Fruit Development and Seed Dispersal

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INITIATION

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Chapter 4 FERTILIZATION AND FRUIT



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Biological standpoint: the success of higher plants hinges on reproduction strategies and the dissemination of viable progeny into habitable environmental niches.

the control on fruit initiation appears to be a universally conserved mechanism amongst all angiosperms.



Fruit Types (seeds black). A–E, succulent indehiscent fruit: A, drupe, 1-seeded (in T.S.); B, drupe, 5 seeded (in T.S.); **C, pome (in L.S.)**; D, superior berry (in L.S.); E, inferior berry (in L.S.); F–K, dry dehiscent fruits; F, many-seeded follicle; G, follicle with 2-winged seeds; H, schizocarp; I, legume or pod; J, lomentum; K, siliqua; L–P, capsules: L, loculicidal capsule; M, septicidal capsule; N, poricidal casule; O, circumsciss capsule; P, schizocarp capsule; Q–U, dry indehiscent fruits, with sections showing position of seed: Q, achene from a superior ovary; R, achene from inferior ovary with apical pappus; S, caryopsis; T, nut; U, samara; V–X, aggregate fruits in L.S.: V, rose 'hip', individual fruits drupelets; W, strawberry, individual fruits achenes; X, blackberry, individual fruits drupelets; Y & Z, multiple fruits in L.S.: Y, syconium or 'fig'; Z, syncarp.



Ecological standpoint:





Domestication of angiosperm (12000 y) Seed production



- Reduced gametophyte
- Ovule Production
- Pollen production (microspores)
- (male gametophyte with sporopollenin)
- Waterless environment
- Pollination vectors (cm vs. Km)





The Development of the Individual Rice Grain from Anthesis through Grain Dry-down



Integument and seed coat Endosperm and albumen movement



Reduced size Most tolerant stage dormant

Selection pressure





Economical standpoint: fruit initiation and fruit set are essential processes in many horticultural and agricultural cropping systems.

The economic relevance of pollination and fertilization is clear if we consider the economic costs associated with their potential loss



Breeding topics:

- Photoassimilate partitioning
- ► Higher yields
- Pre-harvest abscission prevention



Several different pillars of research characterize the published knowledge about fruit initiation and how it relates to fertilization ...

Induced parthenocarpy

Quantifying and localizing phytohormones

Genetic analysis

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.





- 2.1. Pollen-stigma recognition and interaction
- Landing

(pollen on compatible stigma)

Adhere

wet vs. **dry** stigma (*grp17-1* mutant without oleosin pr. In pollen) Primary recognition

Hydrate

Aquaporin-like pr. In stigma ----- water absorption/ pollen acceptance

Germinate

(Time consuming- Phalaenopsis orchid)



2.2 Pollen germination and pollen-tube growth

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 <u>gibberellic</u> acid (GA) mediated control.

Similarly, gibberellins are also necessary for pollen-tube growth across different plant species.

The pollen tube grows by tip growth and periodic <u>callose</u> deposition

GA biosynthesis is essential for pollen germination and pollen tube growth

Promotive effects of pollen germination on FI

- In certain orchids, self-incompatible pollinations arose, pollen tubes grew only a small amount but this appeared to stimulate the development of parthenocarpic fruit.
- In cucumbers and eggplants, the base of the style had to be reached by the pollen tube
- Later measurements of growth-promoting hormones (namely auxin) in pollen grains and pollen tubes ruled out these as the possible sources of growth hormones



- The downregulation of the flavonoid biosynthesis pathway genes, CHALCONE SYNTHASE 1 and 2 (CHS1/CHS2), by RNA interference led to parthenocarpic tomato fruit development.
- 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 ('prickle pollination').



Pollen-tube guidance towards the ovules depends on a complex signaling network which involves signals from the female gametophyte and components of the style and ovary

in the parthenocarpic *pat-2* tomato mutant the distorted pollen-tube growth has been related to the high proportion of defective ovules present in the pistil.

3 Female receptivity and the cessation of gynoecial growth

- The female receptive period is an important final component of the floral maturation process and has a direct bearing on fruit set and initiation.
- The receptive period has also been referred to as the effective pollination period (EPP).

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. ?

Factors affecting EPP

► Temp.

As a rule, pollen-tube growth on floral tissues is temperature dependent.

The optima are usually higher for pollen-tube growth.

