged by supplementary lighting to provide a 16-h photoperiod as described in ref. 38. To ensure the best possible representation of all progeny types, seedlings were germinated and raised to at least the third true-leaf stage in sterile culture as described in ref. 60.

Mutagenesis and Screening. Asexually derived seed of the apomict C4D was collected from emasculated capitula (35) and mutated by using a ^{60}Co source. A lethality curve was constructed, and a dose of 400 Gy (40 krad) of γ-irradiation was found to represent an approximate LD_{50} for fresh dry seed. Sufficient seed was treated at this level to ensure the survival of ≈2,500 individual seedlings. Assessment for loss of apomixis was initially made by visual detection of reduced seed set on sectors of M_1 plants. This screen took advantage of the observation that even apomictic forms of *Hieracium* express sporophytic self-incompatibility

(61). To generate stable nonchimeric mutants, tissue segments ≈2–4 mm in length, immediately subtending mutant seed heads, were harvested and used to regenerate plants as described by Bicknell (62).

Regenerants that continued to demonstrate a reduced-seed set phenotype were then assessed for the components of apomixis as described by Bicknell *et al.* (61). Briefly, for each mutant at least five immature capitula were severed above the plane of the developing ovaries, and the resulting progeny were assessed for ploidy by using a flow cytometer. Only plants capable of parthenogenetic development could set seed after this treatment because decapitation removes both anther and style tissue before anthesis and stigmatic receptivity. At least five capitula on each mutant were also cross-pollinated with the orange-flowered accession A4Z, and the progeny was assessed for ploidy and for morphology. In this manner, it was possible to assign each mutant into one of three classes: those that had lost apomeiosis, those that had lost parthenogenesis, and those without either (Table 1). To avoid any possibility of identity by decent within the mutant population, only one mutant sector was progressed from each chimeric M_1 plant.

Segregating Population. A subset of 92 seedlings was randomly selected from a population of *H. pilosella* × *H. caespitosum* F1 hybrids, providing a 90% probability of detecting a mapping distance of 2.5 cm or greater. All were assessed for the components of apomixis as described above.

Molecular Markers and Mapping. Molecular markers were used to identify candidate genomic regions associated with the observed mutant classes. Because the mutants were derived from asexually generated seed and all subsequent operations retained the somatic genotype of the original M_1 plant, the mutants were treated as near-isogenic lines. Furthermore, the deletion mutants were assumed to represent genomic subsets of the wild-type genotype. By using this feature, candidate genomic regions associated with apomixis were defined as regions of marker loss found to occur in at least three independent mutants. The marker class used for this work was secondary digest-amplified fragment length polymorphisms (SDAFLP) (63), which were separated by PAGE (16) and visualized by using either radioisotope exposure to film or by silver staining, or they were examined by using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). SDAFLP was used because it is independent of template methylation.

Early results with simpler AFLP protocols (64) proved to be uninterpretable because the methylation status of many of the γ-irradiation-induced mutants was very different from that of the wild type (data not shown). Discerning marker bands were isolated from silver-stained polyacrylamide gels, amplified by PCR, and sequenced. For most of the central marker bands identified, minisequencing (65) was conducted to clarify band identification, enable the design of more specific AFLP primers, and facilitate band isolation.

SCAR markers were developed from the sequence data and tested against the mutants and segregant plants. A total of 256 AFLP primer combinations were tested against a panel of mutants, the wild type, and a control plant, which was isolated after shoot regeneration from an irradiated plant that had retained apomixis.

To expedite marker screening, a four-step process was conducted utilizing emerging mapping data to refine the mutant panel used to identify centrally positioned markers about commonly lost "consensus" genomic regions. In the first step, 79 mutants were tested by using eight AFLP primer combinations. This step provided 39 markers lost in more than 3 mutants. An analysis of these data led to the selection of 36 mutants that appeared to have discrete deletions about two distinct loci. This group included plants known to have lost either apomeiosis or parthenogenesis and a group of 13 in which neither apomeiosis nor parthenogenesis was expressed. The remaining plants typically demonstrated very large deletions, making them less suitable for mapping. In the second step, a further 20 primer combinations were tested on the 36 selected plants. After analysis of these data, the third step utilized an elite set of 8 mutants and 2 controls to test a final 228 primer combinations for markers close to the mid points of the consensus-deleted regions. This set included plants with small deletions and others with intermediate-sized deletions. In this way, it was possible to assign each marker to one of two classes: those more proximal than the most central breakpoint and those more distal. In the final step, 29 key mutants were used to order the most central markers.

The power of this multistep approach was that it allowed large numbers of primer combinations to be tested rapidly at low cost. The clear risk was that the early selection of mutants representing potential consensus regions could bias the results and possibly lead to the nonidentification of critical marker clusters. Because this effect would be particularly pronounced when the candidate mutant panel was constricted too rapidly, the multistep approach was taken to mitigate the risk.

Mapping was conducted empirically through two-dimensional tabulation. Matrices were constructed in which mutants were assigned to columns, and marker presence was recorded in rows (see Fig. 2). Marker order was discerned based on four principal assumptions: that each loss-of-function mutant had lost some, or all, of a locus responsible for its characteristic phenotype; that a marker close to the locus would be more frequently lost than one positioned more distally; that genomic regions are typically lost contiguously, and therefore adjacent markers would be more likely lost together than markers in nonadjacent positions; and that the most probable solution is the most parsimonious one.

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