Chapter 1

A Comparison of In Vitro and In Vivo Asexual Embryogenesis

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Abstract

In plants, embryogenesis generally occurs through the sexual process of double fertilization, which involves a haploid sperm cell fusing with a haploid egg cell to ultimately give rise to a diploid embryo. Embryogenesis can also occur asexually in the absence of fertilization, both in vitro and in vivo. Somatic or gametic cells are able to differentiate into embryos in vitro following the application of plant growth regulators or stress treatments. Asexual embryogenesis also occurs naturally in some plant species in vivo, from either ovule cells as part of a process defined as apomixis, or from somatic leaf tissue in other species. In both in vitro and in vivo asexual embryogenesis, the embryo precursor cells must attain an embryogenic fate without the act of fertilization. This review compares the processes of in vitro and in vivo asexual embryogenesis including what is known regarding the genetic and epigenetic regulation of each process, and considers how the precursor cells are able to change fate and adopt an embryogenic pathway.

Key words Adventitious embryony, Apomixis, Cell fate, Gametic embryogenesis, *Kalanchoë*, Parthenogenesis, Somatic embryogenesis

1 Introduction

Embryogenesis describes the development of a single cell into an embryo. In plant embryogenesis there is no cell migration, so embryo pattern formation and cell type specification is interrelated with oriented cell division and expansion. Within sexual angiosperm plant species, embryogenesis usually occurs in vivo within floral organs during the events of seed formation. Formation of an embryo can also occur via asexual pathways in seeds, from somatic plant cells in vivo or be induced experimentally from somatic plant explants or gametes in vitro.

This review describes and compares the processes of in vivo and in vitro asexual embryogenesis including what is currently understood regarding the molecular mechanisms underlying each process.

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2 Types of Embryogenesis

2.1 Zygotic (Sexual) Embryogenesis

The most prevalent form of embryogenesis in plants occurs following double fertilization in the female gametophyte (embryo sac) found in the ovule of the flower, which gives rise to the embryo and endosperm compartments of the seed (Table 1; Fig. 1a). Haploid male and female gametes form in the anther and ovule, respectively, via meiosis and subsequent mitosis [1, 2]. Double fertilization initiates when the male pollen tube containing two sperm cells enters the ovule. One haploid sperm cell fuses with the meiotically derived haploid egg cell in the female gametophyte to form the single-celled diploid zygote, which then undergoes cell division and pattern forming events to give rise to the diploid embryo [3]. The other haploid sperm cell fuses with the diploid central cell nucleus of the embryo sac, which initiates divisions to form triploid endosperm that provides resources to the developing embryo [4]. Ovule tissues that surround the embryo and endosperm contribute to the seed coat.

Evolutionary speaking, embryogenesis is a much older process than seed formation and initially resulted from the fusion of two homospores into the zygote, gradually evolving in present day heterospory [5, 6]. The zygote formed following fusion of parental gametes is the first cell evident during sexual reproduction with a competence for embryogenesis. In plants, an "embryogenic" state is not only restricted to the zygote and in the following sections, ways of attaining an embryogenic state other than via fertilization will be discussed (Table 1).

2.2 Asexual Embryogenesis in Seeds: Apomixis and Parthenogenesis in Cereals

Apomixis is a term describing a suite of developmental processes resulting in the formation of an asexual seed. Characteristic features of all apomicts include fertilization-independent formation of an egg cell or another somatic ovule cell into an embryo, and the development of functional endosperm in apomicts occurs either with or without fertilization [7, 8]. As a result, plants germinating

Table 1

Characteristics of each type of embryogenesis considered in this review

Type of embryogenesis	Precursor cell	Mode of embryogenesis	Ploidy of embryo	Biological environment
Zygotic	Egg	Sexual	Diploid	In vivo
Parthenogenesis	Egg	Asexual	Diploid	In vivo
Adventitious embryony	Nucellar/integument	Asexual	Diploid	In vivo
Somatic embryogenesis	Somatic cells	Asexual	Diploid	In vitro/in vivo
Gametic embryogenesis	Egg/sperm	Asexual	Haploid	In vitro