Integrity of the female gametophyte

There are marked differences in female receptivity duration between the ecotypes

Hormone content

Mutations in the Auxin Response Factor 8 gene (ARF8), dramatically shorten the duration of female receptivity and lead to reduced seed set.

the receptivity periods in Arabidopsis ovules are significantly shorter than the period to exposures of 10 nmol GA3

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

Environment

Nitrogen

stigmatic secretions induced by pollination, that can help release carbohydrates from the transmitting tissue and prolong embryo sac viability



While the beginning of female receptivity is demarcated by the period when pollen tubes can grow on the stigma, style and transmitting tissue

the end of female receptivity is onset by an irreversible initiation of floral senescence

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.

removal of stamens ??

anthers producing high concentrations of free auxins.

high auxin levels in the tapetal tissues are critical for pollen development (*YUCCA6* and *YUCCA2 expression*).

Prior to 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.

5. Fertilization





In the vast majority of angiosperms, the mature female gametophyte consists of a seven-cell, eight-nucleate 'Polygonum-type' structure, bounded by amembrane that lacks a plant cell wall.

(A) Scheme of a seven-celled embryo sac arranged in four cell types: three antipodal cells, placed just below the hypostase, and a large central cell with its nucleus polarized toward the chalaza (B). At the micropylar end, the egg apparatus is composed of an egg cell (C) and two synergids (D). (B–D) Are Feulgen-stained z-stack images. a, antipodal cells; ccn, central cell nucleus; ec, egg cell; ecn, egg cell nucleus; sy, synergids; syn, synergids nuclei. Bar in (B,C) = 20 μ m and (D) = 10 μ m.





А





5.1 Signal transduction before fertilization

- 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.

two other processes regulating pollen-tube guidance:

- diffusible ovule-derived attractants from unfertilized ovules
- and
- repellents from fertilized ovules.

Auxin, together with calcium produced in synergids, had long been hypothesized as pollen-tube chemotropic attractants



The synergids and the central cell

<u>also</u> play a role in the guidance of the pollen tube prior to fertilization.

In contrast to the GABA gradient, these appear to be short-range recognition and developmentally regulated.





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.



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.

- Double fertilization begins upon fusion of the tube tip with a synergid cell. 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).

Upon discharge of the sperm cells into the degenerating synergid:

1- 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 microtubules (Ye et al., 2002; Raghavan, 2003).

2- Following double fertilization, development of a diploid zygote and triploid endosperm is initiated.

3- The remaining synergid eventually deteriorates (Kasahara et al., 2005)

4- The integuments expand and divide to accommodate the developing embryo and endosperm.

5- 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.

5.3 Signal transduction during fertilization

- After compatible fertilization, rapid changes in membrane-bound calcium occur.
- A complex signalling network is involved in the coordination of double fertilization
- An early signalling event is the generation of a calcium influx following the fusion of the sperm and egg cell.

Characterization of the *Arabidopsis aca9* mutant has further contributed to the understanding of the role played by calcium signalling during fertilization. *ACA9* encodes a Ca2+ pump that is primarily expressed in pollen. Mutant *aca9* pollens not only display reduced pollen-tube growth but are also defective in sperm cell release

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.
- first observed in the FERTILIZATION-INDEPENDENT ENDOSPERM (FIE).
- It is widely accepted that upon fertilization auxin originating from the seed is generated.
- In cdc2a mutant pollen, a single sperm cell is produced and is able to fertilize the egg cell.

5.3.2 Roles of the integuments in fruit initiation

- selection of male gametes arriving at the ovule
- Providing nutritive support to the developing zygote and endosperm,
- signal transduction that directly stimulates fruit initiation.

Cells remain mitotically active throughout the female receptive period. However, soon after fertilization, the integument cells begin expansion and the mitotic index further increases



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 GF 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.)

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.

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.



