Genetic mechanisms of apomixis

Melissa Spielman, Rinke Vinkenoog and Rod J. Scott*

Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

The introduction of apomixis to crops would allow desirable genotypes to be propagated while preventing undesirable gene flow, but so far there has been little success in transferring this trait from a natural apomict to another species. One explanation is the sensitivity of endosperm to changes in relative maternal and paternal contribution owing to parental imprinting, an epigenetic system of transcriptional regulation by which some genes are expressed from only the maternally or paternally contributed allele. In sexual species, endosperm typically requires a ratio of two maternal genomes to one paternal genome for normal development, but this ratio is often altered in apomicts, suggesting that the imprinting system is altered as well. We present evidence that modification of DNA methylation is one mechanism by which the imprinting system could be altered to allow endosperm development in apomicts. Another feature of natural apomixis is the modification of the normal fertilization programme. Sexual reproduction uses both sperm from each pollen grain, but pseudogamous apomicts, which require a sexual endosperm to support the asexual embryo, often use just one. We present evidence that multiple fertilization of the central cell is possible in *Arabidopsis thaliana*, suggesting that pseudogamous apomicts may also need to acquire a mechanism for preventing more than one sperm from contributing to the endosperm. We conclude that strategies to transfer apomixis to crop species should take account of endosperm development and particularly its sensitivity to parental imprinting, as well as the mechanism of fertilization.

Keywords: apomixis; endosperm; imprinting; fertilization; methylation; polyspermy

1. INTRODUCTION

In most angiosperm species, seed development involves paternal and maternal gametes: one sperm from a pollen grain fertilizes the egg to produce the embryo, while the other fuses with the central cell to generate endosperm. An exception to this mode of reproduction is apomixis, in which the embryo develops without a paternal genome, though in most apomictic species the endosperm still requires fertilization. There are various ways in which embryo sac development and fertilization behaviour are altered to achieve apomixis, with different patterns typical of different taxa (Nogler 1984; Richards 1986; Koltunow 1993; Crane 2001), but one definitive feature is that the embryo is a clone of the mother. Apomixis, therefore, has traditionally been attractive to plant breeders as a means of preserving desirable combinations of genes that would be broken up by recombination and segregation in sexual reproduction. Engineering male sterility into an apomict would have the added benefit of preventing gene flow through pollen, which would, for example, aid in the containment of transgenes.

So far apomixis has not been transferred to an experimental or crop species. We will argue that one explanation is the sensitivity of endosperm to changes in relative maternal and paternal contribution owing to parental imprinting. This is an epigenetic system of transcriptional

regulation by which some genes are expressed only from the maternally or paternally contributed allele, so that male and female gametes transmit complementary sets of potentially active genes to the next generation. Imprinting in angiosperms operates mainly in endosperm, and offers an explanation for the observation that this tissue requires a specific ratio of maternal (m) to paternal (p) genomes for normal development (Haig & Westoby 1991). In most species the central cell nucleus is formed by the fusion of two haploid polar nuclei so the critical ratio in the endosperm is 2m : 1p. Any deviation from this ratio, obtained for instance by crossing plants of different ploidies, often results in seed abortion, presumably owing to an imbalance in the active copies of maternally and paternally imprinted loci in the endosperm.

Apomicts that require fertilization for endosperm development need to cope with parental genomic imbalance, as the bypass of meiosis that is part of apomictic development typically results in doubling of the chromosome number in the central cell as well as the egg. Those with asexual endosperm face an even more serious problem, as these generate seeds without any paternal contribution. Therefore, the existence of imprinting poses a challenge to inducing apomixis in sexual species over and above the basic requirement of inducing embryogenesis without fertilization. Another factor to consider is the fate of the second sperm in each pollen grain. In sexual reproduction, only one sperm is available to fertilize the central cell, as the other is absorbed by the egg. In pseudogamous apomicts, however, there is a 'spare sperm' that could potentially also enter the central cell and affect the parental genomic ratio. Although *in vitro* studies in maize show

*Author for correspondence (bssrjs@bath.ac.uk).

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there is a block to multiple fertilizations of the egg (Faure *et al.* 1994), to our knowledge no such data have been published for the central cell. We will present evidence suggesting that in the sexual species *Arabidopsis thaliana* there is no polyspermy block on the central cell, and will discuss the implications of this for the ‘spare sperm’ problem and induction of apomixis.

2. REPRODUCTIVE DEVELOPMENT IN NATURAL APOMICTS

(a) *Types of apomixis*

Apomixis has been reported in more than 300 taxa distributed over 35 families, and is most common in the Poaceae, Asteraceae and Rosaceae (Richards 1986). There are three main pathways for apomixis—adventitious embryony, diplospory and apospory (figure 1)—with many variations on each. In adventitious embryony, embryos develop directly from sporophytic cells outside the embryo sac. By contrast, both diplospory and apospory involve modifications to female gametophytic development, and are therefore sometimes described as gametophytic apomixis. In diplospory, the MMC either bypasses or fails to complete meiosis, and divides to form an unreduced embryo sac. In aposporous species, the unreduced embryo sac develops from a sporophytic cell that differentiates from the nucellus later than the MMC. In both types of gametophytic apomixis, the circumvention of chromosome reduction before embryo sac formation is known as ‘apomeiosis’. There are numerous reviews of the types of apomixis and their phylogenetic distribution, for example Nogler (1984), Richards (1986), Koltunow (1993), Savidan (2000), Crane (2001) and Grimanelli *et al.* (2001).