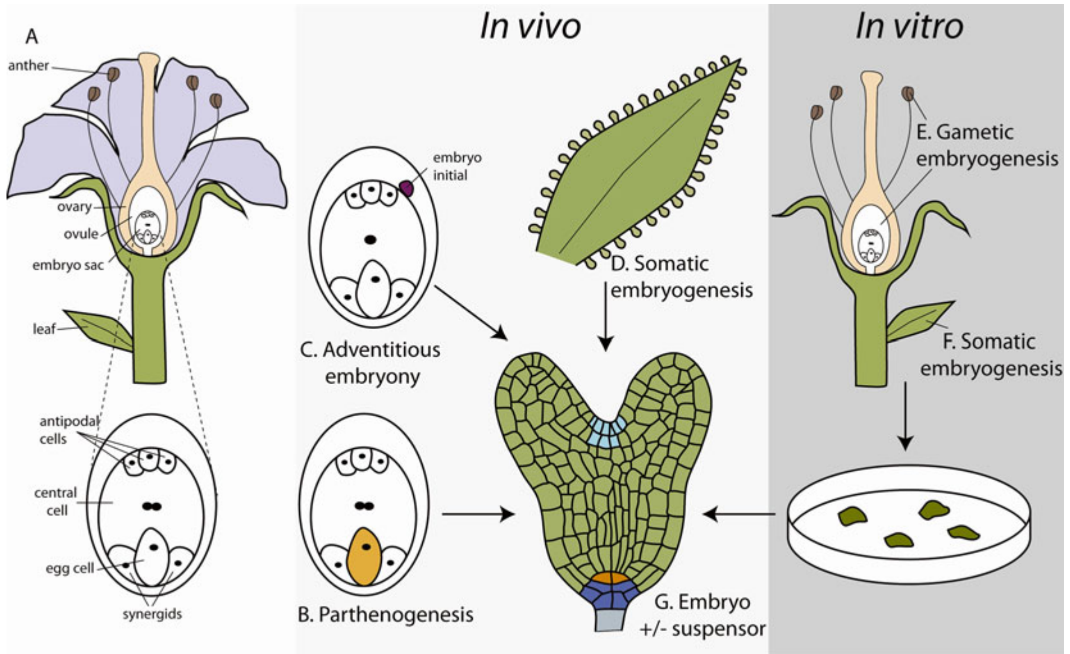

Fig. 1 Asexual embryogenesis occurs in vivo and in vitro from different cell types. (**a**) Floral organs and leaves are some of the source plant tissue for inducing embryogenesis in vitro. Asexual embryos also form in ovules in vivo; (**b**) Parthenogenesis involves the development of a chromosomally reduced or unreduced egg cell (*yellow*) into an embryo without fertilization; (**c**) Nucellar or integument cells (*red*) adjacent to an embryo sac within the ovule develop into embryos through adventitious embryony; (**d**) In vivo somatic embryogenesis is known to occur in species such as *Kalanchoë*, where the embryos develop along leaf margins; (**e**) Gametic embryogenesis involves the experimental induction of embryogenesis from gametic cells such as microspores and ovules; (**f**) Embryogenesis can be induced in somatic cells following experimental treatment; (**g**) Embryos formed via asexual embryogenesis may or may not possess a suspensor. At a heart-shaped stage, the typical plant embryo contains precursor cells for the shoot apical meristem (*blue cells*), and the root apical meristem which consists of a quiescent center (*orange cells*) and columella stem cells (*purple cells*)

from seeds derived via apomixis are genetically identical to the maternal parent.

Apomixis has evolved independently across different angiosperm plant families and genera many times, and has been documented in more than 120 angiosperm genera that belong to approximately 40 families [9]. Apomixis is genetically controlled by dominant loci in studied species and is not prevalent in agronomically important plants [10]. Apomixis mechanisms are generally divided into two categories: gametophytic or sporophytic, based upon the location of the precursor cell which develops into the embryo. In gametophytic apomixis, the embryo develops without fertilization (termed parthenogenesis) from an egg cell found inside an embryo sac that has formed mitotically without prior meiosis, and is thus chromosomally unreduced (Table 1; Fig. 1b).

Two common mechanisms termed diplospory and apospory give rise to such embryo sacs. They are distinguished by whether the starting cell is a megaspore mother cell or another somatic cell in the ovule, respectively (see Hand and Koltunow [7] for further information). Gametophytic apomixis and parthenogenesis are found and studied in species including eudicots *Taraxacum officinale* (dandelion), *Boechera* spp., and *Hieracium* spp. and also in grasses *Pennisetum squamulatum* and *Paspalum simplex* among others [11–14].

During sporophytic apomixis, which is also called adventitious or nucellar embryony, embryos develop without fertilization directly from diploid somatic ovule cells surrounding an embryo sac (Table 1; Fig. 1c). Most commonly, the embryos arise from two different ovule tissues: the nucellus and the inner integument. Nucellar embryony is widespread among *Citrus* species [15, 16]. The embryo initial cells that give rise to the asexual embryos differentiate near the developing embryo sac [17] and they can be specified as early as the 2–4 nuclear stage of embryo sac formation [18, 19]. The embryo initial cells develop and form globular-shaped embryos that can only develop to maturity if the sexually derived embryo sac is fertilized, as the sexual and asexual embryos share the nutritive endosperm. The developing seed therefore consists of one sexual embryo and one or more asexual embryos and is termed polyembryonic. The sexually derived embryo may not develop or survive germination [17].

Asexual embryogenesis is evident within seeds of the "Salmon" system of wheat. In contrast to gametophytic apomixis, a chromosomally reduced embryo sac develops via the usual events of meiosis, spore selection, and mitosis evident in sexually reproducing angiosperms. However, salmon wheat lines are capable of up to 90 % parthenogenesis, whereby the egg is able to initiate embryogenesis without fertilization [20, 21]. Parthenogenesis capability results from translocation of the short arm of wheat chromosome 1B with the short arm of chromosome 1R of rye. This particular translocation results in the loss of two critical loci in wheat: *Suppressor of parthenogenesis* (*Spg*) and *Restorer of fertility* (*Rfv1*), along with the gain of a *Parthenogenesis* (*Ptg*) locus from rye. In addition to this translocation, parthenogenesis is dependent upon organellar DNA from *Aegilops causdata* or *A. kotschyi*, demonstrating the importance of cytoplasmic as well as nuclear factors in asexual embryogenesis in vivo [21]. The existence of fertilization-independent embryo development from different cell types in the ovules of apomicts suggests that multiple cells can acquire an embryogenic state. This contrasts with sexual reproduction where the embryogenic state is suppressed until fertilization and restricted to the egg cell within the female gametophyte. In parthenogenetic cereals the embryogenic state is attained by the egg

in the absence of fertilization whilst embryogenic competency is suppressed in the remaining ovule cell types.