Table 1. Hormone treatments used to induce parthenocarpy and species related

Hormone related	Treatment	Species	Refs
Auxin	Naphthaleneacetic acid (NAA) 2,4,5-Tri-chlorophenoxy propionic acid (2,4,5-TP) Chlorophenoxy-acetic acid (4-CPA) 2,4-Dichlorophenoxy-acetic acid (2,4-D) Indole-3-acetic acid (IAA)	Cucumis sativus, Elaeis guineensis, Elaeis oleifera, Elaeis guineensis Solanum lycopersicum Solanum lycopersicum, Elaeis guineensis, Elaeis oleifera, Pyrus spp. Arabidopsis thaliana	[54,64,85,94–99]
Brassinosteroids	24-Epibrassinolide (EBR)	Cucumis sativus	[54]
Cytokinin	N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) 6-Benzylaminopurine (BA, benzyladenine)	Ctrulus lanatus, Cucumis melo, Ficus carica L, Cucumis sativus, Pyrus spp., Solanum lycopersicum Arabidopsis thaliana	[57,58,94,99–103]
Ethylene	1-Methylcyclo-propene (1-MCP) (ethylene inhibitor)	Solanum lycopersicum	[56]
Gibberellin	GA _B	Malus domestica, Annona spp., Ficus carica L., Vitis vinifera, Solanum lycopersicum	[41,63,64,94,95,104-106]
	GA _{4+7,} GA ₄	Vitis vinitera, Solanum lycopersicum Cucumis sativus, Pyrus spp.	



- Auxin appears to have a primary role during fruit initiation since the <u>genetic analysis</u> of wild-type Arabidopsis fruit initiation with gibberellin biosynthesis and perception mutants shows that auxin-mediated differentiation underlies other signalling pathways.
- transcriptional profiling during fruit initiation also shows directionality in phytohormonal responses with auxin preceding gibberellin responses at 12–14 h period post-fertilization.
- The use of the <u>transgenic DEFH::iaaM</u> construct in a broad range of species 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.

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.
- The ARFs are a family of Aux/IAA interacting proteins that contain a DNAbinding domain (DBD) which recognizes auxin response elements (AuxREs) in DNA sequences.
- FWF/ARF8 transcripts are naturally downregulated within 24 h postpollination.
2 Horticulture Research, 2024, 11: uhae209



Figure 1. Phylogenetic tree of ARFs involved in fleshy fruit development or ripening. Amino acid sequences of ARFs verified to participate in fleshy fruit development or ripening and ARF family members from Arabidopsis thaliana were used to construct a phylogenetic tree based on neighbor-joining. The sequence alignments were performed with MUSCLE. Green and orange rectangles represent genes involved in regulating fruit development and ripening, respectively. Red and purple circles in rectangles indicate genes with positive and negative roles, respectively, controlling fruit development or ripening, and gray stars denote bidirectional regulators performing both positive and negative functions in the processes. The accession numbers of

AUXIN RESPONSE FACTOR8 Is a Negative Regulator of Fruit Initiation in Arabidopsis

Marc Goetz,¹ Adam Vivian-Smith,^{1,2} Susan D. Johnson, and Anna M. Koltunow³

ARF8:GUS expression is switched off soon after fertilization has occurred in wild-type plants, indicating that a fertilization signal deactivates ARF8. The removal of ARF8 activity after fertilization might abolish a developmental block that represses fruit growth and allows initiation of seed and fruit developmental programs.

In unfertilized ovules, expression of the ARF8:GUS marker persists and staining is observed throughout the ovule, indicating that the negative regulation through ARF8 is kept active. These expression patterns are therefore consistent with ARF8 acting as a negative regulator of fruit initiation, and collectively they indicate a central role for the ovules in mediating positive and negative signals involved in fruit development.



Figure 6. Model for the Role of ARF8 in Fruit Development.



Model for the Role of ARF8 in Fruit Development

Figure 6 shows a model for the role of ARF8 during the transition from carpel to fruit growth. Our data suggest that ARF8 represses fruit development, and in the simplest model, ARF8 may do so by directly activating genes that themselves repress fruit development. Alternatively, ARF8 may invoke repression by being a member of a complex of proteins. Protoplast transformation experiments support the concept that the transcriptional activity of the ARF8 protein is regulated by heterodimerization with auxin/indole-3-acetic acid (Aux/IAA) proteins that inhibit this activity (Guilfoyle et al., 1998; Ulmasov et al., 1999a, 1999b; Guilfoyle and Hagen, 2001; Rogg and Bartel, 2001; Liscum and Reed, 2002; Tiwari et al., 2003). Physical interactions between ARF8 and members of both the Aux/IAA repressor and ARF protein families have been demonstrated (Hardtke et al., 2004;

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Mini Review

Auxin response factors: important keys for understanding regulatory mechanisms of fleshy fruit development and ripening Downloaded from https

Bai-Jun Li^{1,2,3,*}, Ruo-Xuan Bao¹, Yan-Na Shi^{2,3,4}, Donald Grierson^{2,5,*} and Kun-Song Chen^{2,3,4}