(b) *Endosperm development in apomicts*

The persistent endosperm of cereals is the major food store in harvested seed, and its development must be taken into account in programmes to induce apomixis in cereal crops. In addition, endosperm is required to support embryogenesis even in species where it is not present in the mature seed. Therefore it is crucial to understand the ontogeny of the central cell, as well as the fertilization regime, in natural apomicts.

Citrus is a model system for studying adventitious embryony. In this genus a reduced, sexual embryo sac develops in the same ovule as the sporophytically derived, parthenogenetic embryo, and fertilization is required for apomictic seed development. The sexual embryo may arrest but the sexual endosperm is required to nourish the asexual embryo (Richards 1986; Koltunow 1993). Therefore the seed contains an endosperm with the normal 2m : 1p genomic ratio, which poses no imprinting-related problems. (It should be noted here that most—possibly all—natural apomicts are polyploid (Savidan 2000), but it is the ratio of maternal to paternal genomes in the seed that matters, not their absolute number (Haig & Westoby 1991; Scott *et al.* 1998). For simplicity, in this paper we will reduce karyotypes to their lowest common denominator.)

Gametophytic apomixis, however, involves the production of an unreduced embryo sac, with consequences

Figure 1. Major apomictic pathways. The three basic types of apomixis are diplospory, apospory and adventitious embryony. Both diplospory and apospory involve apomeiosis, where meiosis is not initiated or not completed, resulting in an unreduced (2n) embryo sac (ES). In adventitious embryony a normal sexual embryo sac (n) is produced while the asexual embryo grows not from an egg but from a sporophytic cell outside the embryo sac. This somatic embryo is nourished by the endosperm produced within the sexual embryo sac, and the sexual embryo may abort. Diplosporous embryo sacs arise from the MMC while aposporous embryo sacs develop from an aposporous initial cell elsewhere in the nucellus. Diplospory can be further divided into the meiotic type, where the MMC initiates meiosis but there is restitution of the products, and the mitotic type, where meiosis is bypassed. There are further variations on the development of embryo sacs arising from apomeiosis, such as the formation of four nuclei rather than eight, which may be linked to maintaining parental genome balance in the endosperm (see § 2b). Apomeiotic apomixis often requires a sexual endosperm to support the parthenogenetic embryo, but in autonomous apomixis the endosperm develops without fertilization.

for the m : p ratio in endosperms of pseudogamous apomicts. If there were no further modifications to gametophyte development or fertilization behaviour, the ratio

would be 4m : 1p, which is often lethal following inter-ploidy crosses within sexual species (Haig & Westoby 1991). Various modifications that may be interpreted as strategies to restore the normal balance are employed in nature. One involves changes to the structure of the female gametophyte: the 'Panicum' type of embryo sac contains four nuclei instead of the eight typical of sexual embryo sacs, so that there is only a single unreduced polar nucleus, and therefore fertilization produces a 2m : 1p ratio (Nogler 1984). Other species modify fertilization behaviour in addition to gametophyte structure. In *Dichanthium annulatum* the unreduced polar nuclei remain separate and either one degenerates or both are fertilized by one sperm each, resulting in two primary endosperm nuclei with a 2m : 1p constitution (Reddy & d'Cruz 1969, cited in Nogler 1984). In *Ranunculus auricomus* both sperm fertilize the unreduced central cell (Rutishauser 1954, cited in Nogler 1984), resulting again in a 2m : 1p ratio. Pseudogamous apomicts do not appear to modify male gametophytic development to produce unreduced sperm (Nogler 1984), although this would be an alternative means of restoring the balance.

There are also apomictic species in which the endosperm develops with a ratio other than 2m : 1p, raising the question of whether the imprinting system is also modified in these plants. It has been reported that in *Tripsacum dactyloides* a single sperm fuses with an unreduced central cell to produce a 4m : 1p endosperm (Brown & Emery 1958, cited in Haig & Westoby 1991). Grimanelli *et al.* (1997) performed flow cytometry on developing endosperms of tetraploid apomictic *Tripsacum*, which were open-pollinated by either diploid or tetraploid plants in the same nursery, and inferred ratios of 8m : 1p or 8m : 2p (= 4m : 1p), suggesting that the imprinting-mediated dosage requirement is less stringent than normal. It should be noted, however, that since immature endosperms were analysed it cannot be certain that all of these ratios would result in viable seeds (Vinkenoog & Scott 2001). Quarin (1999) analysed endosperm ploidy and germination frequency of seeds produced by tetraploid apomictic *Paspalum notatum* after manual pollination by plants of the same species with ploidies ranging from 2x to 8x, and found that all pollen ploidies (and therefore all parental genomic ratios in the endosperm) tested could support development of viable seeds. This also suggests a modification of the imprinting system. The most extreme examples, however, are autonomous apomicts such as *Taraxacum* and *Hieracium*, which have lost the normal requirement for paternal contribution to the endosperm. In § 4b we will return to the question of how the imprinting system might be modified in apomicts.