2.3 Somatic Embryogenesis In Vivo from Leaves

Somatic embryogenesis is known to occur in vivo in nature, where embryos develop on the surface of plant tissue (Fig. 1d) [22]. For example, plants of the genus *Kalanchoë* reproduce asexually through the ectopic formation of plantlets along their leaf margins [23]. The plantlets arise following proliferation of cells described as "dormant meristems" that are found in notches along the leaf margin [24, 25]. Some *Kalanchoë* species require stress to induce plantlet formation while others do not and constitutively form asexual plantlets. Because of this form of multiplication, *Kalanchoë* species are known as "mother of thousands." The embryo resulting from somatic embryogenesis is diploid and genetically identical to the somatic precursor cells from which it was formed.

2.4 In Vitro Somatic and Gametic Embryogenesis

It is possible to induce asexual embryogenesis in vitro from gametic cells including male microspores (termed androgenesis), and from egg cells or the associated accessory cells found in the female gametophytes (termed gynogenesis) (Table 1; Fig. 1e). This process requires gametophytic cells to switch to a sporophytic embryo formation pathway. Application of various stress treatments such as cold/heat shock and starvation are applied to the anther, isolated microspores, cultured ovules, ovaries, or flower buds to induce the switch [26–28]. The resulting embryos are haploid, possessing either maternal or paternal chromosomes depending on the gametophytic precursor cell. The production of haploid plants through in vitro gametic embryogenesis is a powerful mechanism to generate homozygous lines much faster than using conventional breeding. Colchicine induced chromosome doubling of haploid embryos during, or just after, embryogenesis results in homozygous doubled-haploid plants which are useful tools in trait discovery and plant breeding applications [29]. Currently, microspore embryogenesis is favored over gynogenesis as a mode of gametic embryogenesis because of its higher efficiency [30].

In vitro somatic embryogenesis can also be induced in vegetative explants or cells following treatment with plant growth regulators (PGR) or stresses such as osmotic shock, dehydration, water stress, and alteration of pH (reviewed in [31]) (Fig. 1f). A few studies have addressed correspondences and differences between zygotic and somatic embryogenesis and suggest that the patterning and specification events are quite similar [32], with the exception of a lack of the suspensor and dormancy in in vitro cultured somatic embryos [33]. Therefore, the most important step in vegetative cells that undergo somatic embryogenesis must be to first gain the "embryogenic" state. Recent work suggests that a release in suppression of the embryogenic state is a plausible mechanism [6, 34].

3 Attaining an Embryogenic State

A prerequisite for embryogenesis in plants is that the precursor cell must attain an embryogenic state which provides the cellular competence for embryo formation. During gametic embryogenesis, and gametophytic apomixis, the developing gametophyte cells respond to induction signals that switch their fate from gametophytic to sporophytic. During zygotic embryogenesis, the zygote has acquired embryogenic competency following fertilization of the egg cell. In somatic embryogenesis in vitro, and adventitious embryony, the embryo precursor cells are somatic sporophytic cells which first must attain the embryogenic state. Changing the developmental fate of a cell is therefore an important component of both in vitro and in vivo asexual embryogenesis.

It has been proposed that somatic embryogenesis consists of two distinct phases which are independent of each other and are controlled by different factors [35]. The initial stage is induction, which involves the somatic cells attaining the embryogenic state usually by the exogenous application of PGR. The following stage is expression, where the newly differentiated embryonic cells develop into an embryo without any further exogenous signals. It is not yet known whether in vivo embryogenesis via adventitious embryony similarly consists of two separate independent phases. However, such a scenario could be envisaged where the sporophytic ovule cells also first acquire embryonic competence by a particular molecular signal, and then develop into an embryo without fertilization via a separate developmental program.

In the process of in vitro somatic embryogenesis, somatic cells attain the embryogenic state following the application of PGR. Auxin is most commonly used [36], although other PGR, including cytokinin and abscisic acid, have proven capable of inducing embryogenesis [37, 38]. Following treatment with PGR, the cells are cultured on a hormone-free medium. Auxin plays major roles in plant growth and morphogenesis including embryo sac development and embryo patterning [39, 40]. In addition to treatment with auxin, the frequency of somatic embryogenesis induction also depends on the species, genotype, tissue, stage of development, and endogenous hormone levels [35, 41]. Therefore although auxin is a universal induction molecule, other factors must be involved in the induction of embryonic competence. The role of cellular stress responses in the induction of somatic embryogenesis is increasingly being recognized. The process of culturing explants for somatic embryogenesis involves wounding, sterilization, and culturing of the explant, all which undoubtedly apply stress to the cells involved. Furthermore, exogenous stresses such as osmotic, heavy metal ion, temperature, and dehydration stresses can enhance

somatic embryogenesis [42–46]. The induction of somatic embryogenesis through the application of auxin or stresses may imply an interaction between auxin and stress signaling. Auxin may therefore activate a stress signaling response, which is involved in inducing embryogenic competence. Many stress-related genes are up-regulated during the early phases of somatic embryogenesis, which supports this theory [47, 48].

