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Chapter 4

FERTILIZATION AND FRUIT INITIATION

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Abstract: Angiosperms have evolved the unique processes of double fertilization and fruit development as key steps of their survival and dispersal strategies. In this chapter, we will examine fertilization and fruit initiation as central restriction points to fruit and seed development. Pollination and fertilization appear essential for fruit initiation, since angiosperm flowers universally enter terminal senescence and abscission phases if pollination is prevented. We review key developmental processes, pathways and genes that were recruited to control and restrict the reproductive growth of the carpel and ovule until fertilization is achieved. Ever since the discovery that exogenous application of phytohormones results in the development of seedless fruit without fertilization (termed parthenocarpy), most research has concentrated on the role of endogenous phytohormones as triggers for fruit initiation after fertilization. We will highlight how uncoupling of fruit initiation from fertilization through mutational studies can further contribute to the understanding of these complex processes. Initial analysis shows that strict local control of auxin signalling, through a transcription factor network, forms one of the decisive and primary events that leads to the hierarchical control over gibberellin metabolism and perception. Testing and challenging these assumptions will provide further knowledge indispensable for controlling fruit set and yield in agriculture.

Keywords: double fertilization; ovule; female receptivity; fruit initiation; vascular development; phytohormonal signalling cascades

4.1 Introduction

From a purely biological standpoint, the success of higher plants hinges on reproduction strategies and the dissemination of viable progeny into

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habitable environmental niches. Fertilization and fruit initiation are essential processes in angiosperm plant reproduction and play a part in maximizing the success and variation. This is evident since the conquest and rise of the angiosperm plant lineage in the dominance of land environments can be directly attributed to the developmental innovations of the flower, ovule, carpel and fruit as vital components of propagation and dispersion. Selection pressure has lead to large variation in fruit development; however, the control on fruit initiation appears to be a universally conserved mechanism amongst all angiosperms. Without pollination and successful fertilization, the ovary and sometimes the accessory tissues, cease to grow and the flower begins a terminal phase of senescence that ends in floral abscission. Alternatively, when pollination and fertilization take place, a cascade of events is triggered, leading to growth and development of the fruit and seed.

Speculation about the evolutionary origin of the fruit has largely been focused on the role of the carpel in initiating and providing protection around the developing ovules and seeds. The development of the carpel and integuments presumably also played a role together with the stigma and style in the selective discrimination of male gametes during pollination and, thus, constitutes an important mechanism in the control of outbreeding (Mulcahy, 1977, 1979). The development of the fleshy fruit most likely co-evolved with extinct megafauna and avifauna where the function was to enable vectorial dispersion. The whole seed would be swallowed at the end of fruit development, and subsequent excretion would ensure a fertile environment for seeds to germinate and colonize. Various mechanisms arose to strictly manage and restrain carbon partitioning to optimal levels in developing flowers and fruits in order to match fitness with success of the zygote in the environment.

From an economical standpoint, fruit initiation and fruit set are essential processes in many horticultural and agricultural cropping systems. Shortly after fruit initiation commences, large diversions in plant resources often occur and the fruit actively recruits photoassimilates and nutrients into the reproductive tissues. Plant breeders, both past and present, have sought to maintain and stabilize high yields and prevent premature fruit drop, while on the other hand, they concentrated selection on plant varieties which maximize their resource allocation into fruits and seeds to provide larger and more various fruit forms (Paran and Van der Knaap, 2007). The economic relevance of pollination and fertilization is clear if we consider the economic costs associated with their potential loss. In 1998, reduced pollination of crops and harvest loss, examined in a combination of 30 crops, comprised US $54.6 billion, a total loss of 46% in harvest yield (Kenmore and Krell, 1998). Broader estimates in 2005, placed the worldwide economic value of pollination alone at €153 billion, although no loss in harvest yield was calculated (Gallai *et al.*, 2008). More recent studies did not support a hypothesis that pollinator decline has yet affected crop yield at a global scale (Ghazoul, 2005; Aizen

et al., 2008); it has been proposed that the increase in pollinator-dependent crops may result in important ecological and economic consequences if pollinator decline is to further continue (Aizen *et al.*, 2008). On the other hand, fruit initiation and fruit development are also strong determinants of plant weed invasiveness which has detrimental effects on cropping systems and the natural environment. In Australia, the economic cost of plant weed invasiveness, facilitated by fruit development and seed dispersal, was estimated at an annual cost of AUD $ 4.039 billion (Sinden *et al.*, 2004; Keller *et al.*, 2007). Thus, it is easy to envisage how mechanisms of altering fruit initiation and retention may offer a way to stabilize and increase yields in crops or, alternatively, become an Achilles' heel for the manipulation and control of invasive plant species.

Several different pillars of research characterize the published knowledge about fruit initiation and how it relates to fertilization. Early research focused on induced parthenocarpy (Noll, 1902), whereby fruit was artificially stimulated independent of fertilization by application of plant-growth regulators (PGRs; Gustafson, 1936) or by various pollination treatments that restricted fertilization or compatibility (Noll, 1902; Yasuda, 1930, 1935). As success with artificial growth regulators gained momentum (Gustafson, 1939a,1939b, 1942; Nitsch, 1952), another area of research was directed at quantifying and localizing phytohormones in specific tissues of the fruit based on the hypothesis that fruit development was initiated and sustained by the developing seeds (Talon *et al.*, 1990a, 1992; Kim *et al.*, 1992; van Huizen *et al.*, 1995; Fos *et al.*, 2000, 2001). Both areas of research now cover a vast number of agricultural and horticultural crops (Schwabe and Mills, 1981), but often the relationship remained obscure between phytohormonal activity and fruit initiation. In contrast, heritable parthenocarpy that occurs naturally or through induced genetic lesions has provided valuable breakthroughs in crops and several genetic loci involved in fruit initiation are now known (Lin, 1984; Rotino *et al.*, 1997; Vivian-Smith *et al.*, 2001; Yao *et al.*, 2001; Bassel *et al.*, 2008; Marti *et al.*, 2008). Recent advances in understanding fruit initiation and the intrinsic linkage to fertilization are now being completed by genetic analysis and transcriptome profiling.

In this chapter, we examine the role of female receptivity in fruit set and the key pathways and genes that control fruit initiation together with their complex relationship with fertilization, and with flower maturation. We present data that reinforce the idea that fruit initiation occurs in a very short period of time, characterized by hours and minutes, and not necessarily days. The contribution of various phytohormones such as auxin and gibberellins is also examined, as is the molecular genetic study of parthenocarpy as a tool to interrogate the early and immediate steps in fruit initiation. Through the course of understanding the molecular basis of fruit initiation, the evolution of the angiosperm fruit structure is also addressed, since extensive conservation of candidate regulatory genes exists.

4.2 Pollination

4.2.1 Pollen-stigma recognition and interaction

The landing of pollen on a compatible stigma marks the beginning of pollination. The pollen will then adhere, hydrate and germinate to produce the pollen tube, a structure specialized in the delivery of the sperm cells to the ovule (Fig. 4.1).

Stigmas can be divided into two broad types, wet and dry, depending on the amount of exudate present (Heslop-Harrison and Shivanna, 1977). It has been suggested that in plants with dry stigmas (e.g. crucifers), the pollen coat plays a more active role in the adhesion and hydration of the pollen grain (Heslop-Harrison and Shivanna, 1977; Heslop-Harrison, 1992). The role of the pollen coat in hydration has been further elucidated thanks to the *grp17-1 Arabidopsis* mutant, where the loss of a single oleosin protein from the coat resulted in a significant delay in pollen hydration (Mayfield and Preuss, 2000). Pollen coat substances are also involved in early pollen-stigma recognition events such as the Brassicaceae-type self-incompatibility system (for more detailed reviews on this subject see Lord and Russell, 2002; Takayama and Isogai, 2005). In the stigma surface, aquaporin-like proteins have been suggested to play a major role in the control of pollen hydration (Tyerman *et al.*, 2002) as well as in pollen acceptance (Lord and Russell, 2002). Additionally, the female determinants of various self-incompatibility systems have also been identified on the stigma surface (Takayama and Isogai, 2005).

Pollen-stigma recognition is an active process which subsequently leads to pollen-tube germination (Fig. 4.1). Nevertheless, there is no evidence suggesting that recognition alone is sufficient to trigger fruit initiation (Zhang and O'Neill, 1993). The time period between pollen landing and pollen-tube germination varies greatly among plants. In *Phalaenopsis* orchids, pollen germinates 4 days after landing (Duncan and Curtis, 1942) providing a unique system for the study of the effect of pollen landing on fruit initiation. Orchids are also unusual among flowering plants in that the ovary and ovules of many orchid species mature after pollination (Withner, 1974). Zhang and O'Neill (1993) showed that physical contact of pollen alone is sufficient to trigger ovary maturation in *Phalaenopsis* orchids. Nevertheless, this interaction failed to induce fruit initiation (Zhang and O'Neill, 1993).

4.2.2 Pollen germination and pollen-tube growth

After pollen hydration, germination occurs which results in the emergence of the pollen tube (Fig. 4.1). The pollen tube is formed by a generative cell which contains the two sperm cells and the vegetative nucleus. Both pollen germination and pollen-tube growth are subjected to gibberellic acid (GA)-mediated control. It has recently been shown in rice that de novo synthesis of

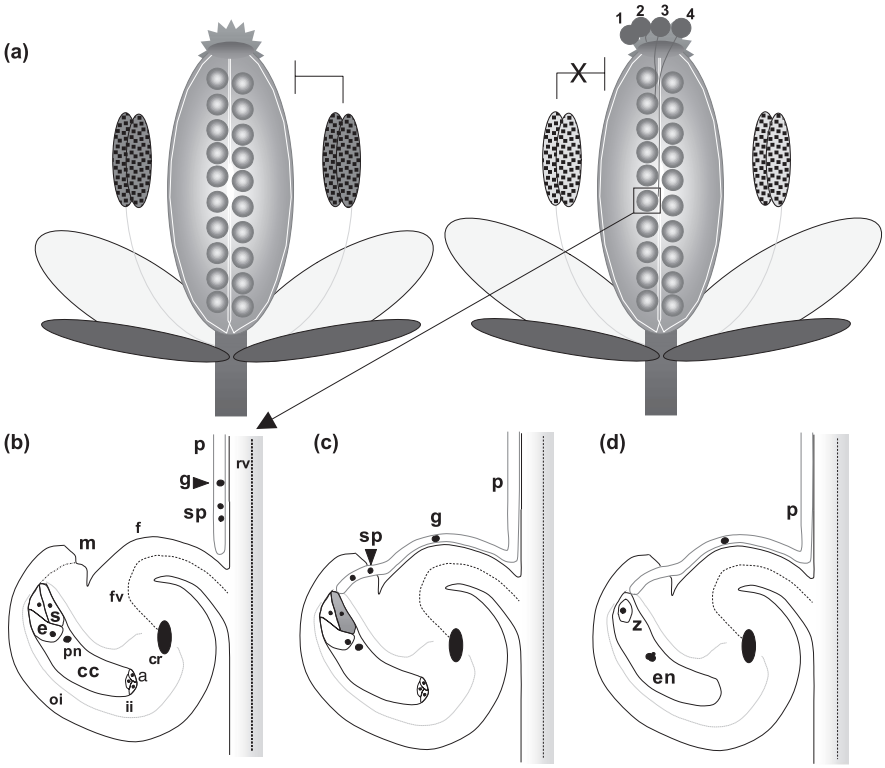


Figure 4.1 Pollination and fertilization in *Arabidopsis* and Brassicaceae. (a) Flower at anthesis stage (left) and during pollination (right) showing several rows of ovules within the pistil. During pollination, the stages of adhesion and hydration (1), recognition and pollen tube emergence (2), tube growth (3) and guidance (4) are shown (right). When anthers dehisce, the inhibitory stimulus for fruit development is removed (see the cross symbol). (b) A pollen tube containing two sperm cells is guided to the ovule micropyle by signals emanating from a fertile female gametophyte and the surrounding sporophytic tissue. (c) The tube tip enters the micropyle of the ovule and unites with a synergid cell that degenerates upon fusion. Two sperm cells migrate to combine with the egg cell and polar nuclei of the central cell, respectively. (d) Synergid cells degenerate and the diploid zygote and triploid endosperm begin development. The outer and inner integuments undergo cell expansion and division to form the seed testa. a, antipodal cells; cc, central cell; cr, chalazal region; e, egg cell; en, endosperm; f, funiculus; fv, funiculus vascular tissue; g, generative cell; ii, inner integument; m, micropyle; p, pollen tube; pn, polar nucleus; oi, outer integument; rv, replum vascular tissues; s, synergid cell; sp, sperm cells; z, zygote.

GA in the pollen grains is required for pollen germination (Chhun *et al.*, 2007). Similarly, gibberellins are also necessary for pollen-tube growth across different plant species (Singh *et al.*, 2002; Cox and Swain, 2006; Chhun *et al.*, 2007). The pollen tube grows by tip growth and periodic callose deposition (for a detailed review on this subject see Krichevsky *et al.*, 2007). Directional tube

growth towards the egg cell is guided by several mutual signalling processes in the pistil, style, funiculus, ovules and female gametophyte (Fig. 4.1).

Several studies have focused on the role played by the pollen tube in fruit initiation. It was first observed by Hildebrand (1865) that in certain orchids, where self-incompatible pollinations arose, pollen tubes grew only a small amount but this appeared to stimulate the development of parthenocarpic fruit. Fruit initiation has also been related to the degree of penetration into the style by the pollen tube. It was concluded that to stimulate fruit initiation in cucumbers and eggplants, the base of the style had to be reached by the pollen tube (Yasuda, 1936). These early observations led to the hypothesis that pollen-tube growth resulted in the transfer of growth-promoting substances from the pollen to the ovary and 'stimulated' the growth of the fruit (Gustafson, 1939b). Later measurements of growth-promoting hormones (namely auxin) in pollen grains and pollen tubes ruled out these as the possible sources of growth hormones. However, it was suggested that pollen tubes may secrete an enzyme responsible for the activation of auxin precursors in the style and ovary (van Overbeek *et al.*, 1941; Muir, 1942). Recently, Schijlen *et al.* (2007) provided further evidence of the possible contribution of pollen-tube growth on fruit initiation. In this case, the downregulation of the flavonoid biosynthesis pathway genes, *CHALCONE SYNTHASE 1* and *2* (*CHS1/CHS2*), by RNA interference led to parthenocarpic tomato fruit development (Schijlen *et al.*, 2007). Downregulation of the flavonoid pathway arrested pollen-tube development in self-pollinated gynoecia. Although the pollen tubes failed to reach and fertilize ovules, their initial growth appeared to be sufficient in triggering fruit set and produce stimulatory parthenocarpy (Schijlen *et al.*, 2007). The role of the flavonoid pathway in the gynoecium and the stimulatory effect of pollen-tube growth in tomato fruit initiation require further examination since several interactions between polar auxin transport and flavonoids have been identified. Most notably the loss of flavonoid biosynthesis in *Arabidopsis* led to increased polar auxin transport (PAT; Murphy *et al.*, 2000; Brown *et al.*, 2001; Lazar and Goodman, 2006; Santelia *et al.*, 2008), leading to the hypothesis that PAT could stimulate fruit initiation. Roles for PAT in fruit initiation are considered in sections below.

The use of ionizing radiation has also provided data about the role of the pollen tube in fruit initiation. Fruit initiation can occur even when pollen samples are treated with high doses of ionizing radiation (Denissen and Den Nijs, 1987; Knox *et al.*, 1987; Sniezko and Visser, 1987; Polito, 1999; Peixe *et al.*, 2000). This technique, commonly known as 'prickle pollination', stimulates parthenocarpic fruit growth and has been documented in various crops including *Cacao*, *Cotton*, *Pistacio*, *Capsicum* and other Solanaceaous species. High-irradiation treatments do not always impair pollen-tube growth but prevent fertilization or steps immediately after fertilization (Speranza *et al.*, 1982; Denissen and Den Nijs, 1987; Peixe *et al.*, 2000). Although the degree and precise stage of impairment requires clarification, arrest is characteristically earlier and different from the late post-zygotic arrest observed in

stenospermocarpic seedless grapes (Cain *et al.*, 1983; Emershad and Ramming, 1984; Hanania *et al.*, 2007) or in *Capsicum* fruit development triggered by interspecific crosses (Tong and Bosland, 2003). The results from prickle pollination suggest that pollen-tube growth and/or processes immediately after fertilization but prior to zygotic development are able to trigger fruit initiation.