(c) Genetic control of apomixis

Gametophytic apomixis involves several modifications to the sexual reproduction pathway, including apomeiosis, parthenogenetic embryo development, and in some cases changes to central cell derivation and fertilization behaviour associated with endosperm development. Evidence from several taxa indicates that apomeiosis is transmitted as a dominant Mendelian factor, though penetrance is limited, explaining the occurrence of facultative apomicts. In some species, such as *R. auricomus*, *Pennisetum maximum* and *Hieracium piloselloides*, apomeiosis along

with the other elements of apomictic reproduction are inherited as a single trait. However, in *Taraxacum officinale*, *Poa pratensis* and *Erigeron annuus*, apomeiosis does not always cosegregate with other features of apomixis. In all species tested there is strong suppression of recombination around the apomeiosis locus, suggesting that apomixis may be controlled by many genes that are inseparably linked in one or two coadapted complexes (Grimanelli *et al.* 2001; Grossniklaus *et al.* 2001).

3. ENGINEERING APOMIXIS

Strategies to introduce apomixis into sexual plants have followed the same pathways as efforts to breed any other desirable trait. They include the following:

- (i) hybridization of related sexual and apomictic species;
- (ii) mutation followed by selection for autonomous seed development in a normally sexual species;
- (iii) identification of the alleles that confer apomixis in nature with a view towards using these for transformation; or
- (iv) construction of 'best guess' artificial apomixis genes, again followed by transformation.

Some progress has been made with all of these methods but so far no sexual species has been engineered to produce embryos within seeds without fertilization.

The Poaceae include a high proportion of both apomictic and economically important species and there are several breeding programmes to transfer pseudogamous apomixis to crops through wide hybridization, for example by crossing *T. dactyloides* with its relative maize, or *Pennisetum squamulatum* with pearl millet. However, all have met obstacles, such as loss of the apomictic trait during introgression, or failure of seed set, the latter possibly owing to parental imbalance in the endosperm caused by the fusion of an unreduced central cell with a reduced sperm (Morgan *et al.* 1998; Savidan 2001; Grimanelli *et al.* 2001; Spillane *et al.* 2001). Introgression of autonomous apomixis, which could circumvent the endosperm problem, is infeasible in cereals as this pathway is almost exclusively restricted to the Asteraceae.

There is a long history of mutagenesis of crop plants, and some lesions have turned out to confer elements of apomictic pathways such as the production of unreduced gametes or parthenogenetic embryos, though none have resulted in crops that produce asexual embryos at a high frequency (Praekelt & Scott 2001). Mutagenesis and screening for apomixis in the model sexual plant *A. thaliana* has been conducted in a more directed way, with some success. Most strategies employed a one-step screen for seed development without fertilization after a single round of mutagenesis. Interestingly, the three *fis* (fertilization-independent seed) mutations found in this way, *fis1/mea* (*medea*), *fis2* and *fis3/fie* (*fertilization-independent endosperm*) (Ohad *et al.* 1996; Chaudhury *et al.* 1997; Grossniklaus *et al.* 1998), allow some autonomous development of the central cell; however, none of these mutations alone induces full differentiation of autonomous endosperm (Vinkenoog *et al.* 2000). In addition, there is little or no development of the egg, possibly

because the *FIS* genes are relatively late acting (Chaudhury *et al.* 1997).

As outlined, cloning natural apomixis genes is proving difficult for several reasons, including suppression of recombination near apomeiosis loci, as well as polyploidy and reduced sexuality of apomicts. Although apomixis loci have been mapped in several species (Grimanelli *et al.* 2001; Grossniklaus *et al.* 2001), none has yet been sequenced. Genes involved in apomixis are also being pursued by other techniques, such as differential display to isolate mRNAs expressed in apomictic rather than sexual flowers (Pessino *et al.* 2001).

Several ‘best-guess’ genes for inducing embryogenesis without fertilization have been identified, such as *AtSERK1* (this is expressed in pre-fertilization ovules and early embryos, and overexpression *in vivo* increases the embryogenic potential of callus (Hecht *et al.* 2001)) and *BABY BOOM* (expressed during seed development and overexpression induces embryogenesis on parts of the plant such as leaf margins and shoot apex (Boutilier *et al.* 2002)). Although the ectopic expression of these genes stimulates somatic embryogenesis in culture or on vegetative organs, there is so far no evidence that they would induce parthenogenesis within a seed, which is a requirement for apomixis.