Whether somatic cells in vitro and nucellar, integument cells and unreduced egg cells in apomicts in vivo acquire an embryogenic state via the same mechanism is currently unknown. Unlike somatic embryogenesis, embryos formed through parthenogenesis and adventitious embryony in apomicts are subject to the developmental influences of the ovule which may produce alternate cues that induce an embryogenic state. Stress and alterations in ovule pattern formation lead to a deregulation of apomixis in *Hieracium* where embryos form ectopically in different ovule positions [49]. Although no genes have yet been identified that are responsible for inducing adventitious embryony, genes related to stress signaling have been implied in the process of nucellar embryony in *Citrus*. Kumar et al. [50] used suppression subtractive hybridization (SSH) and microarray to detect genes that were differentially expressed during asexual embryo initiation and discovered genes related to stress signaling, including heat shock proteins.

Some similarities exist in the morphology of the embryo precursor cell for in vitro somatic embryogenesis and in vivo adventitious embryony. In *Citrus* species that undergo adventitious embryony, those nucellar cells that ultimately differentiate into embryos are distinguished from surrounding nucellar cells by their large nuclei and dense cytoplasm [51]. These nucellar initial cells also have very thick callosic cell walls and later become thinner walled, rounder, larger, and with a prominent nucleus prior to cell division [17]. Histological observations of embryonic somatic cells cultured in vitro from various species show that these embryonic cells are relatively small and also contain large nuclei and dense cytoplasm when compared to other somatic cells (reviewed in Namasivayam [52]). Large nuclei and dense cytoplasm are also characteristic of cells that are precursors of the female gametophyte, including the aposporous initial cell in aposporous apomictic plants, distinguishing them from surrounding somatic cells [1, 53].

4 Embryo Morphology

Zygotic embryogenesis within angiosperms passes through a series of sequential stages to give rise to the mature differentiated structure. In *Arabidopsis* and some other angiosperms, the first division

of the zygote produces an apical cell that continues to be embryogenic, while the second basal cell is no longer embryogenic and continues to form the multicelled suspensor. Further divisions of the apical cell produce a globular embryo, and differentiation and expansion of the cotyledons leads to heart and torpedo-shaped embryos [54]. Only the suspensor derived hypophyseal suspensor cell continues to form the quiescent center and the columella stem cells of the root meristem (Fig. 1g) [55]. Variation in early cell division patterning exists between different dicotyledonous species, although the typical globular, heart, and torpedo morphological stages still usually occur [54]. Zygotic embryogenesis in monocotyledonous species differs from dicots mostly with respect to planes of symmetry and the position of the shoot apical meristem [56]. Variation in embryo formation also exists between monocot species. The embryo is the only plant structure in which both the root and shoot apical meristem is formed simultaneously. This requires a highly complex series of pattern forming and specification events, including establishment of small populations of stem cells. These cells continue to support the formation and activity of meristems during the remainder of the plant life cycle (for a recent review see [57]). Extensive studies have revealed molecular details of the formation of the major tissue types as well as the meristems themselves during embryogenesis [6].

The processes of asexual embryogenesis, both in vivo and in vitro, often differ from the regular divisions and patterning events that define zygotic embryogenesis. Embryo pattern formation during apomictic embryogenesis (parthenogenesis) can be irregular compared to zygotic embryogenesis in related sexual species. In aposporous *Hieracium*, for example, embryogenesis frequently commences earlier than in sexual plants as once the egg differentiates, it transits rapidly to embryogenesis, and in some cases altered division planes can result in a different embryo appearance. Multiple embryos can also form in aposporous *Hieracium* embryos in either the same or a secondary embryo sac [58]. Although most *Hieracium* parthenogenetic embryos resemble those formed by zygotic embryogenesis in sexual plants, embryos with one or three cotyledons have also been observed. Despite developmental alterations in the primary pattern of embryos formed in aposporous *Hieracium* species, the resulting germinated seedlings eventually exhibit normal plant growth when grown on hormone free media in vitro [58].

In vivo asexual embryogenesis in *Kalanchoë* species proceeds through the typical globular, heart and torpedo stages from meristematic cells along leaf margins [24]. However unlike zygotic embryos, *Kalanchoë* asexual plantlets resemble shoots that then grow adventitious roots from a hypocotyl structure [23]. Once the root system has developed, *Kalanchoë* plantlets detach from the mother plant, fall to the ground and become new plants.

In vitro embryogenesis could also be described as heterogeneous, as multiple developmental pathways are possible which occur at varying frequencies within a single species and even the same culture [33, 59, 60]. Detailed characterization of in vitro embryogenesis pathways has been performed using time-lapse tracking from embryonic cell suspensions [33, 61]. Early development of most microspore derived embryos involves a globular embryo with little cellular organization that undergoes symmetrical division and does not resemble a typical zygotic embryo [54]. Other microspore-derived embryos appear to form via a developmental pathway that involves asymmetric division and consequently more closely resemble zygotic embryos. Recently, microspore embryogenesis systems have been developed that consistently produce such embryos [59, 62]. These systems involve a heat stress period that is either shorter or at a much lower temperature than is usually applied.