Pollen-tube guidance towards the ovules depends on a complex signalling network which involves signals from the female gametophyte and components of the style and ovary (Higashiyama *et al.*, 1998; Higashiyama, 2002; Dresselhaus, 2006; Palanivelu and Preuss, 2006; Escobar-Restrepo *et al.*, 2007; Rotman *et al.*, 2008). Disruption to this complex signalling network can lead to the disorientation of pollen tubes and, thus, prevent fertilization. For example, specific fruit initiation mutants can affect pollen-tube polarity and reduce seed set. This is the case in the parthenocarpic *pat-2* tomato mutant where the distorted pollen-tube growth has been related to the high proportion of defective ovules present in the pistil (Mazzucato *et al.*, 2003) and associated with increased levels of endogenous gibberellins (Olimpieri *et al.*, 2007). Altered seed set patterns as a result of defective pollen-tube growth are also found in *Arabidopsis* plants overexpressing the *GA2ox2* gene (Singh *et al.*, 2002; Cox and Swain, 2006). In the light of previous observations, it is tempting to suggest that the impaired pollen-tube growth could also play a part in enhancing parthenocarpic fruit development in the *pat-2* background. On the other hand, even though stimulatory parthenocarpy has been observed in a few crops, it has not been detected yet in *Arabidopsis* mutants where pollen or ovules have subtle defects disrupting fertilization (Vivian-Smith, 2001; Vivian-Smith *et al.*, unpublished data).

4.3 Female receptivity and the cessation of gynoecial growth

Often in horticulture, two or more varieties are planted together in a specific planting or orchard design to maximize cross-pollination for hybrid production or yield. The female receptive period is an important final component of the floral maturation process and has a direct bearing on fruit set and initiation, since viable female and male components must both exist in space and time, while the plant sufficiently conserves essential resources. The receptive period has also been referred to as the effective pollination period (EPP) and is the mutual or partial sum of the longevity for the stigma, style and ovule, while taking into account the time taken for the pollen tube to grow and fertilize the ovule (Williams, 1966; reviewed Sanzol and Herrero, 2001; Page *et al.*, 2006). During the maturation and receptive periods, specific molecular pathways restrict the growth of the pistil and accessory tissues and, thus, stop them from developing into fruit.

Despite the wealth of data in crop species and extensive analysis of female gametophyte development, female receptivity has not been the subject of any significant genetic analysis. Female receptivity can be quantified by emasculating flowers before anthesis and allowing a sample of flowers to be pollinated on successive days post-anthesis (Williams, 1966). Final seed set reflects the period when pistils were most receptive to pollination. As a rule, pollen-tube growth on floral tissues is temperature dependent and different results would be expected if pistils were incubated at sub-optimal temperatures for stigmatic receptivity, pollen-tube development or ovule longevity (Sanzol and Herrero, 2001). Often the optimum for pollen-tube elongation and the optimum for support on the female component do not coincide, and the optima are usually higher for pollen-tube growth (Hedhly *et al.*, 2005a,b).

In *Arabidopsis*, female receptivity, as assessed by seed set, lasts up to 3 days post-anthesis and effectively correlates with the integrity of the female gametophyte which deteriorates shortly thereafter (Christensen *et al.*, 1998; Vivian-Smith and Koltunow, 1999; Vivian-Smith and Offringa, unpublished). There are marked differences in female receptivity duration between the ecotypes Landsberg and Columbia (Vivian-Smith and Koltunow, 1999). Nevertheless, the receptivity periods in *Arabidopsis* ovules are significantly shorter than the period the pistil remains receptive to exposures of 10 nmol GA_3 that stimulate fruit development (Vivian-Smith and Koltunow, 1999). A longer period of gibberellin perception suggests that the viability of the gametophyte and ovule is completely independent to the perception and signalling of a GA_3-mediated growth in the pistil and that the gibberellin-mediated restriction maybe directly occurring in the carpel.

Mutations in the *Auxin Response Factor 8* gene (*ARF8*), which lead to parthenocarpic fruit initiation, dramatically shorten the duration of female receptivity and lead to reduced seed set (Vivian-Smith *et al.*, 2001). *ARF8* mutants also initiate fruit development precociously and the pistil protrudes far enough to prevent proper contact between the stigma and anthers to effect proper self-pollination (Vivian-Smith *et al.*, 2001). Taken alone, however, the *arf8* mutant data may suggest an indirect link with female receptivity. On the contrary, mutations in *ARF8* together with the related gene *ARF6* lead to complete sterility and dramatically prevent flower maturation in numerous aspects (Nagpal *et al.*, 2005; Wu *et al.*, 2006). This data suggest a global role for both genes in flower maturation, female receptivity and pollen-tube growth.

Distinct genetic pathways halt further development of the egg cell and the central cell at maturity and this has been demonstrated with the use of *Arabidopsis* gametophytic and sporophytic mutants (see sections below). Evidence that the female gametophyte reciprocally exerts control over the developing sporophyte comes from transcriptional profiling studies where mutants lack a viable gametophyte (Johnston *et al.*, 2007). Significant modulation of the sporophytic genes has been observed for *SUPERMAN* (SUP), *Small Auxin Upregulated RNA* (*SAUR*), *C3HC4-type RING finger proteins*, the homeobox gene *SHOOT MERISTEMLESS* (*STM*) and the *STYLISH2* (*STY2*)

transcription factor. However, these genes are just a few examples amongst 527 genes identified (Johnston *et al.*, 2007). Many of these genes could be candidates linking female receptivity, the female gametophyte and the regulation of fruit initiation.

Other studies have also implicated the phytohormone cytokinin in gametophyte development and maintenance of receptivity. Pischke *et al.* (2002) and Hejatko *et al.* (2003) demonstrated that *CKI* is expressed in the female gametophyte until fertilization and it is essential for gametophyte viability. Previous research showed that overexpression of *CKI* results in cytokinin independency in somatic tissues (Kakimoto, 1996; Glover *et al.*, 2008). If *CKI* functions in a similar manner in the female gametophyte, it may play a significant role in maintenance of gametophyte viability via a cytokinin-related pathway. Female receptivity can also be positively influenced by the application of nitrogen fertilizer (Williams, 1965; Tromp *et al.*, 1994), and by stigmatic secretions induced by pollination, that can help release carbohydrates from the transmitting tissue and prolong embryo sac viability (Herrero, 1992).

While the beginning of female receptivity is demarcated by the period when pollen tubes can grow on the stigma, style and transmitting tissue (Kandasamy *et al.*, 1994), the end of female receptivity is onset by an irreversible initiation of floral senescence (O'Neill, 1997; O'Neill and Nadeau, 1997; Lewis *et al.*, 2006).

4.4 Additional restraints on flower development and fruit initiation

Prior to pollination, the floral whorls surrounding the pistil may play a role in repressing or slowing ovary growth (Vivian-Smith, 2001; Vivian-Smith *et al.*, 2001; Fig. 4.1). Accordingly, the specific removal of stamens in *Arabidopsis thaliana* has been shown to promote pistil growth slightly in wild-type plants, but moreover, the effect is significantly pronounced in genetic backgrounds that display parthenocarpy (*fwf/arf8*) or fertilization-independent seed development (*fis2-2*; Vivian-Smith, 2001). Combinations of these mutants with the conditional male sterile *pop1/cer6-1* mutant do not alleviate the retardation in silique growth and emasculation of *pop1/cer6-1* flowers is still required to achieve full comparative silique elongation (Vivian-Smith, 2001; Vivian-smith *et al.*, 2001). From these experiments, stamens and pollen have been pinpointed as being fully responsible for the retardation in fruit initiation (Fig. 4.1; Vivian-Smith and Offringa, unpublished). The basis of both the *FWF/ARF8* and anther dehiscence pathways is to ensure that wild-type plants are successfully synchronized in dehiscence, self-pollination and fruit initiation, but taken separately, the anther acts independently to prevent precocious pistil growth.

The restraint on ovary growth during female receptivity could be mediated in part by anthers producing high concentrations of free auxins (Aloni *et al.*, 2006), since auxin flow in anther filaments and high auxin levels in the tapetal tissues are critical for pollen development (Feng *et al.*, 2006; Cecchetti *et al.*, 2008). High auxin levels appear to be mediated in part by the auxin biosynthesis genes *YUCCA6* and *YUCCA2* (Feng *et al.*, 2006; Cecchetti *et al.*, 2008) which are regulated by the *SPOROCYTLESS/NOZZLE* gene (*SPL/NZZ*) that controls gametophyte development (Li *et al.*, 2008). Before the female receptive period, a strong auxin maximum is formed in stamens as judged by the auxin transcriptional response reporter, *DR5::GUS* (Aloni *et al.*, 2006; Feng *et al.*, 2006; Cecchetti *et al.*, 2008; Li *et al.*, 2008). Prior to wild-type pollen dehiscence, the auxin maximum declines and is absent upon dehiscence, providing a natural mechanism to decrease the growth of the anther filaments through reduced PAT (Aloni *et al.*, 2006; Cecchetti *et al.*, 2008). The strongest evidence for pathways facilitating the restraint of *Arabidopsis* fruit growth from the anther comes from double mutant analysis where several genes have been isolated (Vivian-Smith and Offringa, unpublished). Serendipitously, one was found during the map-based cloning of the *fwf-1/arf8-4* mutant (Vivian-Smith, 2001; Vivian-Smith *et al.*, 2001). Mutations in the *aberrant testa shape-1* (*ats-1*) mutant, also known as *kanadi4-1* (*kan4-1*; McAbee *et al.*, 2006), were observed to enhance silique development in the *pop1/cer6-1 ats-1/kan4-1 fwf-1/arf8-4* background independently of anther emasculation (Vivian-Smith, 2001; Vivian-Smith *et al.*, 2001). Defects in *ATS/KAN4* cause incomplete separation and growth of the ovule integuments. *ats-1/kan4-1* mutant ovules consist of three cell layers that have a shared unitegmic identity, as opposed to two outer and three inner integuments in wild type (Léon-Kloosterziel *et al.*, 1994; McAbee *et al.*, 2006). Importantly, total mesocarp cell counts from fully developed siliques of *pop1/cer6-1 ats-1/kan4-1 fwf-1/arf8-4* and wild-type-pollinated siliques were the same (Vivian-Smith *et al.*, 2001) suggesting that together *ATS/KAN4* and *FWF/ARF8* control a large portion of the fruit initiation pathway. The identity or reduced integumentary cell layers in *ats-1/kan4-1* appear to disrupt a key parallel signalling pathway that does not alone trigger fruit initiation but does link signalling with the restriction of fruit growth facilitated by the anther (Vivian-Smith *et al.*, 2001).

The study of MADS box gene mutants has further contributed to the understanding of the restraint imposed by the other floral whorls in ovary growth. For example, loss of function mutation in the *MdPI* (apple *PISTILLATA* homologue) causes parthenocarpic fruit development in apple (Yao *et al.*, 2001) which could also be attributed in part to the disappearance of the restraint imposed by the third whorl organs (namely stamens) and to the replacement of ovule identity. Similarly, the parthenocarpic fruit development observed in tomato transgenic plants with low expression levels of *TM29* (tomato *SEPALLATA* homologue) could also be linked to the disruption of petal and stamen identity (Ampomah-Dwamena *et al.*, 2002). However, this phenotype can also be attributed to the altered expression levels of *TM29* in the ovaries

(Ampomah-Dwamena *et al.*, 2002) and, thus, the correlation between outer floral whorls disruption and the observed ovary growth remains to be further clarified in these transgenic plants. In *Arabidopsis*, carpelloid identity replaces ovule primordia in alleles of the *bel1-1* mutants (Reiser *et al.*, 1995; Western and Haughn, 1999; Brambilla *et al.*, 2007) and in the *knuckles* mutant (Payne *et al.*, 2004). Both mutants develop fruit independent of fertilization but the length is completely correlated with the total number of carpelloid ovule structures produced in the carpel (Vivian-Smith, 2001). Similar occurrences are observed in *Capsicum* and tomato species (Gray-Mitsumune *et al.*, 2006; Tiwari *et al.*, 2006).

4.5 Fertilization

In the vast majority of angiosperms, the mature female gametophyte consists of a seven-cell, eight-nucleate 'Polygonum-type' structure, bounded by a membrane that lacks a plant cell wall (Fig. 4.1; Christensen *et al.*, 1998; Yadegari and Drews, 2004). This type of gametophyte has two synergid cells and an egg cell located at the micropylar pole, thus comprising the three-celled egg apparatus (Fig. 4.1, note that one synergid is hidden behind the other). Three antipodal cells are positioned at the chalazal pole of the ovule. Two nuclei of the central cell form the polar nuclei that locate adjacent to the egg cell (Fig. 4.1b; Yadegari and Drews, 2004).

Considerable variation exists on the general architecture of the female gametophyte, however, the basal angiosperm *Amborella* has a similar structure to higher angiosperms and consists of an eight-celled, nine-nucleate female gametophyte (Friedman, 2006). In *Amborella,* an egg cell is derived from a division of one of the three synergid cells to form a four-celled egg apparatus, unlike the *Arabidopsis* female gametophyte where the egg cell is specified from a designated nucleus and remains in association with the two synergids. Other basal angiosperms frequently contain a four-celled 'Nuphar/Schisandra-type' gametophyte that contains an egg cell, two synergids and a uninucleate central cell at maturity (Friedman and Williams, 2003; Williams and Friedman, 2002; Friedman, 2008). However, the majority of higher angiosperms presents a seven-cell polygonum-type gametophyte and, thus, this type of gametophyte is used as a reference point for the remainder of this review. Further information on gametophyte development is extensively covered elsewhere (Drews and Yadegari, 2002; Punwani and Drews, 2008).

4.5.1 Signal transduction before fertilization

The delivery of two sperm cells to the mature female gametophyte by the pollen tube relies on a robust mutual communication (Fig. 4.1; Hülskamp *et al.*, 1995). Palanivelu *et al.* (2003) showed that pollen-tube growth in stigma,

style and ovule is guided by gradients in γ-aminobutryic acid (GABA). Pollen tubes utilize a GABA-transaminase, encoded by the *POP2* gene, to provide pollen directionality by degrading the GABA stimulant. Guidance towards the female gametophyte occurs since the GABA gradient peaks at the micropylar integument cells. Degradation of GABA is probably involved in ensuring that only a single tube enters the micropyle (Fig. 4.1). As such, GABA maybe a contact-mediated guidance mechanism (Palanivelu and Preuss, 2006). Palanivelu and Preuss (2006) also defined two other processes regulating pollen-tube guidance. These include diffusible ovule-derived attractants from unfertilized ovules and repellents from fertilized ovules. The male gametophytic *tepitzin1* mutant indicates a requirement for the auxin-inducible homeobox gene *WOX5* for *Arabidopsis* pollen-tube growth (Gonzali *et al.*, 2005; Dorantes-Acosta and Vielle-Calzada, 2006). Auxin, together with calcium produced in synergids, had long been hypothesized as pollen-tube chemotropic attractants (Van Went and Willemse, 1984; Chaubal and Reger, 1990; Raghavan, 2003).

The synergids and the central cell also play a role in the guidance of the pollen tube prior to fertilization (Higashiyama *et al.*, 2001; Kasahara *et al.*, 2005; Chen *et al.*, 2007; Rotman *et al.*, 2008). In contrast to the GABA gradient, these appear to be short-range recognition and developmentally regulated (Palanivelu and Preuss, 2006). For instance, the plasma membrane-associated GEX3 protein is expressed in the female gametophyte and required for micropylar pollen-tube guidance (Alandete-Saez *et al.*, 2008). Boisson-Dernier *et al.* (2008) also show that the *AMC* gene, that encodes peroxisomal protein, functions at short range in both female and male gametophytes through potential diffusible signals. Mutations in the *MYB98* gene specifically prevent proper differentiation of the synergid cells which fail to differentiate the structural filiform apparatus that facilitates the reception of pollen tubes (Kasahara *et al.*, 2005). As a consequence, most *myb98* ovules fail to attract pollen tubes, suggesting that *MYB98* plays a role in the transcriptional activation of the network of genes involved in signalling or in the structural differentiation required for signalling (Kasahara *et al.*, 2005). Transcriptional profiling has validated transcriptional networks regulated by *MYB98* and identified small secreted peptides/proteins as *MYB98* targets (Jones-Rhoades *et al.*, 2007; Punwani *et al.*, 2007). Our reanalysis of supplementary data from *myb98* (Jones-Rhoades *et al.*, 2007) and female gametophyte transcriptional profiles (Yu *et al.*, 2005) shows that the cytochrome P450 *CYP78A9* gene is upregulated in *myb98* female gametophytes. This is of potential interest since overexpression of *CYP78A9* with the 35S promoter provides strong sterility and fruit initiation (Ito and Meyerowitz, 2000). However, the fact that viable *myb98* homozygotes are generated indicates double fertilization per se is not defective (Kasahara *et al.*, 2005).

In the context of pollen-tube signalling and fruit initiation, *Arabidopsis* contrasts with reports of stimulatory parthenocarpy in horticultural crops.

From *Arabidopsis* research alone, one could be convinced that pollen-tube development does not produce a stimulus for fruit initiation. Further analysis of the many *Arabidopsis* mutants that avoid fertilization (Pagnussat *et al.*, 2005) should illuminate this aspect.