4. APOMIXIS AND THE ENDOSPERM PROBLEM REVISITED

(a) *Interploidy crosses and imprinting in endosperm development*

Flowering plants easily tolerate polyploidy, but crosses between plants of different ploidies result in abnormal seed development often followed by abortion, with reciprocal endosperm phenotypes depending on the direction of the cross (Haig & Westoby 1991). In *A. thaliana*, crosses between 2x and 4x plants in either direction produce viable seeds with 3x embryos. However, in crosses between a 4x seed parent and 2x pollen parent (4x × 2x), resulting in an endosperm with a parental genomic balance of 4m : 1p (maternal excess), the endosperm divides very little and the mature embryo is small; while the reciprocal 2x × 4x crosses, producing 2m : 2p endosperms (paternal excess), result in overproliferation of the endosperm and ultimately large embryos (figure 2; Scott *et al.* 1998). Crosses between 2x and 6x plants give more extreme maternal and paternal excess phenotypes, depending on the direction of the cross, and seeds from these crosses abort. Although the maternal : paternal balance of the embryo is also disturbed in interploidy crosses, there is evidence from maize that it is the 2m : 1p ratio in the endosperm, not the ratio in the embryo, that is crucial for normal seed development (Lin 1984).

The 2m : 1p requirement is commonly interpreted as an indication that parentally imprinted genes are involved in endosperm development (Lin 1984; Haig & Westoby 1991; Alleman & Doctor 2000). Because imprinting silences some alleles in the maternally contributed genome and others in the paternally contributed genome, each parent transmits a different set of potentially active alleles to the endosperm. In both mammals and flowering plants, the two taxa where imprinting has been observed, there is phenotypic and molecular evidence that many (though not

Figure 2. The importance of the parental genome ratio in endosperm development. (a) In *A. thaliana*, as in most flowering plants, crosses between individuals of the same ploidy result in an endosperm with a ratio of two maternal genomes (represented by dark circles) to one paternal genome (white circles) (2m : 1p). Interploidy crosses disturb this ratio, with increasing imbalance having increasingly severe effects on seed development, as measured by endosperm size, seed weight and ultimately seed viability. Extra maternal relative to paternal genomes (4m : 1p, 6m : 1p) inhibit mitosis in the endosperm and result in small seeds, while extra paternal genomes (2m : 2p, 2m : 3p) have the opposite effect. (b) Confocal micrographs of seeds at 8 days after pollination show developmental differences generated by parental genomic imbalance. In the control seed from a 2x × 2x cross (middle), the embryo has reached torpedo stage, and the PE is cellularized. In a 6x × 2x cross resulting in lethal maternal excess (left) the endosperm has barely proliferated and has cellularized early, while a 2x × 6x cross generating lethal paternal excess (right) results in the overproliferation of the endosperm and cellularization failure.

all) active paternal alleles of imprinted loci promote growth of offspring, while the active maternal alleles inhibit growth (Haig & Westoby 1991; Moore & Haig 1991; Tilghman 1999; Alleman & Doctor 2000). This explains why increasing the relative dose of paternal genomes is correlated with increase in endosperm prolifer-

ation and seed size, while increasing the relative maternal dose has the opposite effect.

(b) Solutions to the endosperm problem: modifications to imprinting

(i) Pseudogamous apomicts

A. thaliana can produce viable (though small) seeds with a 4m : 1p ratio in the endosperm. However, many other species, including crops where apomixis would be desirable, are less tolerant. In maize, crosses between 4x seed parents and 2x pollen parents produce mainly shrunken kernels that rarely germinate (Cooper 1951), and 4x × 2x crosses in *Brassica campestris* (=rapa) similarly produce inviable seeds with small endosperms (Håkansson 1956). As described in § 2a,b, one consequence of apomeiosis is an unreduced embryo sac, so fertilization by a single reduced sperm typically produces a 4m : 1p ratio in the pseudogamous endosperm. What is the mechanism by which species that cannot survive deviation from the normal 2m : 1p ratio adapt to pseudogamy?

In previous work we discussed evidence that the epigenetic state of a genome affects the degree to which it functions as paternal or maternal in the endosperm. We proposed the term 'epigender' to denote the paternal or maternal character of a genome as defined by the sex-specific marking of its genes for imprinting (Spielman *et al.* 2001). The 'm' quality of polar nuclei and 'p' quality of sperm are epigender states (figure 3). There is strong evidence from mammals and also some from plants that monoallelic silencing of imprinted genes is maintained by epigenetic regulatory mechanisms such as DNA methylation (Surani 1998; Alleman & Doctor 2000). Therefore, the epigender of a polar nucleus or sperm nucleus can in theory be modified by disrupting the regulatory mechanisms associated with imprinting.

We tested this hypothesis using *Arabidopsis* plants with reduced DNA methylation owing to the presence of a *MET1* a/s construct (Finnegan *et al.* 1996). Crosses where only one parent had reduced methylation were particularly instructive. When the pollen parent was hypomethylated (2x × 2x*MET1* a/s), the resulting seeds were small, with endosperm phenotypes characteristic of maternal excess, but when the seed parent was hypomethylated (2x*MET1* a/s × 2x), the endosperm overproliferated and seeds were large (Adams *et al.* 2000). This is consistent with a model in which hypomethylation of one parental genome prevents silencing of alleles that would normally be active only when inherited from the other parent—thus phenocopying the effects of extra genomes. Significantly, these experiments show that the epigender of the central cell or sperm, and therefore the effective ratio of genomes in endosperm, can be altered.