Figure 2. Roles of ARFs in fleshy fruit development. The deduced roles of ARFs reported to participate in fleshy fruit development in tomato and strawberry are shown. Red arrows and green lines indicate activation and inhibition, respectively. ARF, auxin response factor; IAA, AUX/IAA protein; AG, AGAMOUS1; AGL, AGAMOUS-LIKE 6; MADS, MADS-BOX; XTH, xyloglucan endotransglucosylase/hydrolase; GA20ox, GA 20-OXIDASE; GID, GIBBERELLIN INSENSITIVE DWARF; GA, gibberellin; RGA, DELLA protein; CYCD, Cyclin D; SAUR, small auxin upregulated RNA; EXP, EXPANSIN Based on research discussed in the text.



Three important processes that regulate auxin action in the flower and fruit are its biosynthesis, transport and catabolism.

iaaM is involved in the conversion of tryptophan to indole-3-acetamide, which is the hydrolyzed to IAA.

In an 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...



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

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.



a chemiosmotic model was proposed in which the noncharged, lipophilic IAA molecule enters the cell through diffusion, or through the action of a saturable auxin import carrier.

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.

The specific location of AECs at the basal side of the cell was hypothesized to be the ratelimiting transport step of PAT.

The *PIN1* gene encodes a transmembrane protein that has similarity to bacterial-type transporters and data suggest that PIN proteins function as AECs.

The *PIN* family in *Arabidopsis* comprises eight members six of which have been functionally characterized through genetic analysis.

Genetic analysis has also uncovered partial redundancy and functional compensation amongst PIN family members. 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.

6.1.3 Auxin signalling and feedback regulation

Auxin rapidly induces early auxin response genes including two other major classes, SAURs and GH3s.

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.



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/IAA protein, and enhanced by MYB77-ARF interaction with the transcriptional response elements adjacent to the AuxRE. (d) The synthetic auxin-responsive reporter, *DRSrev::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.



- Gibberellins form a large family of tetracyclic diterpernoid compounds of which only a small number are active PGRs.
- 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 fruitsetting compounds.



In higher plants, GA biosynthesis can be divided into three stages:

- 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).
- In the second stage, GA12 and/or GA53 are produced as a result of the action of ent-kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO) in the case of GA12 and an extra 13-hydroxylation step in the case of GA53.
- The final stage results in the synthesis of active GAs through two parallel pathways (Fig. 4.5):

the non-13-hydoxylation pathway (leading to GA4) and the early 13-hydroxylation pathway (leading to GA1 and GA3 in some cases). Enzymes involved in this final stage include 2-oxoglutarate-dependent dioxygenases, GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox).



Figure 4.5 The GA biosynthesis pathway in higher plants. The pathway is shown from the common precursor geranylgeranyl diphosphate to the active gibberellins GA₁ and GA₄. The names of the enzymes catalyzing each step are shown in italics.



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.

- in pea, removal of seeds reduced the activity of GA 20-oxidase in the pericarp.
- In tomato, pollination/fertilization results in increased expression levels of GA 20-oxidases on the ovary.

2 recent studies:

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-typepollinated 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).



6.2.2 Gibberellin signalling

Similarly to the GA biosynthesis pathway, GA response and signalling path-ways 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.



<u>The SPINDLY (SPY) locus</u> 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)





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 *asSIDELLA* transgenic fruit and hand-pollinated *asSIDELLA* fruit (adapted from Marti *et al.*, 2007). Used with permission of the publisher and authors.

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.
- Like numerous other phytohormones, cytokinins have also been identified as potential phytohormonal components produced in developing seeds that could promote fruit development.
- ▶ Indeed, exogenous cytokinin application to pistils can result in parthenocarpic fruit development in *Arabidopsis*, *Brassica napus* and in pea amongst many other species.
- Additionally, stimulation of cytokinin biosynthesis by using fruit and ovary-specific expression of the *ipt* gene from Agrobacterium produces parthenocarpy in tomato.
- 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.



- Cytokinin downregulated ARF8, PIN2 and an auxin biosynthesizing nitrilase gene.
- These three genes are characterized as late responders to cytokinin treatment since the modulation of expression occurred after 120 min.
- This matches well with data that cytokinin treatment to pistils can trigger an auxin response in unfertilized Arabidopsis ovules.
- 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 (<3h). 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.