4.5.2 Double fertilization

Double fertilization, first described by Guignard in 1899, involves one sperm cell uniting with the egg cell, while the second sperm cell fuses with the central cell and undergoes karyogamy with the polar nuclei (Fig. 4.1; Weterings and Russell, 2004; Yadegari and Drews, 2004). It has been extensively reviewed (Raghavan, 2003; 2006; Weterings and Russell, 2004; Dresselhaus, 2006) and recently the technical limitations of live visualization have been resolved by confocal laser scanning microscopy (CLSM; Ingouff *et al.*, 2007; Fig. 4.2; Vivian-Smith and Offringa, unpublished data). Double fertilization begins upon fusion of the tube tip with a synergid cell (Fig. 4.1c; Higashiyama *et al.*, 2000; Weterings and Russell, 2004; Sandaklie-Nikolova *et al.*, 2007). At this point, the pollen tube stops growing and discharges the two sperm cells. In some species, synergid degeneration occurs well before pollen tube arrival (Raghavan, 2003) while in *Arabidopsis* it has been reported that synergid cell death occurs upon pollen tube contact (Sandaklie-Nikolova *et al.*, 2007). Bidirectional communication can however increase cell permeability well before contact, since preferential propidium iodide (PI) staining occurs in the selected synergid before the pollen tube has arrived (Vivian-Smith and Offringa, unpublished data). This is also the case in ~15% of ovules from the *pop1/cer6-1* male sterile mutant, suggesting that a long-range pollen-synergid signalling triggers events prior to synergid cell death and double fertilization.

Upon discharge of the sperm cells into the degenerating synergid, migration of sperm cells to the egg and central cells occurs (Faure *et al.*, 2002; Weterings and Russell, 2004; Ingouff *et al.*, 2007). Movement towards their respective nuclei is facilitated by remnant F-actin coronas and microtubles (Ye *et al.*, 2002; Raghavan, 2003). Following double fertilization, development of a diploid zygote and triploid endosperm is initiated (Fig. 4.1; Faure *et al.*, 2002). The remaining synergid eventually deteriorates (Kasahara *et al.*, 2005) and the integuments expand and divide to accommodate the developing embryo and endosperm. In *Arabidopsis* and other angiosperms, the integuments differentiate post-fertilization to form the seed coat or testa that protects the seed and facilitates the transfer of nutrients and photoassimilates to the seeds (Fig. 4.1; Bowman, 1993; Wittich, 1998). Many steps are required to enable gamete fusion and karyogamy (Jensen, 1964; Faure *et al.*, 2002), but in *Arabidopsis* this occurs within 2–3 h after the arrival of the pollen tube (Berger *et al.*, 2008; Fig. 4.1c).

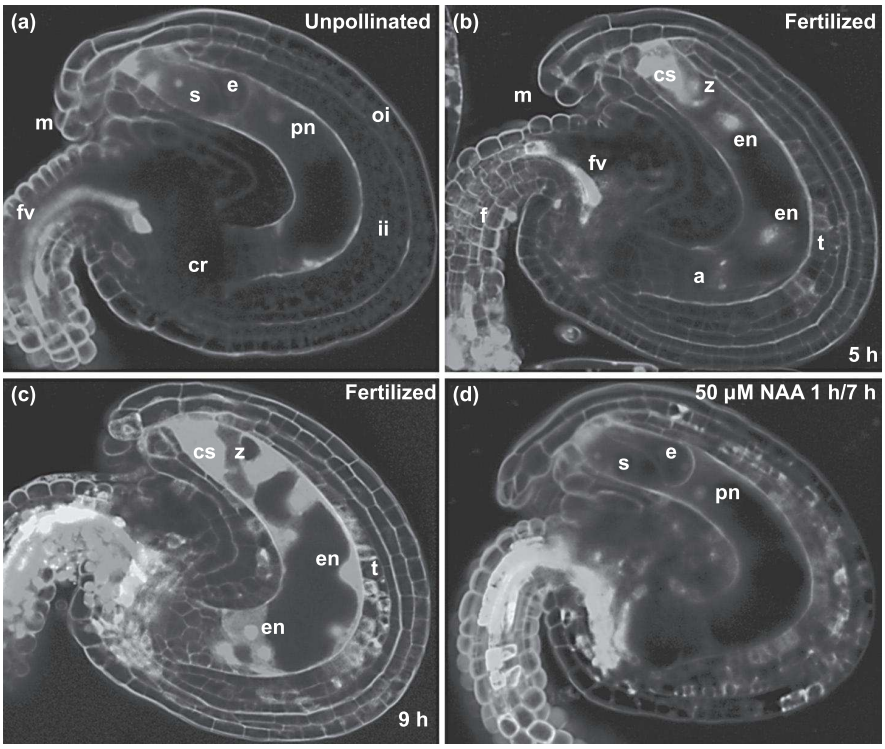


Figure 4.2 Confocal laser scanning microscopy (CSLM) images of unfertilized and fertilized ovules of *Arabidopsis* expressing the synthetic auxin-responsive reporter gene *DR5rev::eGFP*. (a) Unfertilized anthesis ovule with minimal GFP expression. (b) Post-fertilized ovule at 5 h. The first nuclear endosperm division has occurred and GFP expression is observed in the endothelium, the chalazal domain and adjacent to the funiculus vascular strand. (c) Ovule after the third endosperm division (9 h post-fertilization) with eight endosperm nuclei and an elongated zygote. Strong GFP expression occurs in the endothelium, the chalaza and funiculus. Weaker expression is observed in the outer integument. (d) Treatment of detached pistils with NAA (50 µM) for 1 h, with subsequent washing for 7 h, induces strong GFP activation in the funiculus and chalaza, and moderate activation in the inner integument and weaker expression in the outer integument. a, antipodal cells; cc, central cell; cr, chalazal region; e, egg cell; en, endosperm; f, funiculus; fv, funiculus vascular tissue; g, generative cell; ii, inner integument; m, micropyle; p, pollen tube; pn, polar nucleus; oi, outer integument; rv, replum vascular tissues; s, synergid cell; sp, sperm cells; t, endothelium; z, zygote. (For a colour version of this figure, please see Plate 2 of the colour plate section.)

4.5.3 Signal transduction during fertilization

After compatible fertilization, rapid changes in membrane-bound calcium occur and the female gametophyte changes cellular polarity, forming the zygote and endosperm (Russell, 1993). A complex signalling network is involved in the coordination of double fertilization (for detailed reviews see Dumas and

Gaude, 2006; Berger *et al.*, 2008). An early signalling event is the generation of a calcium influx following the fusion of the sperm and egg cell (Antoine *et al.*, 2000). This Ca^{2+} influx will subsequently spread throughout the egg cell and it is believed to contribute, at least partially, to the transient elevation of cytosolic Ca^{2+} observed shortly after (Antoine *et al.*, 2001). The release of high concentrations of Ca^{2+} during the synergid cell degeneration (Chaubal and Reger, 1990, 1992a,b) is also likely to contribute to this process (Digonnet *et al.*, 1997). The increase in cytosolic Ca^{2+} concentration has been proposed to trigger egg cell activation (Digonnet *et al.*, 1997; Antoine *et al.*, 2000). Kranz (1999) also reported that isolated gametes that were fused using in vitro fertilization techniques required pulses of auxin (25–40 mg L–1 2,4-D) to initiate cell division in the newly formed zygote.

Characterization of the *Arabidopsis aca9* mutant has further contributed to the understanding of the role played by calcium signalling during fertilization. *ACA9* encodes a Ca^{2+} pump that is primarily expressed in pollen (Schiott *et al.*, 2004). Mutant *aca9* pollens not only display reduced pollen-tube growth but are also defective in sperm cell release (Schiott *et al.*, 2004). Disruption in sperm cell release was also previously reported in the *sirene* (Rotman *et al.*, 2003) and *feronia* (Huck *et al.*, 2003) female gametophyte mutants. In both *siréne/feronia* and *aca9* mutants, pollen tubes fail to initiate the release of sperm cells into the synergid and, consequently, pollen tubes continue to grow inside the female gametophyte (Huck *et al.*, 2003; Rotman *et al.*, 2003; Schiott *et al.*, 2004; Escobar-Restrepo *et al.*, 2007). However, while *sirene/feronia* are female gametophyte mutants (see section below), *aca9* mutation affects the male gametophyte (Huck *et al.*, 2003; Rotman *et al.*, 2003; Schiott *et al.*, 2004). More recently, a similar phenotype to that observed in *siréne/feronia* and *aca9* mutants was also described in the *Arabidopsis amc* mutant (Boisson-Dernier *et al.*, 2008). In this mutant, sperm cell release is only impaired when an *amc* pollen tube reaches an *amc* female gametophyte, resulting in the pollen-tube outgrowth previously described (Boisson-Dernier *et al.*, 2008). AMC functions as a peroxin in reproductive tissues and it has been postulated that mutations in this gene may result in the loss of a molecule originating from the peroxisome required for female and male gametophyte communication (Boisson-Dernier *et al.*, 2008).

Undoubtedly, coordination of an intrinsically complex and costly process such as double fertilization must rely on a robust regulatory network, and miscommunication during female gametophyte development can lead to fertilization-independent fruit initiation.

4.5.3.1 Roles of the egg cell and central cell in fruit initiation

The role of the endosperm and egg cell in the control of seed development has been extensively reviewed (Pien and Grossniklaus, 2007) and several mutants have been isolated that uncouple fruit initiation from fertilization. Autonomous endosperm development in the absence of fertilization was first observed in the *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*)

mutant (Ohad *et al.*, 1996) and in the *FERTILIZATION-INDEPENDENT SEED* (*FIS*) mutant class (Chaudhury *et al.*, 1997). *FIE*, *FIS2* and *MEDEA/FIS1* (*MEA*; Grossniklaus *et al.*, 1998) are all part of the *FIS* mutant class (Ohad *et al.*, 1996; Grossniklaus *et al.*, 1998; Luo *et al.*, 1999) and derived from a genetic screen that searched for silique elongation in a male sterile background (Chaudhury and Peacock, 1994). The screen additionally produced the *fwf* mutant (Vivian-Smith *et al.*, 2001). The *fis* class mutants encode members of the Polycomb group (PcG) proteins and together with *MSI1* and *RBR1* form various multiprotein complexes involved in transcriptional regulation through chromatin remodelling (Spillane *et al.*, 2000; Sørensen *et al.*, 2001; Kohler *et al.*, 2003; Guitton *et al.*, 2004; Ingouff *et al.*, 2005; Jullien *et al.*, 2008). Mutations in *FIS1/MEA*, *FIS2*, *FIE*, *MSI1* or *RBR1* in the female gametophyte result in fertilization-independent endosperm development which triggers fertilization-independent silique elongation (Ohad *et al.*, 1996; Grossniklaus *et al.*, 1998; Luo *et al.*, 1999; Kohler *et al.*, 2003; Jullien *et al.*, 2008). Additionally in *fis* and *msi* mutants, autonomous endosperm development can also give rise in some cases to seed-like structures containing aborted embryos arrested at an early stage (Chaudhury *et al.*, 1997; Guitton and Berger, 2005), suggesting that the central cell plays an important role in the control of both fruit and embryo initiation. Recently, autonomous endosperm development was also observed in *sirène* (*srn*) and *scylla* (*syl*) mutants (Rotman *et al.*, 2008). The *FERONIA/SIRÈNE* receptor-like kinase is expressed in the two synergids and it is involved in the control of the release of the sperm cells (Huck *et al.*, 2003; Rotman *et al.*, 2003; Escobar-Restrepo *et al.*, 2007). In *fis* loss of function mutants, endosperm proliferation in the absence of fertilization is caused by the relief of the restraint imposed by *FIS* genes in the central cell; however, the origin of endosperm proliferation in *srn* and *syl* mutants remains to be clarified (Rotman *et al.*, 2008).

The study of *cdka:1/cdc2a* mutants has also provided useful insights into autonomous endosperm and fruit initiation (Nowack *et al.*, 2006). In *cdc2a* mutant pollen, a single sperm cell is produced and is able to fertilize the egg cell (Nowack *et al.*, 2006). Although selfing of heterozygous mutant plants as well as reciprocal crosses with wild-type plants showed that *cdc2a* pollen caused seed abortion (Nowack *et al.*, 2006), initial egg cell fertilization was able to promote autonomous endosperm proliferation (Nowack *et al.*, 2006).

It is widely accepted that upon fertilization auxin originating from the seed is generated (Fig. 4.2). CLSM images have shown that initial endosperm division is enough to trigger the auxin signal (Fig. 4.2b). It would be interesting to investigate whether fertilization of the egg cell by *cdc2a* pollen can indeed trigger auxin responsiveness to the same extent that double fertilization does.

4.5.3.2 Roles of the integuments in fruit initiation

Apart from selection of male gametes arriving at the ovule and providing nutritive support to the developing zygote and endosperm, the integuments have a role in signal transduction that directly stimulates fruit initiation.

Analysis of the cell cycle marker $P_{CycB1;2}$*:GUS* in integuments indicates that cells remain mitotically active throughout the female receptive period (Ingouff *et al.*, 2006). However, soon after fertilization, the integument cells begin expansion and the mitotic index further increases (Ingouff *et al.*, 2006). Significantly, the expression of auxin-responsive *DR5rev::eGFP* marker also begins in the integuments around 5 h post-fertilization (Fig. 4.2b; Vivian-Smith and Offringa, unpublished data). Considering that it takes 2–3 h for green fluorescent protein (GFP) expression from the *DR5rev::eGFP* reporter to become visible by CLSM (Sauer *et al.*, 2006), auxin responsiveness must at least occur 2–3 h post-fertilization.

Another indication that integument development can trigger fruit initiation independent of fertilization comes from two other sources. The knockout of the *MET1* DNA methyltransferase enzyme, in the *met1-3* mutant, stimulated both the differentiation of the seed coat testa and also that of fruit development without fertilization (FitzGerald *et al.*, 2008). These results imply control of DNA methylation in integument morphogenesis. In another case, the genetic analysis of the *bel1-1* mutant also uncouples fruit initiation from fertilization and ovule development suggesting that in wild type, the control of integument development, possibly together with the nucellar identity, is linked to the control fruit initiation (Western and Haughn, 1999; Vivian-Smith, 2001). Associated pseudo-integument development has also been reported in parthenocarpic tomatoes (Mazzucato *et al.*, 2003; Goetz *et al.*, 2007; De Jong *et al.*, 2008) and in the *Arabidopsis fwf/arf8* mutants, albeit much weaker than in tomato (Vivian-Smith *et al.*, 2001).

4.6 Hormonal cues during fruit initiation

In 1936, Gustafson discovered that application of synthetic auxins to emasculated flowers of several different plant species resulted in parthenocarpic fruit development and, thus, established the initial linkage between fruit initiation and plant-growth regulators (Gustafson, 1936). At present, three main types of plant-growth regulators are recognized as having phytohormonal properties that can potentially induce fruit setting and fruit development (Gillaspy *et al.*, 1993). Application of auxin, gibberellins or cytokinin, either alone or in combination, has been shown to trigger parthenocarpy across a wide variety of plant species (Gustafson, 1936; King, 1947; Srinivasan and Morgan, 1996; Vivian-Smith and Koltunow, 1999; Ozga *et al.*, 2002, 2003). Application of optimal combinations of plant-growth regulators to emasculated pistils can often promote elongation to the extent observed in fully seeded fruits (Vivian-Smith *et al.*, 2001). These results have led to a long standing belief that fruit initiation is sustained by phytohormone biosynthesis occurring during the stages of seed development, although often this assertion remains unchallenged.

Understanding the genetics and molecular genetics behind natural parthenocarpic mutants permits further investigation of the phytohormonal

signalling and the relationship to pollination, fertilization and fruit initiation. Uncoupling fruit initiation from fertilization, with parthenocarpy, offers a unique method to examine these relationships. For example, a role for the ovule in fruit initiation during parthenocarpy has been uncovered (Vivian-Smith, 2001; Koltunow *et al.*, 2002). However, many questions remain to be answered. To what degree does the ovule contribute in signalling? How are phytohormonal responses initiated and then propagated through the entire flower? Which are the sites of biosynthesis and signal transduction?

4.6.1 Auxin

Auxin plays a crucial role in plant development by directing basic processes such as division, elongation, phyllotaxy, organ primordia differentiation, apical dominance, tropic responses and response to shading (Sauer *et al.*, 2006; for a more detailed review see Benjamins and Scheres, 2008). Auxin also appears to have a primary role during fruit initiation since the genetic analysis of wild-type *Arabidopsis* fruit initiation with gibberellin biosynthesis and perception mutants shows that auxin-mediated differentiation underlies other signalling pathways (Vivian-Smith and Koltunow, 1999; Vivian-Smith *et al.*, 2001). Furthermore, transcriptional profiling during fruit initiation also shows directionality in phytohormonal responses with auxin preceding gibberellin responses at 12–14 h period post-fertilization (Vriezen *et al.*, 2007). The use of the transgenic *DEFH::iaaM* construct in a broad range of species (Rotino *et al.*, 1997; Ficcadenti *et al.*, 1999; Mezzetti *et al.*, 2004; Yin *et al.*, 2006) additionally suggests a universal role for auxin in triggering fruit set. Accordingly, auxin-mediated signalling is an early response in the *Arabidopsis* ovule (Fig. 4.2). Auxin-responsive reporters show transcriptional activation 2–3 h post-fertilization expression, when the nuclear endosperm has undergone only one division (Fig. 4.2). Auxin responses continue to increase after the third endosperm nuclear division, primarily in the integument tips, the endothelium and the chalazal region. Activation at the base of the funiculus is observed less than 12 h after fertilization (not shown). Apart from expression data, portrayed in Fig. 4.2, parthenocarpic mutants and quantitative trait loci (QTLs) have also been characterized and these clearly support roles for auxin as a primary fruit initiation cue (Vivian-Smith *et al.*, 2001; Wang *et al.*, 2004; De Jong *et al.*, 2008; Gorguet *et al.*, 2008).