Various ways in which apomicts cope with the endosperm problem are shown in figure 4. As discussed in § 2b, some modify embryo sac development, fertilization behaviour or both to generate a 2m : 1p endosperm from an unreduced central cell. However, those that allow a 4m : 1p (or greater) ratio in the endosperm presumably must modify their imprinting systems. Based on our uniparental hypomethylation experiments we propose that paternalization of the polar nuclei, through a mechanism such as hypomethylation or modification to other components of gene silencing complexes, could be a way of

Figure 3. The model for epigender states in endosperm development. Plant somatic cells appear to be insensitive to parental imprinting so are represented as both maternal and paternal, as all imprinted genes are potentially active. During gametogenesis, imprinted genes that will be expressed only from the paternal genome during endosperm development are silenced in the polar nuclei, giving them maternal epigender, while genes that will be maternally expressed are silenced in the sperm nuclei, giving them paternal epigender. In a typical embryo sac with two polar nuclei, fertilization produces 2m : 1p endosperm, meaning that the maternally active imprinted loci and paternally active imprinted loci are in a 2 : 1 ratio.

restoring the effective balance to allow seed development. Ideally, such modifications would be targeted to specific cell types and sets of genes to prevent possible general effects of global changes to gene expression on seed development.

(ii) Autonomous apomicts

If imprinting is modified in pseudogamous apomicts to counteract the effects of maternal excess in endosperm, the ability of autonomous apomicts to develop endosperm without any paternal contribution suggests that these species are no longer responsive to parental genomic imbalance. The possibility that DNA methylation might also be involved in the adaptation of autonomous apomicts is supported by experiments in which hypomethylation was combined with the *fie-1* mutation of *Arabidopsis* (Vinkenoog *et al.* 2000).

Maternally transmitted mutations in the *FIS3/FIE* gene cause ectopic central cell proliferation in unfertilized ovules and overproliferation of sexual endosperm in fertilized seeds (Ohad *et al.* 1996; Chaudhury *et al.* 1997). *FIS3/FIE* encodes a Polycomb group protein that participates in a complex involved (like DNA methylation) in gene silencing (Luo *et al.* 1999; Ohad *et al.* 1999). If an ovule carrying a *fie-1* mutation is pollinated, there is dramatic overproliferation of endosperm reminiscent of the lethal paternal excess phenotype seen in 2x × 6x crosses (figure 5a), and the embryo arrests by torpedo stage. According to the epigender scheme, this suggests that the two polar nuclei are paternalized by the *fie* mutation, and the addition of a sperm brings the genomic ratio in the endosperm over the threshold of paternal excess that *Arabidopsis* can tolerate. If the pollen donor is hypomethylated by the *MET1* a/s transgene, however, overproliferation of

Figure 4. Effects of embryo sac (ES) morphology and fertilization strategy on parental genome ratio in the endosperm. In different pseudogamous apomicts (left) endosperms may contain varying ratios of maternal to paternal genomes. Some pseudogamous apomicts modify embryo sac development or fertilization behaviour to achieve a 2m : 1p parental genomic balance, while others apparently tolerate imbalance. Autonomous apomicts (right) tolerate the most extreme imbalance as there is no paternal contribution to the endosperm. In the diagrams of embryo sacs, polar nuclei are represented by black circles, and sperm (in fertilized embryo sacs) by white circles.

the endosperm is attenuated, and viable—though large—seeds are produced (Vinkenoog *et al.* 2000). We interpret this as showing that hypomethylation of the pollen donor provides sufficient maternalization of the paternalized *fie-1* endosperm to rescue seed development.

Hypomethylation also alters the phenotype of unfertilized *fie-1* mutant ovules (Vinkenoog *et al.* 2000). Although *fie-1* mutant central cells undergo some degree of autonomous development, they do not produce differentiated, cellularized endosperms without fertilization (figure 5*b*). The pollinated *fie-1* phenotype suggests that the effective genomic ratio in the autonomous *fie-1* endosperm would be approximately 1m : 1p (figure 5*a,b*)—the same found in 2x × 4x crosses, which *Arabidopsis* can tolerate (Scott *et al.* 1998)—so there must be some other block to full endosperm development in these mutants. We found that combining the *MET1* a/s transgene with the *fie-1* mutation allowed endosperms to follow many features of the normal endosperm developmental programme (figure 5*c*; Vinkenoog *et al.* 2000; Vinkenoog & Scott 2001). Therefore, the *fie-1* mutation and DNA hypomethylation combined can at least to some extent substitute for the lack of a paternal genome in autonomous endosperm development, suggesting a mechanism by which autonomous apomicts could circumvent the requirement for fertilization to make endosperm.