Early during zygotic embryogenesis, a region of the embryo differentiates to become a suspensor that functions to connect the embryo to surrounding tissues, thereby positioning the embryo inside the seed [63]. The suspensor also acts to transport nutrients and hormones to the embryo. When microspore embryogenesis more closely mimics zygotic embryogenesis, a recognizable suspensor is always present, which suggests the suspensor plays a role in supporting early patterning events [59, 62, 64]. A suspensor is also formed during in vivo asexual embryogenesis, although throughout *Citrus* nucellar embryony, the suspensor becomes evident at a much later stage of development than in zygotic embryos [15]. In aposporous *Hieracium*, embryos that develop in the micropylar end of the embryo sac always form a suspensor and embryos that develop within secondary chalazal embryo sacs may or may not form a suspensor and often arrest at the globular stage [65]. The development of suspensors in asexual embryogenesis suggests that fertilization is not required for formation of the suspensor.

Unlike asexual embryos formed in apomictic seeds which undergo desiccation and dormancy as part of seed maturation, embryos formed in vitro and in vivo in *Kalanchoë* develop directly into seedlings. Despite not developing within a seed, in vitro somatic embryos also undergo some form of maturation and accumulate late embryogenesis abundant (LEA) proteins, although sometimes treatment with ABA is first required to induce maturation [66]. In vitro somatic embryos also accumulate seed storage proteins, which are recognized as important for the future development of in vitro somatic embryos into plants. Only those embryos that have accumulated enough storage proteins and have acquired desiccation tolerance will develop into normal plants [60]. A comparison between asexual in vivo and somatic in vitro embryogenesis processes was performed by measuring the

accumulation of citrin seed storage proteins in polyembryonic seeds and in vitro cultured embryos in *Citrus*. This study revealed that in vitro embryos accumulate fewer citrins and at a later developmental stage than within the polyembryonic seed, suggesting that despite not being derived from fertilization events, the nucellar embryos are influenced by the seed environment [19].

Formation of endosperm is a crucial component of seed development which does not accompany in vitro embryogenesis. The precursor of the endosperm is the large diploid central cell of the embryo sac. During sexual seed formation, the endosperm will only develop following double fertilization, when one of the two sperm cells fuses with the two central cell nuclei to produce triploid endosperm. Formation of viable seed via apomixis also requires the formation of endosperm. The majority of apomictic species studied require fertilization to develop endosperm, a process which is termed pseudogamy. In some apomictic species, typically members of the Asteraceae, endosperm can develop without fertilization of the central cell. Maternal (m) and paternal (p) genome ratios in the endosperm are typically 2m:1p in sexual species and disturbance in this ratio may lead to seed abortion. Apomicts tend to tolerate variation in endosperm ploidy and maternal and paternal genome ratios which are not easily tolerated in sexually reproducing plants, and have developed various strategies to ensure seed viability [67].

Apomictic *Hieracium* species are able to form endosperm without fertilization. The polar nuclei fuse prior to the development of nuclear and then cellular endosperm, in the absence of fertilization and the resulting endosperm exhibits a 4m:0p genome ratio in aposporous *Hieracium*. The trait of autonomous endosperm (AutE) has recently been separated from fertilization-independent embryogenesis in *Hieracium* through two inter-specific crosses [68]. Two individuals were identified that form reduced embryo sacs containing meiotically derived eggs and central cells through the sexual pathway. However, egg cells within these individuals are unable to commence embryogenesis without fertilization although in the absence of fertilization, the fused polar nuclei undergo proliferation and continue to develop cellular endosperm with a 2m:0p genome ratio. This indicates a paternal genome contribution is neither required for endosperm initiation, nor cellularization in both chromosomally reduced and unreduced embryo sacs. When egg cells from these individuals are fertilized, embryogenesis occurs to completion and viable seed is formed. It is currently unclear if the central cell is also able to be fertilized as this would result in a parental genome ratio of 2m:1p ratio as seen in sexual species [68].

5 Genes Implicated in In Vivo and In Vitro Asexual Embryogenesis

Similarities between asexual embryogenesis in vitro and in vivo raise questions regarding whether these processes are controlled by the same molecular mechanisms. Although no genes responsible for embryogenesis have yet been isolated from apomictic plants, a number of gene candidates have been identified through differential gene expression analysis, genetic mapping and study of sexual mutants with phenotypes that mimic asexual embryogenesis. Attempts to understand in vitro somatic and gametic embryogenesis have also resulted in a range of gene candidates that when expressed ectopically, result in embryo formation.