4.6.1.1 Auxin-mediated transcriptional activation

Several genetic lesions in the auxin pathway conferring autonomous fruit initiation have been isolated. Each of these mutants appears to work within the auxin-mediated transcriptional network. The *Arabidopsis* genome encodes 22 functional auxin response transcription factors (ARFs) and 29 Aux/IAA interacting proteins, and each gene appears to have strong sequence conservation in other plant genomes (Remington *et al.*, 2004; Okushima *et al.*, 2005).

The ARFs are a family of Aux/IAA interacting proteins that contain a DNA-binding domain (DBD) which recognizes auxin response elements (AuxREs) in DNA sequences (Figs. 4.3 and 4.4; Kim *et al.*, 1997; Ulmasov *et al.*, 1999a,b). Information about the role of *ARF8* in fruit initiation came from the map-based cloning of the *FWF* locus (Vivian-Smith, 2001). All nullomorphic lesions in the *Arabidopsis FWF/ARF8* gene induce parthenocarpy, but surprisingly the homeologous expression of the *arf8-4* gDNA allele can also provide parthenocarpy in tomato and *Arabidopsis* (Vivian-Smith, 2001; Vivian-Smith *et al.*, 2001; Goetz *et al.*, 2006, 2007). *FWF/ARF8* transcripts are naturally downregulated within 24 h post-pollination (Goetz *et al.*, 2006, 2007), unambiguously similar to the nullomorphic phenotype, suggesting that the removal of the *ARF8* mRNA induces fruit initiation. QTL mapping and microsynteny between *Arabidopsis* and tomato has also uncovered a potential link between natural variations in parthenocarpic tendency and the *SlARF8* locus located on chromosome 4 (Gorguet *et al.*, 2008). Recently, the related *SlARF7* locus has also been implicated in fruit initiation (Vriezen *et al.*, 2007; De Jong *et al.*, 2008). *SlARF7* was initially identified through cDNA AFLPs technology used for transcriptional profiling. This methodology was applied to wild type and GA_3-induced fruit initiation and revealed that *SlARF9* and the Aux/IAA proteins *SlIAA2* and *SlIAA14* are upregulated within 24 h post-fertilization, but that the *SlARF7* transcript was downregulated within 24 h in ovule and placental tissues (Vriezen *et al.*, 2007). On the basis that *SlARF7* may function as a repressor, like *AtARF8*, De Jong *et al.* (2008) specifically silenced *SlARF7* by RNAi. Indeed *SlARF7* silenced lines produced fruit initiation without fertilization, although the morphology was not entirely similar to pollinated fruit. RNAi has also been used to silence the tomato Aux/IAA protein *SlIAA9* (Wang *et al.*, 2005). Silencing *SlIAA9* also caused parthenocarpy, but it also resulted in the expression of simple leaves and altered leaf vascular differentiation (Wang *et al.*, 2005). Single base deletions in *SlIAA9* recapitulated the RNAi phenotype confirming the specificity of the *SlIAA9* RNAi phenotype (Zhang *et al.*, 2007). While the expression of *SlIAA9* is not known, the expression of *SlARF7* in the placenta and ovules (Vriezen *et al.*, 2007) and the expression of *AtARF8* in the endothelium, the female gametophyte, the funiculus and the chalaza (Goetz *et al.*, 2006) clearly indicate a strong involvement with the ovule. The expression of the synthetic auxin-responsive reporter gene shown in Fig. 4.2 correlates precisely with *ARF8* reporters in the unfertilized *Arabidopsis* ovule (as observed in Goetz *et al.*, 2006). Initial expression occurs in the chalaza at <5 h post-fertilization, the endothelium and the integument tips (Fig. 4.2b). Stronger expression occurs with each nuclear endosperm division (Fig. 4.2c). Auxin treatment to the unfertilized *Arabidopsis* pistil, which presumably overrides ARF8-mediated restriction, recapitulates a similar post-fertilization expression pattern (Fig. 4.2d).

Many nullomorphic and antimorphic Aux/IAA mutants have been characterized in *Arabidopsis* (Overvoorde *et al.*, 2005). Mutations in conserved

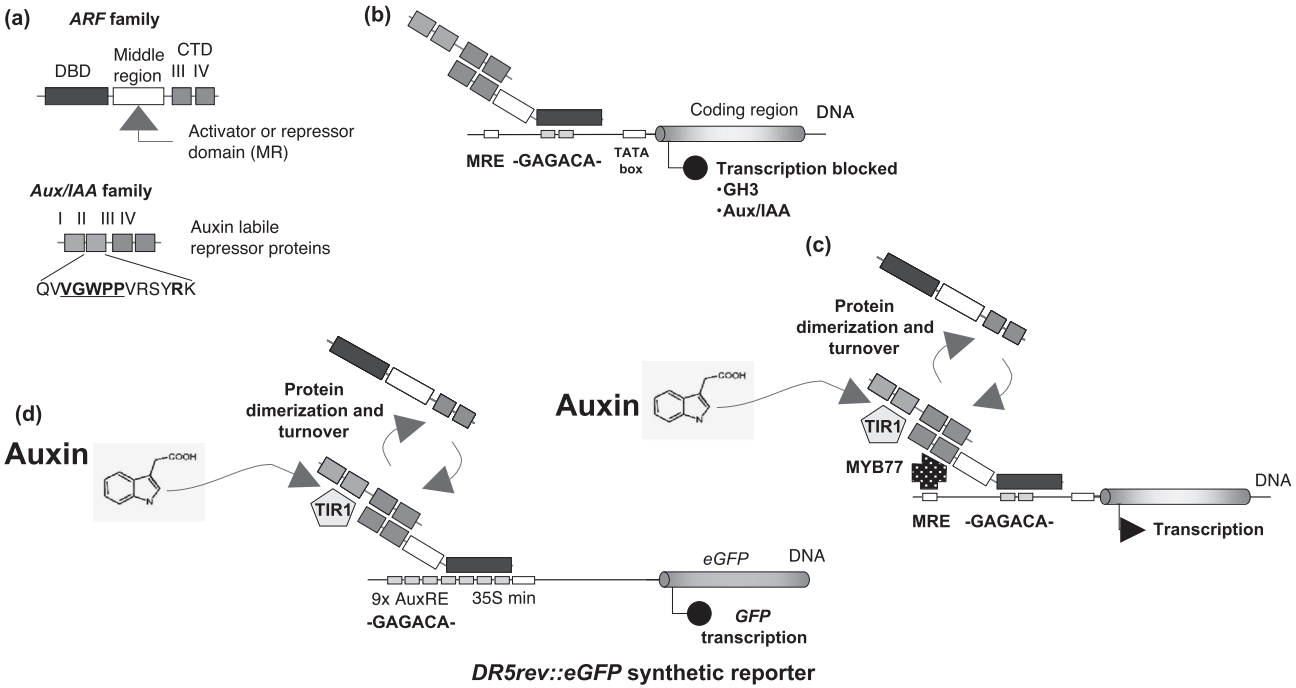


Figure 4.3 Auxin-responsive gene regulation. (a) The structure and function of ARF and Aux/IAA proteins which regulate expression of auxin-responsive genes. ARF proteins contain a DNA-binding domain (DBD) and carboxy-terminal domains (CTD) III and IV. The CTD regions facilitate hetero- and homodimerization amongst other ARF and Aux/IAA proteins as well as binding with MYB77. (b) Repression of downstream coding regions occurs when an Aux/IAA protein interacts with an ARF protein that is bound to auxin response elements (AuxREs). Downstream target genes are often Aux/IAA proteins and *GH3* genes creating a loop of auxin transcriptional responsiveness. (c) Possible transcriptional activation by ARF of auxin response genes containing an AuxRE after free auxin induces lability of the Aux/IAA protein through the TIR1/AFB proteolysis pathway. Free IAA is sandwiched between domain II of the Aux/IAA protein. This enables interaction of the TIR1/AFB auxin receptors with Aux/IAA proteins that shunt Aux/IAAs into the ubiquitin-proteasome pathway. Transcription of the downstream gene occurs once the ARF is depressed by the lability of the Aux/IAA protein, and enhanced by MYB77-ARF interaction with the transcriptional response elements adjacent to the AuxRE. (d) The synthetic auxin-responsive reporter, *DR5rev::eGFP*, consists of multimerized AuxREs within a miminal promoter. This allows the activity of ARF and Aux/IAA proteins to be monitored at the cellular level by observing the output of the eGFP protein.

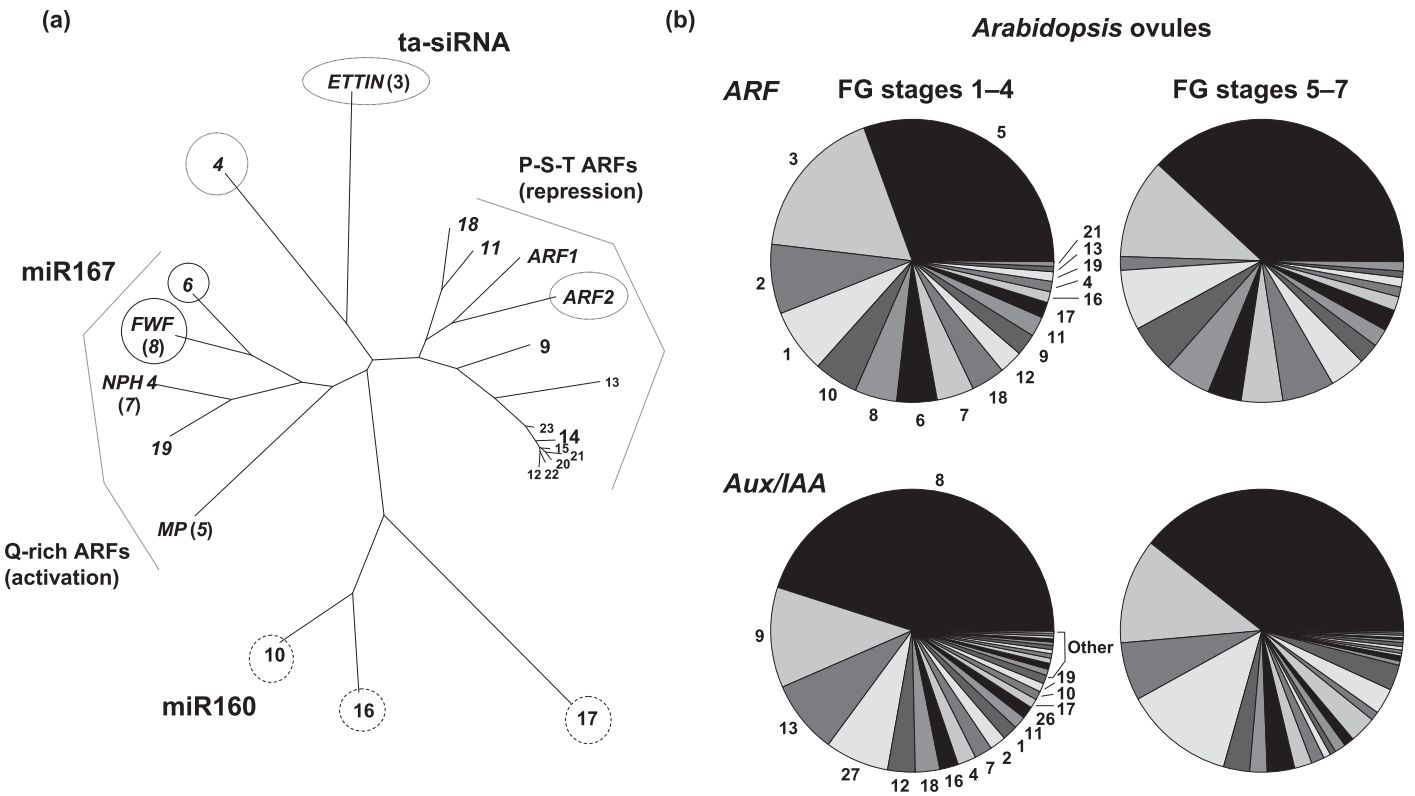


Figure 4.4 (a) Neighbour-joining phylogeny of *Arabidopsis* ARF protein sequences. Activator and repressor ARFs are represented on the left side and right side, respectively. ARFs with non-canonical middle regions (MRs) are centrally located. miR167 targets are highlighted by full circles (*ARF6* and *ARF8*), miR160 targets by broken circles (bottom ARFs) and tasi-RNA targets by short-dashed circles (top-right). Distances were determined using the Juke–Cantor algorithm and displayed using the TreeView programme (Page, 1996). (b) Expression profiles of *ARF* and *Aux/IAA* genes from *Arabidopsis* ovules at female gametophyte (FG) stages 1–4 and 5–7 derived from microarray data (adapted from Yu *et al.*, 2005).

domain II often produce antimorphic behavior due to increased stability of the IAA protein (Muto *et al.*, 2007) and several mutants provide insight into their functions during fertilization and fruit development. BDL/IAA12, for instance which interacts with MP, has a crumpled fruit phenotype and fails to elongate properly (Hamann *et al.*, 2002), suggesting that auxin-induced development is impaired. In the dominant mutants *AXR5/IAA1*, *IAA28*, *IAA19/MSG2* and *CRANE/IAA18* sterility is observed (Rogg *et al.*, 2001; Yang *et al.*, 2004; Muto *et al.*, 2007; Uehara *et al.*, 2008). So far, construction of double and triple mutant nullomorphs has provided only very subtle phenotypes, underscoring heavy redundancy amongst the 33 proteins in this family (Overvoorde *et al.*, 2005). In fact, *iaa8-1 iaa9-1* and *iaa5-1 iaa6-1 iaa19-1* mutants appear wild type (Overvoorde *et al.*, 2005). By contrast, promoter and domain swap experiments amongst Aux/IAA proteins have uncovered redundancy, but importantly promoters and Aux/IAA proteins themselves provide specific interactions that control plant development (Muto *et al.*, 2007). The specific spatial and temporal distribution, that has been determined for some of the ARFs and IAA pairs, suggests that specific ARF–IAA complexes mediate developmental responses to auxin, but that Aux/IAA proteins tend to be promiscuous (Weijers *et al.*, 2005; Muto *et al.*, 2007). This contrast to some extent with the result in tomato for *SlIAA9* where a single knockout specifically induces fruit initiation (Wang *et al.*, 2005). The reduction of *SlIAA9* in either the carpel or the ovule may be specific enough to perturb auxin responses and induce parthenocarpy, and this result may signify specialized function for *SlIAA9* in *Solanaceous* flower and fruits. Experiments whereby antimorphic proteins are specifically expressed within the ovule could provide data on the local modulation by auxin signalling, thereby overcoming redundancy.

Another level of regulation occurs at the level of translation of ARF proteins. Nishimura *et al.* (2005) describe the ribosomal protein L24/SHORT VALVE 1 (RPL24/STV1), which has a role in assisting the re-initiation of translation from small upstream open reading frames (uORFs) and is required for auxin-mediated gynoecium patterning. The ability to regulate translation was demonstrated by the removal of upstream uORFs which led to significantly increased expression of the mORF for *MP* and *ETTIN* (*ETT/ARF3*) (Nishimura *et al.*, 2005; Tran *et al.*, 2008). At this stage, widespread conservation for uORFs in *ARF* 5′UTR regions has not been described, although an examination of rice and *Arabidopsis* has been performed and this shows numerous uORFs in most ARF mRNA transcripts (Nishimura *et al.*, 2005; Tran *et al.*, 2008). *AtARF8* has 10 AUGs, or potential uORFs, in the 627 bases comprising the 5′UTR (Goetz *et al.*, 2007). Expression of the *arf8-4* gDNA allele, which was used to verify the cloning of *ARF8* (Vivian-Smith, 2001), also produces parthenocarpic phenocopies of null *fwf/arf8* alleles. Thus, the action of RPL24/STV1 may potentially account for the antimorphic impact of *fwf-1/arf8-4* gDNA in both *Arabidopsis* and tomato (Vivian-Smith, 2001; Goetz *et al.*, 2007) since RPL24/STV1 may be recruited to the full length mutated *fwf-1/arf8-4* transcripts, thereby reducing transcription upon the native *ARF8* 5′UTRs.

ARF8 expression is also presumably regulated at many other levels. This can be highlighted by the fact that a conserved and functional alternative splice acceptor sequence introduces 5 bp deletion and a premature stop codon within the DBD giving rise to shorter splice variant mRNAs in both *Arabidopsis* and tomato (Goetz *et al.*, 2007).