5. SINGLE VERSUS MULTIPLE FERTILIZATION OF THE CENTRAL CELL: CONSIDERATIONS FOR EVOLUTION AND ENGINEERING OF APOMIXIS

(a) The spare sperm problem

All pollen grains in flowering plants contain two sperm. In sexual reproduction one fuses with the egg and the other with the central cell, but in pseudogamous apomicts the egg is not fertilized, potentially leaving one sperm with no function. In some species the embryo sac or fertilization is modified so that both sperm are used to produce endosperm (§ 2*b*), but in most cases it is assumed that only one sperm fuses with the central cell. Savidan (2000) noted that if ‘dual fusion’ (the use of both sperm to fertilize the central cell) could be systematically induced it should diminish the frequency of (undesirable) sexual embryos that occasionally occur in facultative apomicts (Nogler 1984). However, in general the fate of the ‘spare sperm’ does not appear to have been investigated, as studies of fertilization in apomicts tend to focus on interactions of sperm with the egg apparatus (Vielle *et al.* 1995). This raises several questions (to our knowledge not previously discussed in the apomixis literature) such as evolution of the mechanism (if any) to prevent fertilization of the central cell by both sperm, and whether this might be employed in engineered apomixis as a means of regulating the genomic ratio in endosperm.

Figure 5. Development of fertilized and unfertilized ovules in *fie-1* mutants of *Arabidopsis thaliana*. (a) Light micrograph of a fertilized *fie-1* seed showing massive overproliferation of uncellularized endosperm, typical of the lethal paternal excess phenotype observed in $2x \times 6x$ crosses. We infer from the phenotype and the nature of the *FIE* gene (see § 4b(ii)) that the polar nuclei have lost imprinting to some extent so have paternal as well as maternal epigenetic. (b) Confocal micrograph of an unfertilized *fie-1* ovule. The central cell has proliferated but there is no regional specification or cellularization typical of sexual endosperm. As the phenotype of (a) suggests the presence of a virtual paternal genome in the endosperm, there is apparently another block to complete endosperm development than lack of a paternal genome. The missing factor is denoted 'x'. (c) Confocal micrograph of an unfertilized *fie-1* ovule from a plant transformed with a *MET1* a/s construct, showing a large chalazal endosperm and cellularized PE typical of a sexual seed. We infer that the combination of the *fie-1* mutation and hypomethylation of the genome have removed a block to autonomous endosperm development by adding 'x'.

(b) Multiple fertilization of the central cell in *Arabidopsis*

In vitro experiments in maize show that there is a polyspermy block on the egg (Faure *et al.* 1994), but an investigation of *in vitro* fertilization of the maize central cell did not report any attempts to fuse the central cell with more than one sperm (Kranz *et al.* 1998). However, preliminary experiments indicate that in *A. thaliana* the central cell can be fertilized by multiple sperm.

Our experiments make use of the *tetraspore* (*tes*) mutation (Spielman *et al.* 1997), which abolishes cytokinesis following male meiosis so that all four haploid microspore nuclei remain in the same cytoplasm (figure 6a). Some of the microspore nuclei fuse during pollen development, and mature pollen grains contain a variable number of sperm with a range of different ploidies (figure 6b,c). Female meiosis in *tes* mutants is normal, and when either *tes* mutants or wild-type plants are pollinated by a *tes* mutant father, a mixture of aborted and viable seed is produced, with the latter falling into several size and phenotypic classes characteristic of normal development or paternal excess (figure 6d; Spielman *et al.* 1997; Scott *et al.* 1998). Taken together, the evidence suggests that seed abnormalities could result from fertilization of the central cell by high ploidy sperm, with $2x$ sperm producing large but viable seeds, and those $3x$ and over causing seed abortion.

There is a significant discrepancy, however, between the high frequency of seed abortion and the low frequency of sperm with measured DNA contents indicating a ploidy of $3x$ or more (Spielman *et al.* 1997). This raises several possibilities: some of the abortion could be caused by sperm abnormalities resulting from the *tes* mutation—or the central cell could be absorbing more than one sperm, increasing the paternal content of the endosperm to lethal levels. To choose between these possibilities we attempted rescue of the seed abortion by hypomethylating the *tes* mutant pollen donors, which as discussed in § 4b would be expected to have a maternalizing influence on endosperm and so neutralize the paternalizing effect of extra sperm genomes in the seed. All seeds from crosses between wild-type seed parents and *tes* *MET1* a/s pollen parents were plump and viable (R. Scott, unpublished data). This suggests that sperm abnormalities are unlikely to be the cause of abortion in *tes* mutant seeds, as there is no obvious mechanism by which hypomethylation could compensate for changes such as loss of chromosomes that might accompany mitosis and nuclear fusion in coenocytic *tes* pollen grains. It is formally possible that paternal hypomethylation could correct hypothetical epigenetic defects in *tes* mutant sperm, but we have also been able to rescue seed sired by a *tes* mutant by using a $4x$ wild-type seed parent to maternalize the endosperm (R. Scott, unpublished data). Taken together, the evidence suggests that the abortion of seeds produced by *tes* mutant pollen parents is partly due to the multiple fertilization of the central cell. We are continuing to investigate this phenomenon using genetic and cytological approaches.