One of the first genes associated with somatic embryogenesis was *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) when its involvement was demonstrated in carrot cell cultures [69]. *SERK* was identified as a marker for cells transitioning from a somatic to an embryogenic state, due to its transient expression in established suspension cell cultures [69]. *SERK* is a leucine-rich repeat (LRR) receptor-like kinase that is also expressed in developing ovules and embryos *in planta* and may therefore influence somatic embryogenesis through the same mechanisms of the sexual pathway [69, 70]. Overexpression and downregulation of *SERK* increases and decreases the efficiency of somatic embryogenesis, respectively [70, 71]. Interestingly, a *SERK* gene has also been implicated in asexual reproduction within an apomictic grass, *Poa pratensis.* cDNA-AFLPs differentially expressed between apomictic and sexual lines of *P. pratensis* revealed a *SERK* gene that displays differential expression [72]. Apomixis in *P. pratensis* involves development of an embryo sac not from the megaspore mother cell (MMC) which is the typical precursor cell for the sexual pathway, but from a diploid somatic cell positioned nearby the MMC. These somatic precursor cells are found in the nucellar ovule tissue. Within *P. pratensis*, *SERK* is expressed in embryo sac precursor cells: the MMC in sexual plants, and somatic nucellar cells in apomictic plants [72]. The same expression profile was also observed in apomictic and sexual lines of *Paspalum notatum* [73]. *SERK* expression was also examined in apomictic *Hieracium* where it was detected throughout the ovule, and expression was not restricted to the nucellar region or MMC in *Hieracium*. *SERK* expression was also observed in developing *Hieracium* embryos [74]. *SERK* is therefore thought to play an important role in changing developmental fate of cells, both in stages of apomixis and in somatic embryogenesis. *BABYBOOM* (*BBM*) is another gene that has been associated with both in vitro and in vivo asexual embryogenesis. *BBM* is an APETELA2 (AP2) transcription factor that was originally identified following subtractive hybridization of cDNA from *Brassica napus* microspores undergoing

embryogenesis [75]. Ectopic expression of *BBM* in *Arabidopsis* or *B. napus* induces somatic embryos, and constitutive expression of *BBM* genes from other species also results in the emergence of ectopic embryos [75–77]. *BBM* expression was also observed in developing *Arabidopsis* zygotic embryos [75]. These results suggest that *BBM* has a conserved role in the induction and/or maintenance of embryo development. *BBM* genes have also been identified within a genomic region essential for apomixis in the apomictic grass *Pennisetum squamulatum* [78]. The apospory-specific genomic region (ASGR) of *Pennisetum* was identified following marker analysis of a selection of apomictic and sexual plants, which revealed a set of apomixis-specific markers that define the ASGR [79]. Sequencing of BAC clones from within the ASGR revealed putative protein coding regions, including two of which had similarity to *BBM* of rice [78]. The ASGR is thought to contain genetic elements responsible for both the formation of a diploid embryo sac, and the process of parthenogenesis. The *BBM* genes within the ASGR are therefore candidate apomixis genes with strong potential to have a role in the induction or maintenance of asexual embryogenesis in *Pennisetum* apomicts. However, confirmation of a role for *BBM* in parthenogenesis has not yet been reported.

The involvement of common genes in zygotic and asexual embryogenesis implies that despite arising from different activation signals and different tissues, each embryogenesis process converges on a similar developmental pathway. Genes with a known involvement in zygotic embryogenesis have therefore been studied in asexual embryogenesis systems to understand whether such genes are also involved in asexual embryogenesis. The *LEAFY COTYLEDON* (*LEC*) family of transcription factors is crucial for regular embryogenesis and is also implicated in somatic embryogenesis. *Arabidopsis* contains three *LEC* genes: *LEC1*, *LEC2,* and *FUSCA3* (*FUS3*), and each of these genes is expressed exclusively in the embryo [80–82]. Ectopic expression of each of the three *LEC* genes leads to vegetative cells adopting characteristics of maturation-phase embryos, and hence this gene family is associated with the process of somatic embryogenesis [80–82]. The *LEC* genes have been linked to auxin production, as *LEC2* is known to activate the auxin biosynthesis genes *YUCCA2* and *YUCCA4* [83]. *FUS3* expression also increases in response to auxin [84]. This interaction with auxin signaling is thought to be responsible for the ability of *LEC* gene expression to induce embryonic competence.

LEC1 has been studied in *Kalanchoë* species and is implicated in the process of asexual plantlet formation in these species. Compared to *Arabidopsis*, the *LEC1* gene of *Kalanchoë daigremontiana* (*KdLEC1*) is truncated and does not rescue the *Arabidopsis lec1* mutation, suggesting it functions differently to

LEC1 in *Arabidopsis* [23]. A functional full length copy of *LEC1* was created by replacing the deleted nucleotides in *KdLEC1* with the corresponding nucleotides from *Arabidopsis* and transformation of *Kalanchoë daigremontiana* with this synthesized *LEC1-LIKE* gene results in disrupted asexual reproduction and in some instances abortion or absence of plantlet formation [85]. This study strongly supports the involvement of *LEC1* in in vivo asexual embryogenesis in *Kalanchoë* and furthermore suggests that the switch from sexual to asexual propagation in the evolution of *Kalanchoë* was probably activated following truncation of the *KdLEC1* gene [85].

Another gene that appears to be involved in the induction of an embryogenic state is the RWP-RK domain containing (RKD) transcription factor RKD2, which is preferentially expressed in the egg cell of *Arabidopsis* and wheat [86]. Ectopic expression of *RKD2* results in ovule integument cells that become enlarged and densely cytoplasmic with prominent nuclei, suggesting these cells have become pluripotent [87]. Ectopic *RKD2* expression also results in some integument cells adopting an egg cell identity, and a low frequency (ca. 0.1 %) of embryo-like structures also appear outside of the embryo sac [87]. This observation is reminiscent of adventitious embryony and may indicate that RKD2 is involved in the induction of embryogenesis from ovule tissue during adventitious embryony.