4.6.1.2 Auxin biosynthesis and transport

Three important processes that regulate auxin action in the flower and fruit are its biosynthesis, transport and catabolism. The role of de novo auxin biosynthesis is certainly vital during fruit initiation and fruit development. However, few studies have considered auxin biosynthesis in ovules or developing fruits in detail. Direct proof of the significance for localized auxin biosynthesis in fruit initiation was first obtained by Rotino *et al.* (1997). In this experiment, the *iaaM* gene from *Pseudomonas syringae* pv *savastonoi* was placed under the control of the placental and ovule-specific promoter *DEFH9*. Eggplants and tobacco transformed with this construct had parthenocarpic fruit development. Since *iaaM* is involved in the conversion of tryptophan to indole-3-acetamide, which is then hydrolyzed to IAA, this results in increased levels of localized IAA (Gaudin and Jouanin, 1995) suggesting that a rate-limiting step during fruit initiation could be the production of free auxin. Many other species have now been successfully transformed and all develop parthenocarpic fruit (Ficcadenti *et al.*, 1999; Mezzetti *et al.*, 2004; Yin *et al.*, 2006; Chapter 9 in this book). Although these results highlight the importance of the ovule during fruit development, the potential use of this chimeric transgene in obtaining additional data regarding the control of wild-type fruit initiation is limited. Additionally, *DEFH9* specificity to the ovule and or placenta is often not reported for these other species. Nevertheless, experiments where *iaaM* expression is specifically targeted to different parts of the ovule or female gametophyte may further clarify the role that these tissues play during fruit set.

Auxin biosynthesis in *Arabidopsis* is partly carried out by flavin monooxygenases encoded by the *YUCCA* gene family (Zhao *et al.*, 2001; Cheng *et al.*, 2006). Dramatic reductions in vascular tissue occur when multiple members are knocked out, consistent with a reduction in auxin biosynthesis and redundancy amongst family members. While *yuc2 yuc6* mutants develop normal vascular and floral morphologies, the *yuc1 yuc2 yuc6* triple mutant develops severe vascular deficiencies (Cheng *et al.*, 2006). Significantly, floral morphology and fruit development could be restored by a *YUC1::iaaM* chimeric construct (Cheng *et al.*, 2006). *YUC1*, *YUC2* and *YUC4* are apparently expressed in the carpel, while *YUC6* is expressed in pollen (Cheng *et al.*, 2006), however, examination of ovule expression data suggests *YUC* specificity in anthesis ovules (supplemental data, Yu *et al.*, 2005).

Experiments with PAT inhibitors show that flower morphology and apical–basal carpel polarity can be severely disrupted by PAT inhibitor application (Nemhauser *et al.*, 2000). Timed applications of PAT inhibitors can

also induce parthenocarpy, suggesting that the anthesis ovules and pistil may exert control over PAT, either directly or indirectly, and this has an impact on the regulation of fruit initiation (Beyer and Quebedeaux, 1974; Schwabe and Mills, 1981; Kim *et al.*, 1992). This appears to contradict the proposed mechanism for auxin action in fruit initiation, since PAT is required to transport auxin from the ovule to the carpel to trigger growth and development. Therefore, the mechanism by which PAT inhibitors triggers fruit initiation may rely on a balance between PAT and AuxRE transcriptional activation. To explain the apparent paradox, one must consider that auxin is also prevalent in the target cells. Therefore, the end concentration in target cells is important and may normally be restricted to a low level through potentiation of PAT and a reduction in transcriptional activation. Once PAT is inhibited, AuxRE would be activated due to the increased net auxin accumulation within the cell that would trigger fruit initiation through cross-talk with other phytohormonal pathways.

To explain PAT, a chemiosmotic model was proposed in which the non-charged, lipophilic IAA molecule enters the cell through diffusion, or through the action of a saturable auxin import carrier (Rubery and Sheldrak, 1974; Raven, 1975). Once inside the cell, the IAA molecule is deprotonated at the higher cytoplasmic pH and only can exit through active export by auxin efflux carriers (AECs) and endosomal/vessicle transport (Dhonukshe *et al.*, 2008). The specific location of AECs at the basal side of the cell was hypothesized to be the rate-limiting transport step of PAT (Lomax *et al.*, 1995). The *PIN1* gene encodes a transmembrane protein that has similarity to bacterial-type transporters and data suggest that PIN proteins function as AECs (Gälweiler *et al.*, 1998). The *PIN* family in *Arabidopsis* comprises eight members six of which have been functionally characterized through genetic analysis (Blilou *et al.*, 2005; Wisniewska *et al.*, 2006). Genetic analysis has also uncovered partial redundancy and functional compensation amongst PIN family members (Friml *et al.*, 2003). As a functional AEC, the PIN1 protein is localized to the basal end of xylem parenchyma and cambial cell files in the *Arabidopsis* inflorescence and root axis (Gälweiler *et al.*, 1998). Microarray analysis also shows that *PIN1*, *PIN2*, *PIN3* and *PIN6* are the most predominant AECs in the anthesis ovule (supplementary data, Yu *et al.*, 2005).

Important new insights into flower development have been obtained through molecular characterization of the *Arabidopsis pin-formed* (*pin1*), *pinoid* (*pid*) and the weak *mp* mutants (Benjamins *et al.*, 2001; Hardtke *et al.*, 2004). In contrast to mutations in *PIN2*, *PIN3* and *PIN6*, *pin1* mutants severely disrupt ovule development at early stages (Sauer *et al.*, 2006), implying an essential role for these proteins in ovules. The mutant also develops pin-like inflorescences, with few infertile flowers, a characteristic phenotype of wild-type plants grown in the presence of high levels of PAT inhibitors (Gälweiler *et al.*, 1998; Nemhauser *et al.*, 2000). Phenocopies have also been observed in the weak *mp* mutant (Hardtke *et al.*, 2004) and the *pid* protein kinase mutant (Benjamins *et al.*, 2001), suggesting a commonality in pathway components.

In this aspect, PINOID (PID) regulates subcellular PIN polarity (Friml *et al.*, 2004) and is rapidly induced by auxin (Benjamins *et al.*, 2001). Intriguingly, *PID* is expressed in chalazal and funiculus during fruit development (Benjamins *et al.*, 2001). This overlaps directly with the areas where *FWF/ARF8* activity restricts fruit initiation (Vivian-Smith, 2001; Koltunow *et al.*, 2002) and the area where vascular development between the ovule funiculus and carpel is later observed (see Section 4.8).

Organization of PAT appears to occur also through KANADI (KAN) proteins since PIN1-dependent patterning and subcellular PIN1 polar localization during early *Arabidopsis* embryogenesis are dependent on redundant actions of the KAN protein class (Izhaki and Bowman, 2007). This is of interest since strong parthenocarpy is elicited by the introduction of the *kan4-1/ats-1* mutant into the *fwf/arf8* background (Vivian-Smith *et al.*, 2001). KAN4/ATS could set up, maintain or directly act to control PAT in specific tissues of the ovule. KAN1 appears to act through *ETT* and *ARF4* to control laminar growth and polarity in lateral organs (Pekker *et al.*, 2005) and a model has been presented for the control of apical–basal carpel patterning by PAT and *ETT* (Nemhauser *et al.*, 2000). It is tempting to speculate that the KAN4 protein in ovules works in a similar manner to KAN1 in support of this model, it has been observed that the gene trap expression for *ETT* does indeed show increasing restriction to ovules and the replum vasculature as the flower approaches anthesis (Nakayama *et al.*, 2005).

4.6.1.3 Auxin signalling and feedback regulation

Auxin rapidly induces early auxin response genes including two other major classes, *SAURs* and *GH3s* (Hagen and Guilfoyle, 2002). The *GH3* gene family in *Arabidopsis* represents an important network component in feedback signalling, auxin homeostasis and in the homeostasis of jasmonic acid (Liu *et al.*, 2005; Terol *et al.*, 2006). GH3 enzymes catalyze bidirectional conjugations of indolic compounds and jasmonic acids with amino acids (Liu *et al.*, 2005). Many *GH3* genes are responsive to environmental stimuli, but most are also primary auxin response genes and are induced in response to auxin treatments to pistils and have altered expression in *fwf/arf8* and *arf6* mutant backgrounds (Nagpal *et al.*, 2005). The genes *GH3.5*, *GH3.6* and *GH3.17* appear to be targets of ARF8 and ARF6 (Tian *et al.*, 2004; Nagpal *et al.*, 2005) and plants overexpressing *ARF8* showed a decrease in free IAA content possibly due to *GH3* expression (Tian *et al.*, 2004). Regulation through *ARF8* and *GH3* genes at anthesis may therefore be a mechanism that constrains auxin responses further since free auxin would be removed and this would potentiate Aux/IAA proteins to form inactive complexes with ARF proteins.

Auxin is involved in many crucial roles in plant development and there are many interconnected processes with auxin signalling. The challenge with investigating auxin-mediated fruit initiation will be to break up the signalling in specific tissues and to independently interrogate these tissues with either mutational genetics, in vivo reporters or through transcriptional profiling that

reveals transcriptional networks. Alternatively, one could employ chemical genetics to perturb specific auxin signalling pathways related to fertilization-induced fruit initiation.

4.6.2 Gibberellins

Gibberellins form a large family of tetracyclic diterpernoid compounds of which only a small number are active PGRs. Bioactive gibberellins are known to be involved in diverse developmental processes including stem elongation, seed germination, leaf expansion, trichome development, de-etiolation and flower and fruit development (Olszewski *et al.*, 2002; Swain and Singh, 2005). Several lines of evidence have shown that fertilization results in increased levels of GA in the ovary (Eeuwens and Schwabe, 1975; Mapelli *et al.*, 1978; Ozga *et al.*, 1992; van Huizen *et al.*, 1995; Ben-Cheikh *et al.*, 1997; Serrani *et al.*, 2007b, 2008; Vriezen *et al.*, 2007). Due to their high gibberellin content, fertilized ovules have long been considered the source of growth-promoting and fruit-setting compounds (García-Martínez *et al.*, 1991). However, a far more complex GA-mediated control of fruit initiation and development is being unveiled, partially as a result of the characterization of the GA biosynthesis and signalling pathways.

4.6.2.1 Gibberellin biosynthesis

In higher plants, GA biosynthesis can be divided into three stages (Olszewski *et al.*, 2002). The first stage results in the synthesis of ent-kaurene by the action of two cyclases, ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS; Fig. 4.5), which in anthesis flowers is located adjacent to vascular bundles, such as the funiculus, medial and lateral vasculature bundles (Silverstone *et al.*, 1997). ent-Kaurene is also highly volatile suggesting mobility at this point of synthesis (Otsuka *et al.*, 2004). In the second stage, GA_{12} and/or GA_{53} are produced as a result of the action of ent-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO) in the case of GA_{12} and an extra 13-hydroxylation step in the case of GA_{53}. The final stage results in the synthesis of active GAs through two parallel pathways (Fig. 4.5): the non-13-hydoxylation pathway (leading to GA_4) and the early 13-hydoxylation

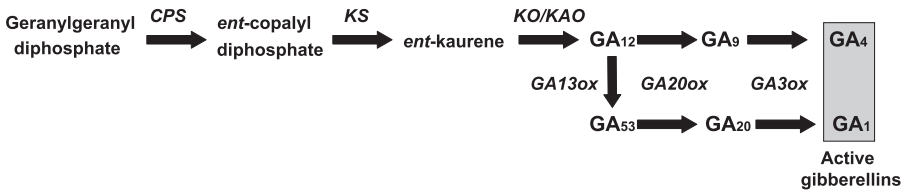


Figure 4.5 The GA biosynthesis pathway in higher plants. The pathway is shown from the common precursor geranylgeranyl diphosphate to the active gibberellins GA_1 and GA_4. The names of the enzymes catalyzing each step are shown in italics.

pathway (leading to GA_1 and GA_3 in some cases). Enzymes involved in this final stage include 2-oxoglutarate-dependent dioxygenases, GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox). Active GAs can be converted to inactive forms by the action of GA 2-oxidases which catalyze the introduction of a hydroxyl at the 2β position (for more detailed review on GA biosynthesis see Olszewski *et al.*, 2002).

Our present understanding of the role of GA biosynthesis in fruit initiation and fruit development is based mainly on the study of the final steps of the biosynthetic pathway. Several studies have shown that pollination/fertilization results in increased expression levels of enzymes catalyzing the last steps of GA biosynthesis (Ozga *et al.*, 1992; Ben-Cheikh *et al.*, 1997; Ngo *et al.*, 2002; Olimpieri *et al.*, 2007; Serrani *et al.*, 2007b; Hu *et al.*, 2008). It was first shown in pea that removal of seeds reduced the activity of GA 20-oxidase in the pericarp (Ozga *et al.*, 1992). In tomato, the study of the early 13-hydroxylation pathway (the main GA metabolic pathway in this species) has also shown that pollination/fertilization results in increased expression levels of GA 20-oxidases on the ovary (Serrani *et al.*, 2007b). GA 20-oxidases are responsible for the conversion of GA_{19} to GA_{20} which will then be metabolized to GA_1 (the main active GA in tomato) by GA 3-oxidases. Serrani *et al.* (2007b) showed that while transcript levels of GA 3-oxidases remained constant in both unpollinated and pollinated ovaries, a marked increase in GA 20-oxidase transcript levels was detected upon pollination/fertilization which suggested that fruit initiation in unpollinated tomato ovaries is perhaps partially limited by the low activity of GA 20-oxidases. This is in accordance with the studies carried out in *pat* tomato mutants where the parthenocarpic phenotype can at least partially be explained by the constitutive expression of *GA20ox1* (Fos *et al.*, 2000; Olimpieri *et al.*, 2007). Although in wild-type-pollinated tomatoes as well as in parthenocarpic *pat* tomatoes GA 20-oxidases were found to be expressed throughout the ovary, higher expression levels were observed in the seeds and ovules, respectively (Olimpieri *et al.*, 2007; Serrani *et al.*, 2007b). Therefore, it is possible that seeds (in wild-type plants) and ovules (at least in some parthenocarpy conferring mutations) may be the origin of growth promotion. Similarly, a recent study of *GA3ox* genes in *A. thaliana* has also concluded that developing seeds are likely to be sites of GA biosynthesis (Hu *et al.*, 2008). Analysis of the expression pattern of *GA3ox* genes in developing siliques showed that *GA3ox1* expression is limited to the replum, funiculi and silique receptacle while *GA3ox2*, *GA3ox3* and *GA3ox4* are expressed in developing seeds (Hu *et al.*, 2008). Despite these expression patterns, further mutant analysis concluded that only *GA3ox1* and *GA3ox4* are likely to be involved in the control of fruit initiation and fruit development in *Arabidopsis* (Hu *et al.*, 2008). Furthermore, it was shown that *GA3ox1* and *GA3ox4* gene expression increased after anthesis indicating that pollination/fertilization is required to induce GA biosynthesis and, thus, fruit growth promotion (Hu *et al.*, 2008). It has been suggested that *GA3ox1* may promote fruit initiation by acting in maternal tissues while the

activity of *GA3ox4* is thought to be limited to the seed endosperm (Hu *et al.*, 2008). The role of maternal tissues as potential sites for GA biosynthesis during fruit initiation has also been underlined by Rieu *et al.* (2008a,b) who showed that pollination of the *Arabidopsis ga20ox1 ga20ox2* double mutant with wild-type pollen fails to restore normal silique elongation, while down-regulation of the main GA inactivation pathway (C_{19}-GA 2-oxidation pathway) induced parthenocarpic fruit development. These findings are in agreement with those by Ozga *et al.* (1992), Olimpieri *et al.* (2007), Serrani *et al.* (2007b) and Hu *et al.* (2008) and, taken together, suggest that fertilization results in the upregulation of GA biosynthesis both in the ovary and ovules.

4.6.2.2 Gibberellin signalling

Similarly to the GA biosynthesis pathway, GA response and signalling pathways have also been intensively studied in the past few decades and many molecules involved in these pathways have been characterized. However, few components of the GA response and signalling pathways have been studied in the context of fruit initiation and fruit development.

The *SPINDLY* (*SPY*) locus of *Arabidopsis* was one of the first molecular players of the GA signalling pathway shown to be involved in the control of fruit initiation and fruit development (Jacobsen and Oleszewski, 1993). Although doubts still persist about the precise role played by *SPY* in the control of GA-mediated responses, particularly in relation to other components of the signalling pathway (Silverstone *et al.*, 1998), it is generally considered to be a negative regulator of the GA response pathway (Jacobsen and Oleszewski, 1993). During the initial characterization of the SPY locus, it was reported that emasculation of *spy* mutant pistils resulted in parthenocarpic silique elongation. This and other phenotypes of the *spy* mutants were suggested to be the consequence of the constitutive activation of the GA perception and/or GA signal transduction. Nevertheless, attempts to repeat the parthenocarpic silique elongation observed by Jacobsen and Oleszewski (1993) have failed and, thus, the role of SPY in the control of fruit initiation and/or fruit development remains to be clarified (Vivian-Smith *et al.*, 1999).