What does the apparent lack of a polyspermy block on the central cell of a sexual species mean for apomicts? It indicates that pseudogamous apomicts that do not use both sperm may have evolved a mechanism for preventing multiple fertilization. It also points to another aspect of

Figure 6. Pollen and seed morphology in *tetraspore* (*tes*) mutants. (a) Transmission electron micrographs of tetrads from wild-type and *tes* mutant plants, each showing three of the four microspores produced by male meiosis. In wild-type tetrads the microspores are separated by cross-walls, but in *tes* mutants there is no cytokinesis following male meiosis and all microspore nuclei develop in the same cytoplasm. (b) Mature *tes* mutant pollen grain stained with DAPI to show DNA. Two pairs of sperm (small and bright) and one vegetative nucleus (diffuse) are visible. (c) *In vitro* germinated pollen tubes from wild-type and *tes* mutant plants stained with DAPI. *tes* mutant pollen tubes can transmit multiple pairs of sperm. The difference in the size of sperm is correlated with the difference in DNA content, and by inference ploidy (Spielman *et al.* 1997). (d) Seeds from a self-pollinated *tes* mutant. Many are aborted, and the plump, viable seeds fall into different size classes (two examples circled). Large viable seeds are correlated with moderate paternal excess and aborted seeds with lethal paternal excess (see § 5b).

reproduction that should be considered when introducing apomixis into a sexual species: for example, it is possible that problems caused by creating 4m : 1p endosperm in a species with a strong imprinting system could be addressed by encouraging multiple fertilization of the central cell. Clearly, more work is needed on this aspect of endosperm development in apomicts.

6. CONCLUSION

Programmes to introduce apomixis into sexual species have focused on circumventing meiosis and inducing par-

thenogenesis, without necessarily accounting for more subtle characteristics of apomictic reproduction such as the imprinting status of the endosperm and behaviour of sperm. This is possibly one of the reasons why apomixis has been so difficult to engineer. We have presented evidence that the modification of DNA methylation could adjust the imprinting system to remove the developmental block on endosperms with insufficient, or no, paternal genomes owing to the bypass of meiosis in pseudogamous apomicts or the absence of fertilization in autonomous apomicts. We are also investigating the possibility of multiple fertilization of the central cell and its effects on endosperm development. The ability to control these features of apomixis should improve the prospects for transferring this mode of reproduction to crops.

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REFERENCES

- Adams, S., Vinkenoog, R., Spielman, M., Dickinson, H. G. & Scott, R. J. 2000 Parental imprinting in *Arabidopsis thaliana* requires DNA methylation. *Development* **127**, 2493–2502.
- Alleman, M. & Doctor, J. 2000 Genomic imprinting in plants: observations and evolutionary implications. *Plant Mol. Biol.* **43**, 147–161.
- Boutillier, K. (and 12 others) 2002 Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *Plant Cell* **14**, 1737–1749.
- Brown, W. V. & Emery, W. H. P. 1958 Apomixis in the Gramineae: Panicoideae. *Am. J. Bot.* **45**, 253–263.
- Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S. & Peacock, W. J. 1997 Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **94**, 4223–4228.
- Cooper, D. C. 1951 Caryopsis development following matings between diploid and tetraploid strains of *Zea mays*. *Am. J. Bot.* **38**, 702–708.
- Crane, C. F. 2001 Classification of apomictic mechanisms. In *The flowering of apomixis: from mechanisms to genetic engineering* (ed. Y. Savidan, J. G. Carman & T. Dresselhaus), pp. 24–43. Mexico: CIMMYT/EU/IRD.
- Faure, J.-E., Digonnet, C. & Dumas, C. 1994 An *in vitro* system for adhesion and fusion of maize gametes. *Science* **263**, 1598–1600.
- Finnegan, E. J., Peacock, W. J. & Dennis, E. S. 1996 Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc. Natl Acad. Sci. USA* **93**, 8449–8454.
- Grimanelli, D., Hernández, M., Perotti, E. & Savidan, Y. 1997 Dosage effects in the endosperm of diplosporous apomictic *Tripsacum* (Poaceae). *Sex. Plant Reprod.* **10**, 279–282.
- Grimanelli, D., Leblanc, O., Perotti, E. & Grossniklaus, U. 2001 Developmental genetics of gametophytic apomixis. *Trends Genet.* **17**, 597–604.
- Grossniklaus, U., Vielle-Calzada, J.-P., Hoepfner, M. A. & Gagliano, W. B. 1998 Maternal control of embryogenesis by *MEDEA*, a polycomb-group gene in *Arabidopsis*. *Science* **280**, 446–450.
- Grossniklaus, U., Nogler, G. A. & Van Dijk, P. J. 2001 How to avoid sex: the genetic control of gametophytic apomixis. *Plant Cell* **13**, 1491–1498.
- Haig, D. & Westoby, M. 1991 Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Phil. Trans. R. Soc. B* **333**, 1–13.