Additional genes including *WUSCHEL* and *AGAMOUS-Like 15* (*AGL15*) are known to induce embryo formation from vegetative tissue when ectopically expressed, and have therefore been implicated in somatic embryogenesis [88, 89]. *WUSCHEL* is known to be involved in specifying and maintaining stem cells in the shoot and root meristem [90] while AGL15 is known to accumulate in developing embryos [91], therefore a role in embryogenesis is to be expected for both of these genes. However, with the exception of *SERK*, most of the genes shown to be involved in zygotic and asexual embryogenesis are not specifically expressed in the egg cell or the zygote. Therefore, whilst important for later stages of embryo development, these genes may not be involved in the process of embryo initiation which is possibly the most important aspect of asexual embryogenesis. It has been proposed that the observed ectopic embryo development associated with misexpression of these genes, is a result of cellular stress, rather than a specific initiation signal expressed by the genes [92]. This hypothesis is consistent with embryonic competence being induced by stress factors, as discussed earlier.

To understand the genetic elements responsible for inducing embryonic competence in both in vitro and in vivo asexual embryogenesis, future experiments will likely focus on comparison of gene expression from embryo precursor cells directly before and after the initiation of embryogenesis. Genetic mapping of apomixis loci

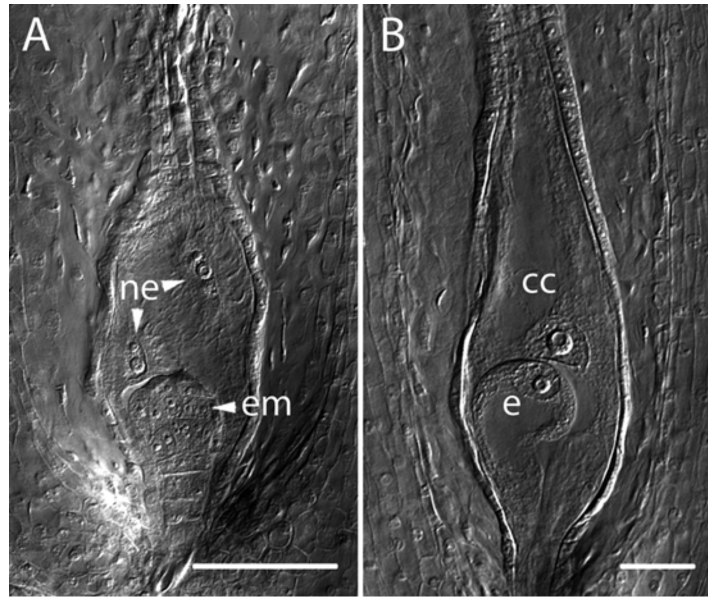

Fig. 2 Cleared ovules from wildtype apomict *Hieracium praealtum* (**a**), and a *H. praealtum lop* mutant (**b**) that has lost the capacity to undergo parthenogenesis and autonomous endosperm development. Within apomictic *H. praealtum* ovules, embryo and endosperm develop from the egg and central cell, respectively, without fertilization (**a**). *H. praealtum lop* deletion mutant m179 (**b**) has lost this capacity and the egg and central cells do not develop further without fertilization. Scale bars = 50 μm. *em* embryo, *e* egg, *cc* central cell, *ne* nuclear endosperm. Ovules were collected at stages 6 (**a**) and 10 (**b**) of capitulum development according to Koltunow et al. [65]

may also reveal which genes are responsible for the initiation of asexual embryogenesis. Genetic analyses of apomicts have shown that gametophytic apomixis is inherited as a dominant trait. In many apomictic species, developmental components of apomixis (meiotic avoidance and parthenogenesis) are controlled by independent loci and further research is underway to isolate the causal sequences that underlie these loci. For example, characterized deletion mutants developed in apomictic *Hieracium praealtum* revealed a genomic region responsible for fertilization-independent embryogenesis and endosperm formation, named *LOSS OF PARTHENOGENESIS* (*LOP*) [93]. Deletion of *LOP* sees the plant become dependent upon fertilization for both embryo and endosperm development (Fig. 2) [13]. Genetic mapping of *LOP* and *AutE* is the focus of current work that may lead to isolation of the causal sequences for both traits.

A genomic locus strongly associated with adventitious embryony in *Citrus* has also been identified [94]. Further characterization of this locus may clarify the mechanism of adventitious embryony and identify the genetic element responsible for

inducing asexual embryogenesis *in planta*. Similarly, the search for genes within the controlling parthenogenesis loci of the salmon wheat system may reveal those genes that are responsible for parthenogenesis in this system. Although controlling genes are currently unknown for the salmon wheat system, it is likely that they have lost those genes required for repressing fertilization independent embryogenesis in sexual plant species.

6 Epigenetic Influence on Asexual Embryogenesis

Various epigenetic marks and pathways have been associated with both sexual and asexual embryogenesis processes, suggesting that the induction and regulation of asexual embryogenesis may involve epigenetic components. For instance, the application of exogenous auxin during somatic embryo induction results in DNA hypermethylation [95], and inhibition of DNA methylation suppresses the formation of embryogenic cells from cultured carrot epidermal cells [96]. Auxin could therefore possibly reprogram gene expression through DNA methylation, leading to the induction of embryogenesis pathways within somatic cells.