DELLA proteins are probably the most intensively studied components of the GA signalling pathway. They are part of the GRAS transcription factors family and, within this larger family, DELLA proteins are characterized by a conserved N-terminal amino acid sequence which appears to be essential for the regulation of GA responses. It has been shown that DELLA proteins act as growth repressors and GA-mediated degradation of these proteins through the 26S proteosome pathway is required in order to promote growth (Fig. 4.6) (for a more detailed review on this subject see Alvey and Harberd, 2005). The restraint in growth imposed by DELLA proteins has recently been considered in the context of fruit initiation by Marti *et al.* (2008) who showed that silencing of the only DELLA protein in tomato (SlDELLA) is sufficient to trigger parthenocarpic fruit development. Earlier studies have indicated that DELLA proteins regulate the expression of GA biosynthesis genes in a

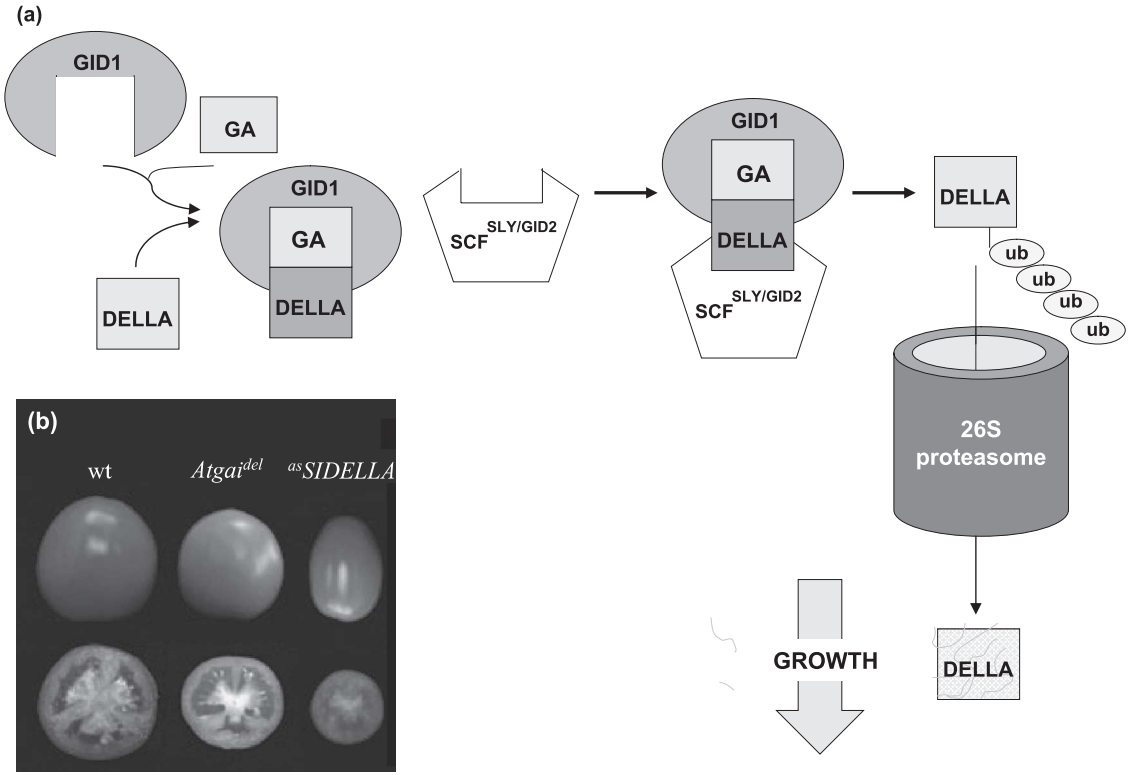


Figure 4.6 (a) Simplified model of GA-mediated DELLA degradation. In the presence of GA, nuclear-localized DELLA proteins associate with the complex form by GA and the GID1 receptor. This association enables the further interaction with the $SCF^{SLY/GID2}$ complex which results in DELLA protein poly-ubiquitination and, ultimately, in growth promotion by the degradation of the DELLA proteins through the 26S proteasome. (b) *DELLA* mRNA silencing causes facultative parthenocarpic fruit development in tomato. From left to right: pollinated wild-type fruit, parthenocarpic *asSlDELLA* transgenic fruit and hand-pollinated *asSlDELLA* fruit (adapted from Marti *et al.*, 2007). Used with permission of the publisher and authors.

feedback manner (Peng *et al.*, 1997; Silverstone *et al.*, 1997). In accordance with these observations, reduced levels of GA biosynthesis genes were recorded in *SlDELLA* tomato fruits (Marti *et al.*, 2008). The role played by endogenous GA levels in *SlDELLA* parthenocarpic fruit set can therefore be dismissed, the simplest explanation being that *SlDELLA* parthenocarpy is due to release of the restraint in growth imposed by DELLA proteins (Marti *et al.*, 2008).

4.6.3 Cytokinin and ethylene perception

The role of cytokinins in fruit initiation and fruit development has been studied to a considerably lesser degree than other phytohormonal processes (for a detailed review of cytokinin signalling see To and Kieber, 2008). Like numerous other phytohormones, cytokinins have also been identified as potential phytohormonal components produced in developing seeds that could promote fruit development (Burrows and Carr, 1970; García-Martínez *et al.*, 1991). Indeed, exogenous cytokinin application to pistils can result in parthenocarpic fruit development in *Arabidopsis* (Vivian-Smith *et al.*, 1999), *Brassica napus* (Srinivasan and Morgan, 1996) and in pea (García-Martínez and Carbonell, 1980), amongst many other species (Bangerth and Schroder, 1994; Yu *et al.*, 2001). Additionally, stimulation of cytokinin biosynthesis by using fruit and ovary-specific expression of the *ipt* gene from *Agrobacterium* produces parthenocarpy in tomato (Li, 2001; Zichao *et al.*, 2002). Strong linkage of cytokinins to the control of cell cycle progression has led to the speculation that cytokinins could be responsible for stimulating carpel cell division post-fertilization (Yu *et al.*, 2001; Li *et al.*, 2003; Vriezen *et al.*, 2007). At this time point, it is impossible to conclude whether fertilized ovules constitute a significant source of cytokinins which either triggers fruit initiation or alters fruit growth. In spite of this, genetic and systems analysis illustrate strong cross-talk between cytokinin, auxin and ethylene signalling pathways.

In microarray experiments carried out on *Arabidopsis* seedlings, cytokinin downregulated *ARF8*, *PIN2* and an auxin biosynthesizing nitrilase gene (Brenner *et al.*, 2005). These three genes are characterized as late responders to cytokinin treatment since the modulation of expression occurred after 120 min (Brenner *et al.*, 2005). This matches well with data that cytokinin treatment to pistils can trigger an auxin response in unfertilized *Arabidopsis* ovules (Vivian-Smith and Offringa, unpublished data). Unfertilized pistils that were treated with benzyl adenine (BA; 1 nmol pistil^{-1}) had a similar auxin response to that observed following pollination (Figs. 4.6b, 4.6c), yet this occurred after a 12 h period. This is significantly greater than the auxin treatment described earlier (<2 h) or even that induced by fertilization (<3 h). Since the cytokinin-induced auxin response occurs outside these timeframes this suggests indirect mechanisms of triggering fruit initiation, or at least that BA treatment had reduced mobility when compared to auxin. The use of a cytokinin-responsive reporter after fertilization (such as *TCS* cytokinin

reporter, Muller and Sheen, 2008) could contribute to clarify whether cytokinin is indeed a primary trigger for the post-fertilization fruit initiation.

Several lines of evidence suggest a role for cytokinin perception in gametophyte development and during stages related to post-fertilization development of the ovule. For example, the *CKI* gene is expressed after the FG5 stage and then it is transiently expressed in the zygote and endosperm until 72 h post-fertilization (Pischke *et al.*, 2002; Hejatko *et al.*, 2003). Evidence for the involvement of the type-A *ARR22* in fertilization and fruit initiation comes from the studies of Gattolin *et al.* (2006) and Horak *et al.* (2008). *ARR22* is expressed in the chalazal region of the ovule shortly after fertilization and may be linked to vascular development since cytokinin negatively regulates protoxylem specification (Mahonen *et al.*, 2006).

In contrast to cytokinin, ethylene has been typically associated with floral and fruit abscission and in fruit ripening (Kendrick and Chang, 2008). Associations of ethylene with fruit initiation have not been intensively investigated, however ethylene precursor molecules, like 1-aminocyclopropane-1-carboxylic acid (ACC), have been associated with actions that stimulate fruit growth (O'Neill, 1997; O'Neill and Nadeau, 1997). Proof of the definitive involvement of the ethylene precursor molecule, ACC, in fruit initiation has been provided by Tang (2003). In these experiments, exogenous application of ACC to unpollinated pistils induced fruit elongation (Tang, 2003). Furthermore, genetic analysis proved that ACC induction was completely dependent on *AXR1*, a ubiquitin-activating enzyme E1 involved in the auxin response pathway (Leyser *et al.*, 1993). The genetic analysis revealing the involvement of *AXR1* in ACC induced fruit elongation, potentially implicates that the degradation of Aux/IAA proteins is involved in parthenocarpic fruit development triggered by the ACC response. These results together with the experiments that show that radio-labelled ACC transport readily occurs in carnation gyneocia (Reid *et al.*, 1984; O'Neill, 1997) may suggest that ACC is an important player in fruit set.

The involvement of ethylene as a positive trigger in fruit initiation has also been demonstrated via other genetic analyses. Combinations of the ethylene perception mutant *ctr1-1* with ovule defective mutations *nzz-2* and *ino-2*, *ats-1/kan4-1* produce a parthenocarpic fruit when emasculated (Vivian-Smith, 2001; Koltunow *et al.*, 2002). These experiments indicate that the *ctr1-1* mutation, which constitutively activates downstream ethylene signalling, can provide a positive stimulus for fruit initiation, but only when components of the ovule that are perceived as repressor elements are removed (Vivian-Smith, 2001). Since the combination of *ctr1-1 ats-1/kan4* gave the strongest elongation when emasculated, PAT within ovule integuments maybe involved (Vivian-Smith, 2001). The ethylene receptor gene *ETR2* is also expressed in the ovule (Sakai *et al.*, 1998) and *ETR2* transcripts appear to be female gametophyte enriched (microarray of Yu *et al.*, 2005; Johnston *et al.*, 2007). This further suggests that ethylene signalling via ETR2 is a potential component in ethylene signal transduction at the time of fertilization.

4.6.4 Hormonal cross-talk

Treatment of unpollinated ovaries with auxins, gibberellins or cytokinins alone does not result in normal fruit development across different plant species (Srinivasan and Morgan, 1996; Vivian-Smith *et al.*, 1999; Ozga *et al.*, 2002) and application of specific hormonal combinations is required to trigger fruit development to the extent observed in fully seeded fruit. For example in *Arabidopsis*, application of gibberellins together with either cytokinins or auxins is required to restore silique length to that of pollinated siliques (Vivian-Smith *et al.*, 2001). These observations suggest that a hormonal interplay is necessary for normal fruit development.

Gibberellin and auxin cross-talk has been the most widely studied hormonal interaction in fruit initiation and fruit development. Growing evidence suggests that the stimulation of fruit initiation and growth by seed origin auxin can at least be partially attributed to the upregulation of gibberellin metabolism. van Huizen *et al.* (1995) showed that the conversion of GA_{19} to GA_{20} in pea pericarp is seed regulated and that application of the auxin 4-Chloroindole-3-acetic acid (4-Cl-IAA) can substitute for the seeds in the promotion of this conversion. Similar conclusions were reached by Ngo *et al.* (2002) who found that treatment of deseeded pea pericarps with 4-Cl-IAA increased GA 20-oxidase gene expression. Recent experiments have also shown that auxin-induced parthenocarpy can be blocked by GA-specific inhibitors (mainly paclobutrazol) (Serrani *et al.*, 2008). On the other hand, analysis of the ovary and ovule transcriptomes induced after pollination or by GA_3 treatment provided data that auxin-induced transcripts were unaffected by GA_3 treatment (Vriezen *et al.*, 2007). These results together with the GA-biosynthesis upregulation observed upon auxin treatment (Van Huizen *et al.*, 1995; Ngo *et al.*, 2002; Serrani *et al.*, 2008) and the lost of auxin-induced parthenocarpy upon PCB treatment (Serrani *et al.*, 2008) suggest that auxin stimulation of fruit set is partially mediated by gibberellins while the opposite appears to be improbable. However, several lines of evidence have also implied that auxin is likely to act independently to gibberellin in many aspects of fruit initiation. For example, clear morphological differences are observed at the tissue level in fruits treated with gibberellins or auxin (Vivian-Smith and Koltunow, 1999; Serrani *et al.*, 2007a). Furthermore, simultaneous application of gibberellin and auxin has an additive effect on fruit development (Vivian-Smith *et al.*, 2001; Serrani *et al.*, 2008). Finally, application of auxin to unpollinated *gai dominant* (*gai-1d*) *Arabidopsis* mutants results in silique growth promotion (Vivian-Smith and Koltunow, 1999). *GAI* encodes one of the five DELLA proteins in *Arabidopsis* (Peng *et al.*, 1997). In *gai-1d* mutants, the GAI mutant protein lacks 17 amino acids critical for GA-mediated degradation but retains its growth repression function (Peng *et al.*, 1997; Harberd *et al.*, 1998). Consequently, *gai-1d* mutants appear to be unable to respond to GA-mediated growth promotion (Peng *et al.*, 1997) which will suggest that the growth promotion observed upon auxin application can be attributed to the

independent effect of auxin in fruit initiation (Vivian-Smith and Koltunow, 1999).

Another level of interaction between gibberellin and auxin during fruit initiation and fruit development can be found in *A. thaliana ga1-3* mutants. These mutants are impaired in an early step of GA biosynthesis (Sun and Kamiya, 1994) and, consequently, produce very low levels of active gibberellins. Vivian-Smith and Koltunow (1999) showed that application of the auxin α-naphthalene acetic acid to *ga1-3* mutant pistils did not cause parthenocarpic fruit development. Similarly, upregulation in the *ga1-3* mutant background of the auxin signal required for fruit initiation by the introduction of the *arf8-4* mutation (formerly *fwf*) did not result in parthecarpic silique elongation either (Vivian-Smith *et al.*, 2001). However, parthenocarpic fruit development was promoted in other GA-biosynthesis mutants such as *ga4-1* and *ga5-1* upon auxin treatment (Vivian-Smith and Koltunow, 1999). Only one of the highly redundant enzymes catalyzing the later steps of GA biosynthesis is impaired in *ga4-1* and *ga5-1* mutants (Talon *et al.*, 1990b; Chiang *et al.*, 1995; Phillips *et al.*, 1995; Sponsel *et al.*, 1997) and, thus, they are weaker GA-biosynthesis mutants than the *ga1-3* mutant. Based on these results, Vivian-Smith and Koltunow (1999) concluded that a threshold of endongenous gibberellins may be required for auxin-induced fruit initiation.

The study of the hormonal regulation of fruit set has mainly focused on the role played by gibberellin and auxin and, thus, relatively little is known about the cross-talk between other hormones during fruit initiation. It has been suggested that SPY may play a pivotal role in the integration of gibberellin and cytokinin pathways by acting as both a repressor of GA responses and as a positive regulator of cytokinin signalling (Greenboim-Wainberg *et al.*, 2005). Nevertheless, without a better understanding of the role of SPY in fruit set (see Section 4.6.2.2), it is difficult to draw any further conclusions regarding the importance of such findings in the context of fruit initiation and fruit development.

A recent study has also shown that application of brassinosteroids can induce parthenocarpic cucumber fruit development (Fu *et al.*, 2008). This is in agreement with previous results by Montoya *et al.* (2005) which showed that Br C-6 oxidase, an enzyme catalyzing what it is thought to be a rate-limiting step in brassinosteroid biosynthesis, is highly expressed in developing seeds in tomato. Both studies certainly point towards a role of brassinosteroids in fruit initiation and fruit development. Furthermore, brassinosteroids have also been shown to act synergistically to auxin in the regulation of several target genes (Goda *et al.*, 2002; Nakamura *et al.*, 2003; Nemhauser *et al.*, 2004; Vert *et al.*, 2008). For example, the brassinosteroid-regulated BIN2 kinase is able to phosphorylate auxin response factor 2 (ARF2) which results in the loss of ARF2 activity (Vert *et al.*, 2008). In the model proposed by Vert *et al.* (2008), brassinosteroids release the repression activity of ARFs (such as AFR2) while auxin increases the expression of activator ARFs. Thus, brassinosteroids and auxin would coregulate gene expression through ARFs activity (Vert *et al.*,

2008). This is in agreement with previous data by Nemhauser *et al.* (2004) who showed that transcript levels of *ARF4* and *ARF8* are negatively regulated by brassinosteroid treatment. Although none of these studies were performed in the context of fruit initiation or fruit development, it is easy to envisage that a similar cross-talk mechanism may be operative during fruit initiation. In addition, *ARF2* interacts with the ethylene pathway (Li *et al.*, 2004) and it is expressed in the integument tip cells (Schruff *et al.*, 2006). Mutations alter flower, seed and fruit development in *Arabidopsis* which could further support the involvement of brassinosteroids and ethylene in fruit initiation (Vivian-Smith *et al.*, 2001; Okushima *et al.*, 2005; Goetz *et al.*, 2006).