- Håkansson, A. 1956 Seed development of *Brassica oleracea* and *B. rapa* after certain reciprocal pollinations. *Hereditas* **42**, 373–396.
- Hecht, V., Vielle-Calzada, J.-P., Hartog, M. V., Schmidt, E. D. L., Boutilier, K., Grossniklaus, U. & de Vries, S. C. 2001 The Arabidopsis *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol.* **127**, 803–816.
- Koltunow, A. 1993 Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. *Plant Cell* **5**, 1425–1437.
- Kranz, E., Von Wiesen, P., Quader, H. & Lorz, H. 1998 Endosperm development after fusion of isolated, single maize sperm and central cells *in vivo*. *Plant Cell* **10**, 511–524.
- Lin, B.-Y. 1984 Ploidy barrier to endosperm production in maize. *Genetics* **107**, 103–115.
- Luo, M., Bilodeau, P., Koltunow, A., Dennis, E. S., Peacock, W. J. & Chaudhury, A. M. 1999 Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **96**, 296–301.
- Moore, T. & Haig, D. 1991 Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet.* **7**, 45–49.
- Morgan, R. N., Ozias-Akins, P. & Hanna, W. W. 1998 Seed set in an apomictic BC₃ pearl millet. *Int. J. Plant Sci.* **159**, 89–97.
- Nogler, G. A. 1984 Gametophytic apomixis. In *Embryology of angiosperms* (ed. B. M. Johri), pp. 475–518. Berlin: Springer.
- Ohad, N., Margossian, L., Hsu, Y. C., Williams, C., Repetti, P. & Fischer, R. L. 1996 A mutation that allows endosperm development without fertilization. *Proc. Natl Acad. Sci. USA* **93**, 5319–5324.
- Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J., Goldberg, R. B. & Fischer, R. L. 1999 Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell* **11**, 407–415.
- Pessino, S. C., Espinoza, F., Martinez, E. J., Ortiz, J. P. A., Valle, E. M. & Quarin, C. L. 2001 Isolation of cDNA clones differentially expressed in flowers of apomictic and sexual *Paspalum notatum*. *Hereditas* **134**, 35–42.
- Prackelt, U. & Scott, R. 2001 Induction of apomixis in sexual plants by mutagenesis. In *The flowering of apomixis: from mechanisms to genetic engineering* (ed. Y. Savidan, J. G. Carman & T. Dresselhaus), pp. 212–228. Mexico: CIMMYT/EU/IRD.
- Quarin, C. L. 1999 Effect of pollen source and pollen ploidy on endosperm formation and seed set in pseudogamous apomictic *Paspalum notatum*. *Sex. Plant Reprod.* **11**, 331–335.
- Reddy, P. S. & d'Cruz, R. 1969 Mechanism of apomixis in *Dichanthium annulatum* (Forsk.) Stapf. *Bot. Gaz.* **130**, 71–79.
- Richards, A. J. 1986 *Plant breeding systems*. London: Allen & Unwin.
- Rutishauser, A. 1954 Die Entwicklungserregung des Endosperms bei pseudogamen *Ranunculus*-arten. *Mitt. Naturforsch. Ges. Schaffhausen* **25**, 1–45.
- Savidan, Y. 2000 Apomixis: genetics and breeding. *Plant Breed. Rev.* **18**, 13–86.
- Savidan, Y. 2001 Transfer of apomixis through wide crosses. In *The flowering of apomixis: from mechanisms to genetic engineering* (ed. Y. Savidan, J. G. Carman & T. Dresselhaus), pp. 153–167. Mexico: CIMMYT/EU/IRD.
- Scott, R. J., Spielman, M., Bailey, J. & Dickinson, H. G. 1998 Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**, 3329–3341.
- Spielman, M., Preuss, D., Li, F.-L., Browne, W. E., Scott, R. J. & Dickinson, H. G. 1997 *TETRASPORE* is required for male meiotic cytokinesis in *Arabidopsis thaliana*. *Development* **124**, 2645–2657.
- Spielman, M., Vinkenoog, R., Dickinson, H. G. & Scott, R. J. 2001 The epigenetic basis of gender in flowering plants and mammals. *Trends Genet.* **17**, 705–711.
- Spillane, C., Vielle-Calzada, J.-P. & Grossniklaus, U. 2001 APO2001: a sexy apomixer in *Coma*. *Plant Cell* **13**, 1480–1491.
- Surani, M. A. 1998 Imprinting and the initiation of gene silencing in the germ line. *Cell* **93**, 309–312.
- Tilghman, S. M. 1999 The sins of the fathers and mothers: genomic imprinting in mammalian development. *Cell* **96**, 185–193.
- Vielle, J.-Ph., Burson, B. L., Bashaw, E. C. & Hussey, M. A. 1995 Early fertilization events in the sexual and aposporous egg apparatus of *Pennisetum ciliare* (L.) Link. *Plant J.* **8**, 309–316.
- Vinkenoog, R. & Scott, R. J. 2001 Autonomous endosperm development in flowering plants: how to overcome the imprinting problem? *Sex. Plant Reprod.* **14**, 189–194.
- Vinkenoog, R., Spielman, M., Adams, S., Fischer, R. L., Dickinson, H. G. & Scott, R. J. 2000 Hypomethylation promotes autonomous endosperm development and rescues post-fertilization lethality in *fie* mutants. *Plant Cell* **12**, 2271–2282.

GLOSSARY

- DAPI: 4,6-diamidino-2-phenylindole
 MET1 a/s: METHYLTRANSFERASE I antisense
 MMC: megaspore mother cell
 PE: peripheral endosperm