Genes within epigenetic pathways have also been implicated in both in vitro and in vivo asexual embryogenesis. One such epigenetic factor implicated in asexual embryogenesis is PICKLE (PKL), a chromatin remodeling protein [97]. *PKL* is responsible for repressing the *LEC* family of transcription factors, as *pkl* mutants display overexpression of *LEC1*, *LEC2* and *FUS3,* and display a phenotype similar to that seen from *LEC1* overexpression [97, 98]. *PKL* activity is therefore acknowledged as an important regulatory mechanism for repressing embryonic identity throughout seedling growth, by suppressing the embryogenic program in somatic cells [99]. For this reason, *PKL* is also a candidate for the induction of asexual embryogenesis. Deregulation of *PKL* in somatic cells or within the egg cell would permit expression of embryogenic genes that are generally only expressed by the developing embryo following fertilization. However to date, *PKL* has not been specifically associated with asexual embryogenesis in any natural apomictic plant.

Strong evidence exists suggesting that epigenetic pathways play a crucial role in asexual embryo and endosperm development during apomixis. Mutants of the Polycomb-Group (PcG) chromatin modeling complex show phenotypes reminiscent of fertilization independent embryogenesis and endosperm formation seen in gametophytic apomixis. In particular, the Polycomb Repressive Complex 2 (PRC2) is known to be involved in the suppression of seed development in the absence of fertilization. The PRC2 is conserved between plants and animals and represses gene expression via trimethylation of histone H3 at lysine 27 (H3K27me3).

Phenotypes of asexual embryo and endosperm development have been observed when core PRC2 genes are mutated in *Arabidopsis*. For instance, the fertilization-independent seed (FIS) PRC2 complex (FIS-PRC2) consists of the genes *MEDEA* (*MEA*), *FIS2*, *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*). Loss of function of any of these genes results in endosperm initiation and proliferation without fertilization. However, the endosperm does not cellularize [100–102]. The role of the FIS-PRC2 complex is therefore considered to inhibit central cell proliferation. In the case of *MSI1* mutants, low levels of parthenogenetic embryo initiation are observed, followed by embryo arrest, so that viable seeds are not formed [103]. The role of some of the FIS-PRC2 genes has been investigated during seed initiation in *Hieracium* spp., one of the few groups of apomicts that develop endosperm without fertilization. Downregulation of *Hieracium* FIE (HFIE), a protein linking multiple PRC2 components inhibiting fertilization-independent endosperm proliferation in *Arabidopsis* does not result in fertilization-independent endosperm proliferation in sexual plants. *HFIE* function is required for completion of both sexual and asexual embryo and endosperm development in examined *Hieracium* species [104]. These results demonstrate that the capacity for embryogenic competence and endosperm formation in apomicts may function via deregulation of other PRC2 complex family members and that additional factors are required to produce viable asexual embryos and endosperm. The identified *Hieracium* AutE plants that form endosperm, but not embryos, without fertilization may help identify and define the roles of genes that regulate the autonomous endosperm mechanism.

The PRC2 complex interacts with other genes implicated in asexual embryogenesis, including the *LEC* gene family. *LEC1*, *LEC2,* and *FUS3* are all overexpressed in *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*) double mutants, which are PcG gene homologues of the PRC2 gene *MEA* [105]. A *cis* regulatory element has been identified within the *LEC2* promoter which is responsible for recruiting the PRC2 complex [106]. These results suggest that the PcG acts to repress embryonic gene expression by histone methylation. Histone acetylation is another epigenetic mark that in contrast to histone methylation, is generally associated with transcriptional activation. Removal of the acetylation is performed by histone deacetylase (HDAC), which consequently results in transcriptional repression. Interestingly, two histone deacetylase genes (*HDA6* and *HDA19*) are partly responsible for repressing the embryonic program during *Arabidopsis* germination [107]. Another HDAC gene (*HDA7*) in *Arabidopsis* is known to be important for normal embryo development [108]. Inefficient or defective histone deacetylation of key embryonic genes may therefore be a candidate mechanism for inducing asexual embryogenesis.

7 Conclusions

While the pathways involved in developing the embryo itself appear common between the various modes of embryogenesis described here, many differences exist between the initiation processes of asexual embryogenesis in vitro and in vivo. Unlike somatic or gametic embryogenesis, apomixis-associated embryogenesis occurs near maternal reproductive tissue, and develops within a seed structure. Despite these differences, in vitro and in vivo asexual embryogenesis share some common factors: a change in the developmental fate of embryogenic precursor cells; and expression of an embryonic pathway in such cells without fertilization. Identifying molecular mechanisms that underlie these processes within in vitro systems may help to understand pathways that lead to apomixis. While some candidate genes for both in vitro and in vivo asexual embryogenesis have been identified, a role in apomixis has not yet been confirmed for any of these genes. One possibility is that embryogenesis related genes are deregulated by epigenetic factors during asexual embryogenesis. Continued research into asexual embryogenesis will yield important findings related to plant cell fate specification and the molecular regulation of embryogenesis.

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