A number of studies have also considered the interplay between auxin and other hormones such as cytokinins, ethylene and abscisic acid (ABA) in fruit initiation. Application of cytokinins to unpollinated *Arabidopsis* pistils increased seed origin auxin, suggesting that cytokinin stimulation of fruit initiation is at least partially mediated by auxins (Vivian-Smith and Offringa, unpublished data). On the other hand, analysis of the tomato ovary transcriptome showed that fruit set either by pollination or by gibberellin application resulted in the downregulation of ethylene and ABA biosynthesis (Vriezen *et al.*, 2007). Based on these results, it was concluded that ABA and ethylene might play an antagonistic role to that of auxin and gibberellin in fruit initiation, possibly by keeping the ovaries protected and/or dormant prior to pollination and fertilization (Vriezen *et al.*, 2007).

Although many different phytohormones and phytohormonal precursors appear to trigger fruit initiation, how this maze of connections is integrated during fruit initiation remains to be further clarified. One particular signalling component which has common elements in ethylene, cytokinin and gibberellin signalling is the MAP kinase pathway. Marcote and Carbonell (2000) described the *PsMAPK3* gene from pea which is an orthologue to the *Arabidopsis AtMPK3*. Interestingly, *PsMAPK3* is upregulated within 30 min of GA_3 and 45 min of cytokinin treatment to unpollinated pea pistils, though no experiments were performed to understand the post-pollination control of *PsMAPK3* transcription (Marcote and Carbonell, 2000). *PsMAPK3* is expressed in the ovule, mesocarp and carpel vascular tissues upon GA and cytokinin treatment. In *Arabidopsis,* much is now known about the role played by *AtMPK3* and the redundant partner protein *AtMPK6* (Ouaked *et al.*, 2003; Takahashi *et al.*, 2003; Miles *et al.*, 2005; Wang *et al.*, 2008; Yoo *et al.*, 2008). Mutations in *AtMPK3* and *AtMPK6* show short integuments when double mutants are constructed (Wang *et al.*, 2008), suggesting that the two proteins support the growth of the integuments during ovule development. It has recently been shown that the two proteins act in a central but pervasive role including signalling for glucose, ethylene, jasmonic acid, reactive oxygen species, floral, abscission, and in both abiotic and biotic stresses. Yoo *et al.*, 2008 showed that AtMPK3 and AtMPK6 proteins translate information from these pathways and provide a distinct output on the EIN3 protein, which is a component of the ethylene signalling pathway. The ACS2 and ACS6 proteins

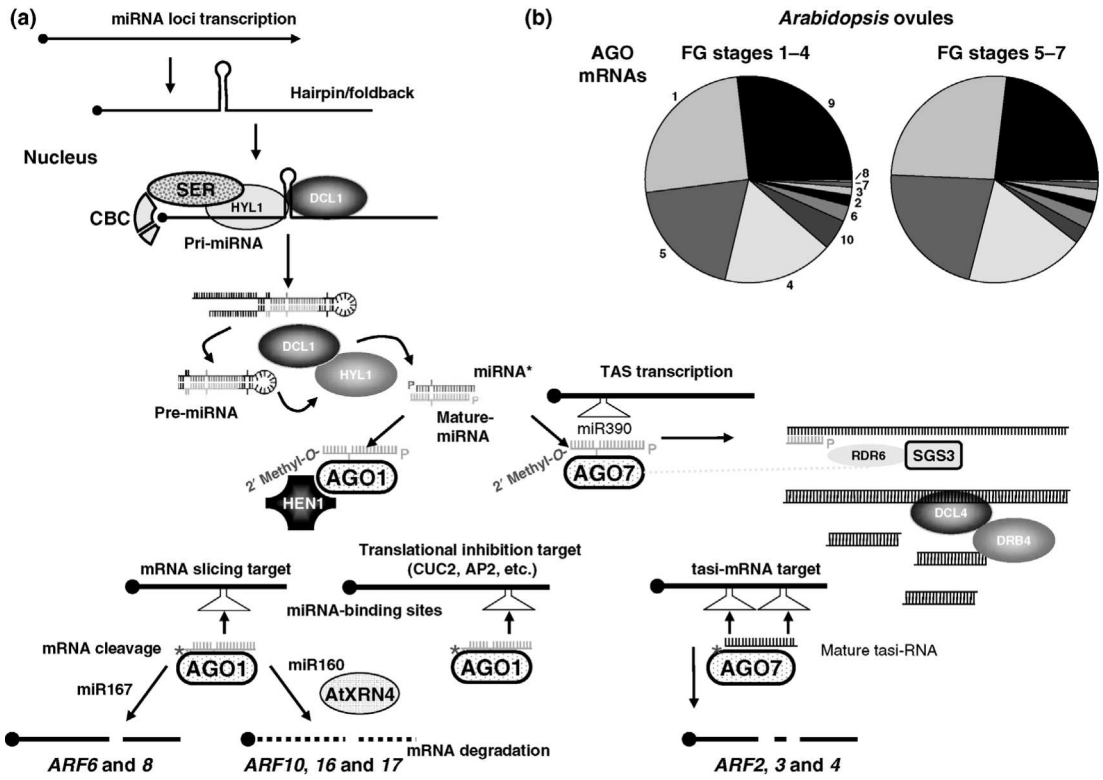
are also directly phosphorylated by this cascade which leads to ACS stabilization and enhanced ethylene signalling (Liu and Zhang, 2004). Therefore, the MPK3 and MPK6 appear to be critical in determining ethylene-signalling specificity amongst many other integrated signals. In the light of the results by Marcote and Carbonell (2000), further analysis of the *MPK3* and *MPK6* genes in fruit development is now needed.

4.7 RNA silencing during fruit initiation

Many ARF mRNAs are subjected to post-transcriptional regulation via endogenous RNA silencing through either miRNA or tasi-RNA regulation (Figs. 4.4 and 4.7; Hunter and Poethig, 2003; Mallory *et al.*, 2005; Williams *et al.*, 2005; Fahlgren *et al.*, 2006; Yang *et al.*, 2006; Liu *et al.*, 2007; Wu *et al.*, 2007). The small RNA molecules generated through these pathways usually encode short 21 nucleotide RNAs that either guide cleavage or translationally inhibit a targeted mRNA transcript via the ARGONAUTE (AGO) proteins (Fig. 4.7; Chapman and Carrington, 2007; Brodersen *et al.*, 2008; for more detailed information about RNA silencing in plants please see Brodersen and Voinnet, 2006). Many miRNAs are under strict spatio-temporal regulation (Valoczi *et al.*, 2006; Wu *et al.*, 2006). For example, miR160 is expressed in the funiculus vasculature and placental tissue (Valoczi *et al.*, 2006), while miR167 is expressed within developing and mature ovules (Valoczi *et al.*, 2006; Wu *et al.*, 2006).

The impact of small RNA regulation on auxin signalling pathway is highly significant both in terms of the number of auxin-related targets as well as in the wide variety of developmental processes affected. Moreover, miRNA-*ARF* post-transcriptional regulation appears to be key in flower maturation and reproductive development in angiosperms and gymnosperms (Ru *et al.*, 2006; Wu *et al.*, 2006; Fujioka *et al.*, 2008; Oh *et al.*, 2008). *ARF2*, *ETT/ARF3* and *ARF4* transcription factors are targeted by miR390 (Montgomery *et al.*, 2008) while the auxin receptors, *TIR1*, *AFB1*, *AFB2* and *AFB3* are under the regulation of miR393 (Navarro *et al.*, 2006). Two key ARF transcription factors in fruit initiation, ARF6 and ARF8, are targets of one of the most abundant miRNAs, miR167 (Lu *et al.*, 2006; Rajagopalan *et al.*, 2006). Undoubtedly, RNA silencing provides the means for relatively rapid mRNA turnover and removal of key transcription factors. All targeted ARFs have short mRNA half-lives (Narsai *et al.*, 2007), particularly when compared to the 3–4 h half-life reported for ARF1 protein turnover (Salmon *et al.*, 2008). Elimination of ARFs through transcript clearance could leave other ARFs, such as *MP*, free to activate auxin responses in ovules (Fig. 4.7).

The formation and biogenesis of miRNAs and ta-siRNAs occur through defined pathways that are dependent on the transcription of precursor non-coding RNAs (pri-miRNAs) from unique *MIR* loci by processes consistent with *PolII*-driven transcription (Fig. 4.7; Xie *et al.*, 2005). Often the number


(a)
miRNA loci transcription
Hairpin/foldback
Nucleus
SER
HYL1
DCL1
CBC
Pri-miRNA
DCL1
HYL1
Pre-miRNA
miRNA*
Mature-
miRNA
2′ Methyl-O-
AGO1
HEN1
TAS transcription
miR390
2′ Methyl-O-
AGO7
RDR6
SGS3
DCL4
DRB4
mRNA slicing target
Translational inhibition target
(CUC2, AP2, etc.)
miRNA-binding sites
tasi-mRNA target
mRNA cleavage
AGO1
miR167
miR160
AtXRN4
AGO1
AGO7
Mature tasi-RNA
mRNA degradation
ARF6 and 8
ARF10, 16 and 17
ARF2, 3 and 4
(b)
Arabidopsis ovules
AGO
mRNAs
FG stages 1–4
FG stages 5–7
1
9
8
7
3
2
6
10
4
5

of *MIR* loci outnumber the target mRNA locus. For example, miR167 has four loci in *Arabidopsis* (miR167a–d; Wu *et al.*, 2007) and larger numbers in other sequenced genomes (*Populus*, 10 miR167 loci; Barakat *et al.*, 2007). In this capacity, site specific and developmental control of miRNA action is achieved. After transcription, precursor molecules are then folded into a hairpin structures (Fig. 4.7) and presented to a complex that includes *DICER-LIKE1* (*DCL1*), *SERRATE* (*SE*) and the double-stranded RNA-binding protein *HYPONASTIC LEAVES1/DRB1* (*HYL1/DRB1*; reviewed, Chapman and Carrington, 2007; Mallory and Bouche, 2008). Processing by DCL1 trims the arms and loop of the miRNA to generate a 5′ phosphorylated 21-nucleotide dsRNA (Fig. 4.7). *HUA ENHANCER1* (*HEN1*), which encodes 2′-*O*-methyltransferase, stabilizes miRNAs by a 3′ methylation (Li *et al.*, 2005; Yang *et al.*, 2007). Following stabilization, this pre-miRNA is loaded into the AGO protein where the sequence is presumably cleaved. This allows the mature miRNA free to guide translational repression or slicing (Fig. 4.7, Brodersen *et al.*, 2008). Translational repression requires the specialist action of other pathway components such as the microtubule-severing enzyme katanin and the mRNA decapping component *VARICOSE* (*VCS*)/Ge-1, which is also involved in vascular biogenesis (Brodersen *et al.*, 2008). AGO proteins therefore are key participants regulating gene expression at the post-transcriptional level and possess both the capability of an irreversible miRNA-guided mRNA slicer or a dynamically reversible translational repressor (Brodersen *et al.*, 2008). The later process may allow rapid changes in gene expression.

Figure 4.7 Biogenesis of miRNAs and post-translational regulation of mRNA targets in the auxin response pathway. (a) miRNAs are generated from non-coding RNA loci and first undergo folding of primary pre-miRNA (top left). The transcript with hairpin foldback structure undergoes processing by the SER/HYL1/DCL1 complex. The CBC complex binds to the mRNA cap and the mRNA is acted upon by the HYL1/DRB protein and DCL1. This generates a 21 nucleotide double-stranded RNA with 5′ phosphorylated two base overhangs and some internal mismatches. The dsRNA is methylated by HEN1 which adds a 2′-methyl group to the 3′ end, increasing the stability of the miRNA. Double-stranded miRNAs are loaded into AGO1 proteins and the miRNA* strand is lost, thereby creating an active miRNA–AGO complex. AGO1 and AGO10/ZWILLE miRNA RISC complexes identify miRNA targets. Either target cleavage or translational inhibition occurs. The *Arabidopsis ARF6* and *ARF8* mRNAs are miR167 cleavage targets and are degraded by an EIN5/XRN4-independent mediated decay. *ARF10*, *ARF16* and *ARF17* are targeted by miR160 and are degraded by an XRN4-dependent process. The ta-siRNA targets *ARF2*, *ARF3* and *ARF4* are processed by a second order miRNA processing mechanism started by miR390. miR390 is preferentially loaded into AGO7 and targets a TAS3 mRNA. Cleavage generates a phased dsRNA priming site. This is enacted upon by RDR6 and SGS3 to generate long dsRNA which is then processed by the DCL4/DRB4 complex into 21 nucleotide siRNAs. These are loaded into AGO7 and processed to target a variety of mRNAs including *ARF2*, *ARF3* and *ARF4*. (b) Representation of relative levels of AGO mRNA transcripts in ovules at female gametophyte (FG) stages 1–4 (left) and 5–7 (anthesis; adapted from Yu *et al.*, 2005, supplementary data).

The strict spatio-temporal accumulation of miR167 in ovules and marginal tissues (Valoczi *et al.*, 2006; Wu *et al.*, 2006; Gifford *et al.*, 2008), combined with the shear miR167 abundance, potentially underlines the importance of miR167 in developmental fate as well as in the control of *ARF6* and *ARF8* and, thus, potentially in the control of fertilization and fruit initiation. In fact, weak *dcl1* alleles cause severe developmental and reproductive defects, including short integuments, incomplete carpel closure and pleiotropy, whereas strong alleles are embryonic lethal (Schauer *et al.*, 2002). Several of these phenotypic effects are recapitulated when genomic versions of the *ARF8* and *ARF6* genes that have mutated miR167-binding sites are introduced into plants (Wu *et al.*, 2006).

Since *ARF8* is downregulated 24 h post-anthesis (Goetz *et al.*, 2006), one may conclude that this occurs either through transcriptional downregulation or by the function of miR167 loci. Although transcriptional downregulation in ovules pre- and post-fertilization cannot be ruled out, a strong case is made for the action of miR167 in triggering target cleavage and clearance, rather than simply refining patterns of *ARF6* and ARF8 transcript distribution during development. For example, overexpression of miR167 recapitulates many of the phenotypes observed in *fwf/arf8* mutants (Ru *et al.*, 2006; Wu *et al.*, 2006). Overexpression studies involving individual miR167 loci also appear to underscore the additional specificity of the miR167 sequence. miR167a phenocopies the *arf6 arf8* null mutants (Wu *et al.*, 2006), while miR167b appears to act specifically on *ARF8* alone (Ru *et al.*, 2006). Furthermore, the expression patterns of individual miR167 loci in *Arabidopsis* (Ru *et al.*, 2006; Wu *et al.*, 2006) provide spatio-temporal information that suggests that miR167 loci regulate the clearance of *ARF8* mRNA transcripts in specific regions of the ovule during development and flower maturation (Wu *et al.*, 2007). Examining miRNA loci expression pre- and post-fertilization should clarify the roles of each loci as to whether they (a) merely maintain transcriptional mRNA lability; (b) they refine expression patterns of transcription factors or (c) take part in triggering development. *ARF8* is apparently self-regulated (Goetz *et al.*, 2006) leading to an alternative theory in which miR167 resolves this function.

miRNA mobility may also be important in the context of fruit initiation and fruit development. Experiments by Tretter *et al.* (2008) have shown that ta-siRNAs, targeting *ARF2*, *ARF3* and *ARF4*, indeed appear to be mobile from cell to cell in vegetative tissues, but miRNAs appear to be limited to cell autonomous action in the same area (Tretter *et al.*, 2008). Given that miR160 and miR167 are located near vascular tissues, evidence for miRNA mobility is less unambiguous. miRNAs, along with various other types of RNAs and RNA-binding proteins, have been isolated and localized in phloem sap (Yoo *et al.*, 2004; Omid *et al.*, 2007; Buhtz *et al.*, 2008; Deeken *et al.*, 2008; Pant *et al.*, 2008). Experimentation with miR399, along with micro-grafting experiments, categorically shows that miR399 is transported in phloem sap of diverse plant species and acts as a systemic molecule that regulates phosphate homeostasis (Deeken *et al.*, 2008). Therefore, although miRNAs mobility in the ovule and

gametophyte has not yet been addressed, it is possible thet the mobility of miR160 and miR167 may be an important factor in their function.

miRNAs and ta-siRNAs regulating ARFs are highly conserved plant gene families (Fahlgren *et al.*, 2007) and a non-neutral selection in miR167 stem-loop structures has occurred with *Arabidopsis* accessions in contemporary times (Ehrenreich and Purugganan, 2008). Strongest purifying selection conserves the short nucleotide sequence of miR167 and the binding sites of exon 13 from both *ARF6* and *ARF8* to gymnosperm and angiosperm lineages (Axtell and Bartel, 2005; Axtell *et al.*, 2007; Axtell and Bowman, 2008). Functional miR167 activity against *ARF8* in gymnosperm female gametophytes and zygotic embryos (Oh *et al.*, 2008) also supports the conservation of the *DCL1-miR167-ARF8* pathway as well as its association with the control of seed development. Several hypotheses about miRNA evolution have been formulated (Chapman and Carrington, 2007) and the roles of miR167-ARF8 in both seed and fruit development could be clarified through the analysis of ancestral miR167 and *ARF8* families in basal angiosperms and gymnosperms.

Often phenotypes of *Arabidopsis* miRNA pathway mutants are affected by the genetics of the ecotype background and this may have a specific bearing on fruit initiation. For instance, there are dramatic modifications of the weak *dcl1* phenotype between the Columbia and the Landsberg *erecta* background (Schauer *et al.*, 2002). Similarly, there are also ecotype-specific modifiers of the *ago1* and *ago10/zwille* mutations (Vaucheret, 2008). Moreover, Landsberg *erecta* shows dramatically increased sensitivity to auxin, parthenocarpy or the *fwf/arf8* mutations compared to the Columbia background (Vivian-Smith, 2001; Vivian-Smith *et al.*, 2001). This leads to the hypothesis that the ecotype-specific modifiers of *AGO1*, *AGO10*, *DCL1* and *ARF8* pathways might be linked to miRNA biogenesis, action or RNA metabolism. The modifier or modifiers, effecting auxin responsiveness and fruit initiation could therefore be dependent on miRNA or AGO-related activities.

Given the important interactions of the non-coding RNA regulation with the auxin response pathway, and the evolutionary conservation of miR167 in seed plants, together with the specific expression of miR160 and miR167 in ovules, one can expect significant new findings concerning non-coding RNA regulation and fruit initiation. This is the case for the *GA2ox2* gene that could be targeted by miR390 activity (Adai *et al.*, 2005). Notably *GA2ox2* provides parthenocarpy when mutated (Rieu *et al.*, 2008a), but conclusive activity by miR390 on *GA2ox2* has not yet been proven, and as such this link must be considered with caution.

4.8 Signal transduction from ovule to carpel and vascular canalization

Fruit initiation involves coordinated intra- and inter-organ signalling between the ovule and carpel. The data by Vriezen *et al.* (2007) highlight that numerous signalling components in the ABA, ethylene, cytokinin, GA and

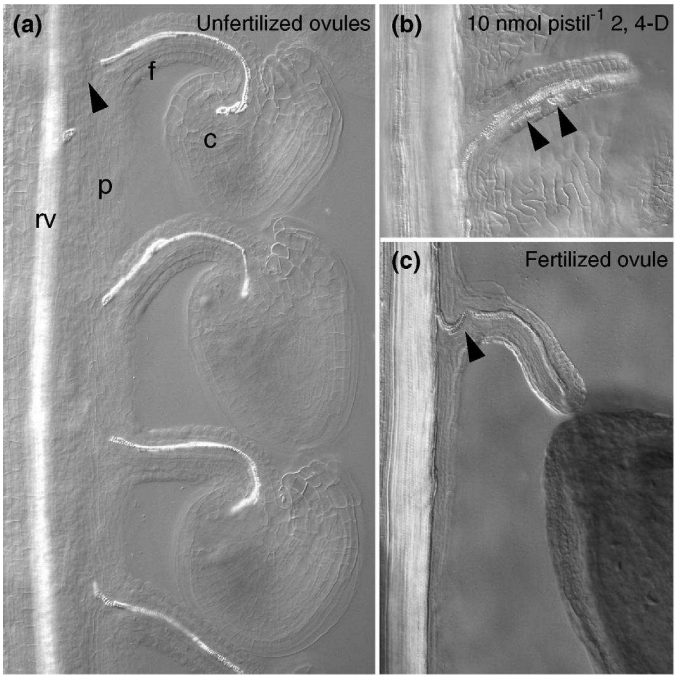


Figure 4.8 Post-fertilization canalization of vascular development in *Arabidopsis* ovules. (a) Development of vascular networks in unfertilized ovules. Unfertilized ovules have a vascular strand that is separated from the replum vascular strand (arrow). (b) Upon treatment with 2–4 D or (c) pollination, vascular biogenesis occurs, thereby joining the replum vascular network with the ovule vascular network. Note that 2–4 D treatment also induces isolated vascular elements throughout the funiculus (arrows). c, chalaza; f, funiculus; p, placental tissue; rv, replum vascular bundle.

auxin pathways are already modulated within 24 h post-pollination. In a short time period, developmental changes also occur and carbon partitioning is established. The development of vascular networks in the ovule, carpel and pedicel of the flower facilitates this process. At anthesis, the vascular network in *Arabidopsis* is separated between the unfertilized ovule and carpel (Fig. 4.8a). A connection is initiated within 54 h post-pollination between the fertilized ovule, which contains a single vascular strand, and the carpel (Fig. 4.8c). This time frame is similar to the *Zinia* in vitro system where tracheid biogenesis occurs within 48 h after auxin and cytokinin treatment (Pesquet *et al.*, 2005). In *Arabidopsis*, treatment with 10 nmol pistil^{-1} 2,4-D (a synthetic auxin) alone was able to induce vascular development in unfertilized ovules (Fig. 4.8b) suggesting that PAT may facilitate the development of this critical vascular junction. In fact, vascular development at this junction occurs precociously in the *fwf/arf8* mutant background supporting the idea that *FWF/ARF8* restricts auxin responses within the ovule (Vivian-Smith, 2001; Vivian-Smith *et al.*, unpublished data). This is compatible with experiments where combinations of the *fwf-1/arf8-4* mutant with mutations in ovule

development show that a signal is generated in the ovules of the *fwf-1/arf8-4* mutant that stimulate the growth of the carpel into a fruit (Koltunow *et al.*, 2002; Vivian-Smith *et al.*, unpublished data).

Vascular biogenesis and development within the ovule may ultimately alter carbon partitioning and source-sink relationships. The processes of vascular biogenesis may not only be restricted to the ovule–carpel vascular junction, but also elsewhere in the flower, since vascular biogenesis, or its absence, in the pedicel has been linked to growth and abscission in citrus (Bustan *et al.*, 1995), *Prunus* (Else *et al.*, 2004) and apple (Drazeta *et al.*, 2004), respectively. Importantly, these observations appear to pinpoint a time when discrimination occurs. Competition begins between fruit and flowers, and causes the fruit abscission often observed in the first week of many important commercial tree crops (Sedgley and Griffin, 1989). Competition also occurs between parthenocarpic fruit and seeded fruit, which are usually stronger (McConchie *et al.*, 1994). Vascular biogenesis presumably reinforces nutrient and photoassimilate allocation to developing fruit affecting retention. Interestingly, the fruit weight locus (*fw2.2*) from tomato is highly expressed in ovules at anthesis and is responsive to adjacent fruit loads (Baldet *et al.*, 2006). This suggests an early role for *fw2.2* in fruit retention. The *Arabidopsis SUC2*, on the other hand, is expressed in the funiculus of the ovule and is required for phloem loading and unloading (Truernit and Sauer, 1995). However, no studies have yet documented *SUC2* expression in enough detail during the pre- and post-fertilization stages to determine whether *SUC2* expression is related to phytohormonal responses in the ovule.

4.9 Current models of fruit initiation

The utilization of PAT inhibitors has provided an excellent understanding into the basis of auxin-mediated vascular network biogenesis in leaves and inflorescences (Mattsson *et al.*, 1999; Sieburth, 1999; Scarpella *et al.*, 2004; Sauer *et al.*, 2006). In leaves, auxin is thought to be synthesized in the marginal tissue and transported away via PAT that is dependent on *MP* and *PIN* efflux carriers (Scarpella *et al.*, 2004; Sauer *et al.*, 2006). However, transport also self-reinforces and stimulates the development of provascular strands that would act as efficient drainage canals, thereby developing the observed vascular networks. In *Arabidopsis* ovules, an analogy can be drawn, with the integuments of the ovule regulating the provision of auxin to the chalaza and funiculus and then to carpel margin. Auxin synthesis after fertilization in the integuments, or integument tips, would not only stimulate the formation of the provascular network in the carpel margin, but also the growth of the carpel into the fruit (Fig. 4.9). Prior to fertilization, *ARF8* is expressed in the female gametophyte, the endothelium of the inner integument and in the chalaza/funiculus regions (Goetz *et al.*, 2006). At this stage, the activity of *ARF8* would restrict the auxin response, possibly through self-reinforcement (Goetz *et al.*, 2006) and through the interaction with Aux/IAA proteins, presumably IAA9 and IAA28, which are expressed in *Arabidopsis* ovules (Fig. 4.9). *KAN4* may function during

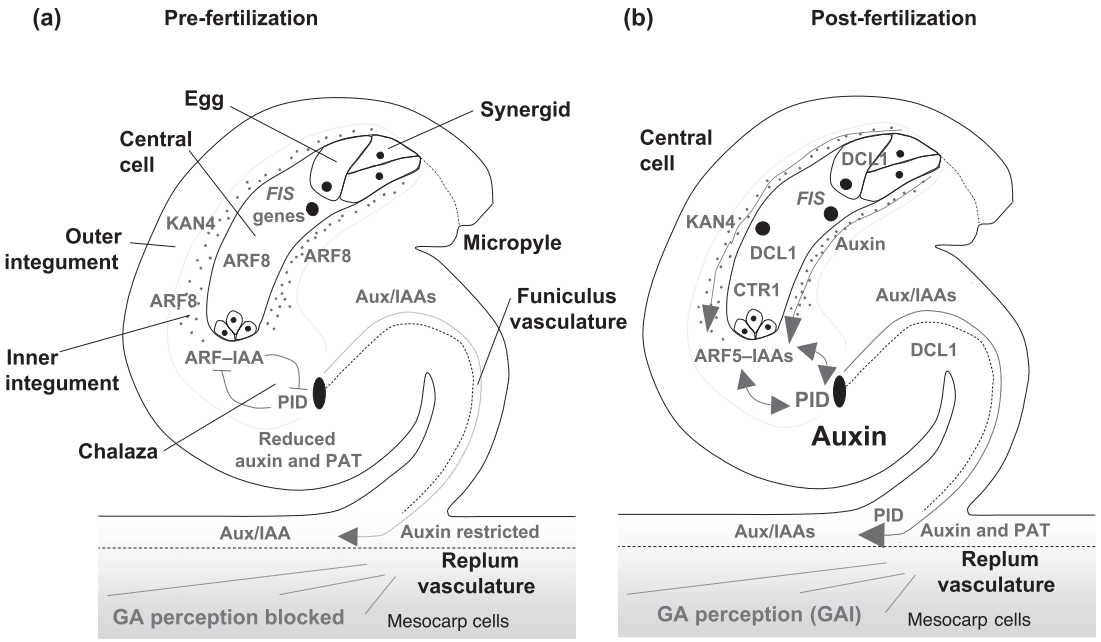


Figure 4.9 Integrated model of fruit initiation. (a) In the absence of fertilization, *FIS* class genes actively restrain central cell growth and autonomous endosperm proliferation. The primary auxin response is also restricted by the activity of ARF8 in the ovules. Specific ovule cells in the pathway shutdown the intracellular auxin response and communication via ARF–ARF and ARF–IAA protein interactions. PID may not play a direct role in this communication but might become important later in the post-fertilization PAT processes. KAN4 may contribute to the synchronization of pistil development before anther dehiscence via control of PAT. In the carpel, GA response remains blocked by the restraint in growth imposed by DELLA proteins. (b) Following double fertilization, zygote and endosperm development is initiated. Concomitant upon the first nuclear division in the endosperm 3–5 h post-fertilization, the primary sporophytic auxin response is initiated in the chalaza and endothelium. The restraint upon auxin response is also eliminated possibly by DCL1-mediated ARF8 removal. Upregulation of the PAT results in the auxin growth response being transmitted to the carpel which in turn, triggers the GA biosynthesis pathway and vascular development. Increased levels of gibberellins cause growth stimulation by DELLA protein degradation.

female receptivity by altering PAT in the ovule (Pekker *et al.*, 2005; Izhaki and Bowman, 2007; Fig. 4.9) and thereby functioning in the pathway that synchronizes pistil growth before anther dehiscence (Fig. 4.9). The stimulation of *GH3* transcription by *ARF8* and possibly *ARF6* (Wu *et al.*, 2007) would further remove free auxin from the ovule through conjugation with amino acids (Woodward and Bartel, 2005) and together with the above mentioned processes restrict vascular biogenesis between the ovule and carpel (Fig. 4.9). miR167 could either refine *ARF8* expression during the pre-fertilization period or actively stimulate the destruction of *ARF8* transcripts post-fertilization. Alternatively, miR167 could act as a 'circuit breaker' by removing any self-reinforcement of ARF8 self-activation during post-fertilization stages. The expression pattern described by Golden *et al.* (2002) for *DCL1*, however, suggests a role in post-anthesis *ARF8* clearance in the female gametophyte and funiculus vasculature. Since *MP* is also expressed in the funiculus vasculature (Hardtke and Berleth, 1998), a role for *MP* would be in the direct activation of auxin responses post-fertilization in this region (Fig. 4.9). PAT and vascular biogenesis would also positively stimulate GA metabolism since both the KS enzyme are localized with vascular tissues (Fig. 4.9; Silverstone *et al.*, 1997; Vivian-Smith and Koltunow, 1999; Vivian-Smith *et al.*, 2001) and PAT has been documented to stimulate the destruction of DELLA proteins (Fu and Harberd, 2003). *GA2ox2*, which would restrict active GAs prior to fertilization (Rieu *et al.*, 2008a), could come under the control of miR390 driven clearance post-fertilization (Adai *et al.*, 2005). miR160 may also have roles during this since this miRNA is localized in the placental and funiculus regions. ZWL/AGO10 and VCS may participate in vascular biogenesis. The roles of ethylene, ethylene precursor molecules, cytokinin and other phytohormones are difficult to place within an integrated model of fruit initiation at this particular point in time, but they may function in the female gametophyte (Fig. 4.9).

The evolution of fruit initiation has largely been absent from review or research. Yet, the fundamental nature of fruit initiation is central and possibly underpins primary characteristics of the angiosperm: closed carpel and ovule development. Processes of flower maturation and synchronization maybe of recent origin, but the aspects relating to canalization of auxin responses and organ initiation are likely to be of older origin and relevant to the ontogeny of fruit initiation. Deducing homologies in structure between non-angiosperms and angiosperms is difficult and often angiosperm and gymnosperm taxonomy is heavily reliant on vascular morphologies of the ovule integuments and the carpel (Frohlich, 2003). The conserved nature of fruit initiation may be elucidated in part by comparable treatments of models relating vascular biogenesis and canalization together with the control of ovule and carpel identity through the transcription factors studied by Alvarez and Smyth (1999) and Herr (1995). It is worthy to note that expression of *ARF8* and *miR167* does actually occur in the integument primordia, and in the leaf and carpel margins (Wu *et al.*, 2006; Goetz *et al.*, 2007). These are the precise locations where auxin is proposed to be biosynthesized (Mattsson *et al.*, 1999; Sieburth, 1999; Scarpella *et al.*, 2004; Sauer *et al.*, 2006).

A model where *ARF8* restricts auxin responses in key marginal tissues to control organ development and canalization may renew interest in vascular biogenesis as a taxonomic morphological marker, while underscoring the relevance for *ARF8* and miR167 expression analysis. Scrutinizing the wide conservation of *ARF8* and *ARF6*, and miR167 families which are restricted to the seed plants, along with the potential derivation of miR167 from an ancestral *ARF6* or *ARF8* gene may also potentially provide crucial pieces of the missing fruit initiation model while providing information on pathways that were recruited during angiosperm flower evolution.

4.10 Concluding remarks

Many studies have contributed to the better understanding of the complex regulatory system controlling fertilization and fruit initiation. Nevertheless, vital questions remain to be answered. Are hormones the initiators or just systemic components of the signalling cascade? Can we isolate the first step triggering fruit set? What are the signals upstream of phytohormonal signalling that are activated directly after fertilization? Which are the sites of endogenous hormone biosynthesis during fruit initiation and are they regulated in the first steps of fruit initiation? What is the nature of the communication events between the female and male gametophyte? The study of fruit initiation in *Arabidopsis* and other species will undoubtedly help to clarify these and other unknowns.

Finally, a number of publications have stated that *Arabidopsis* and *Brassicaceae* appear to be far from optimal models for fruit development and have suggested that plant species bearing large fleshy fruit offer superior advantages for understanding the molecular basis of fruit initiation. However, genes controlling fruit initiation are likely to be conserved throughout angiosperm plant lineages. Furthermore, many commercial fruit crops have been domesticated over thousands of years and show strong selection for consumer traits (e.g. tomato, Nesbitt and Tanksley, 2002; Bai and Lindhout, 2007; Cong *et al.*, 2008; Xiao *et al.*, 2008; Apple, Harris *et al.*, 2002; *Capsicum*, Paran and Van Der Knaap, 2007; *Phaseolus*, *Curcubitaceae*). This is highlighted by the finding that parthenocarpic figures were intentionally planted as early as 11 200–11 400 years ago in the Jordan Valley (Kislev *et al.*, 2006). As a consequence, many of these crops also show a degree of latent parthenocarpy that does not exist in wild accessions. For example in tomato, 23 commercial cultivars were recently tested all of which displayed certain degree of latent parthenocarpy (Goetz *et al.*, 2007). In contrast, *Arabidopsis* is comparatively free from selected potentiation and latent parthenocarpy and, thus, it is likely to provide a more truthful picture of fruit initiation. Further research together with the transfer of mutant trait loci into crop species will undoubtedly lead to an acceptance of *Arabidopsis* as a tractable model for fruit initiation.

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