- In contrast to cytokinin, ethylene has been typically associated with floral and fruit abscission and in fruit ripening.
- 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.
- Proof of the definitive involvement of the ACC in fruit initiation: exogenous application of ACC to unpollinated pistils induced fruit elongation (Tang, 2003).
- Genetic analysis proved that ACC induction was completely dependent on AXR1, (a ubiquitin-activating) enzyme involved in the auxin response pathway.
- 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 may suggest that ACC is an important <u>player in fruit</u> <u>set.</u>

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 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. These observations suggest that a hormonal interplay is necessary for normal fruit development.

Growing evi-

dence 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 GA₁₉ to GA₂₀ 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 GA₃ treatment provided data that auxin-induced transcripts were unaffected by GA3 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.



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.

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



A number of studies have also considered the interplay between <u>auxin</u> and other hormones such as <u>cytokinins</u>, <u>ethylene and abscisic acid (ABA)</u> 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 <u>ABA and ethylene</u> 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). Review

Parthenocarpy, a pollination-independent fruit set mechanism to ensure yield stability

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Box 1. Roles of phytohormones in fruit set and their metabolism

IAA, under its active form, the indoleacetic acid, regulates cell division. The biosynthesis of IAA is catalyzed by YUCCA proteins or indole synthase. Auxin has an autoregulation system: a high level of IAA promotes Aux/IAA genes expression, which downregulates the auxin biosynthesis, whereas a low level of IAA downregulates Aux/IAA gene expression, suppressing the downregulation of IAA synthesis.

GA is mostly responsible for cell expansion. Five GA forms are considered as bioactive in angiosperms: GA₁, GA₃, GA₄, GA₇, and more recently DHGA₁₂. Their binding to *GID1*, a GA receptor, will activate the GA responsive pathways. GA 3β-hydroxylase and GA 20-oxidase (respectively, GA3ox and GA20ox) catalyze the biosynthesis of GA₁ and GA₄, which are then catabolized by GA 2-oxidase (GA2ox), whereas the biosynthesis of GA₃ and GA₇ is still unclear. GA are promoted by IAA response and repressed by DELLA proteins. GAs are responsible of DELLA protein degradation, enabling the activation of DELLA-repressed genes, initiating fruit growth.

ETH mainly impacts fruit maturation and abscission in climacteric fruits. Key enzymes in ethylene biosynthesis pathway, converting S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene, are, respectively, ACC synthase (ACS) and ACC oxidase (ACO). Ethylene receptors such as ETRs initiate ethylene signaling, which leads to the production of ethylene response factors (ERFs). Ethylene downregulation is induced under low levels of the hormone.

ABA is mainly described as regulating fruit maturation in nonclimacteric fruits and as a promoter of sugar accumulation. The main enzyme involved in its biosynthesis is the 9-cis-epoxycarotenoid dioxygenase (NCED), which catalyzes xanthoxin synthesis, a molecule then transported into the cytoplasm and metabolized in ABA. In *Arabidopsis thaliana*, the main catabolism pathway of ABA was characterized and the principal regulator identified is the cytochrome P450 monooxygenase, encoded by a member of the CYP707A family.

CKs are key promoters of cell division and differentiation. They are adenine derivatives, and their main active form in plants, trans-zeatins, are synthetized by three major enzymes: isopentyl transferase (IPT), cytochrome P450 CYP735A, and nucleoside 5'-monophosphate phosphoribohydrolase LONELY GUY (LOG). During flowering, another form, cis-zeatin riboside, is also described as important.

BRs, in fruit development, act in cell elongation, cell division, and fruit ripening. The most active BR identified is called brassinolide. BR biosynthesis begins with campesterol which undergoes a C-6 oxidation and a C-22 oxidation. Its biosynthesis is also catalyzed by a cytochrome P450 (CYP90B1 and CYP85A1/2).



Figure 2. Hormonal and molecular mechanisms involved in fruit setting. (A) Phytohormone dynamics during fruit set initiation. At 1,5 DPA, in pollinated or parthenocarpic flowers, fruit set is initiated by a decrease of ETH and ABA levels or an increase in BR. At 3 DPA, the increase of IAA and CK levels triggers the biosynthesis of GA, leading to fruit development. In the nonfertilized and nonparthenocarpic flower, after anthesis, ABA and ETH levels remain high and inhibit the increase of IAA, CK, and GA levels, leading to flower abortion. Abbreviations: DAA, days ante-anthesis; DPA, days postanthesis. (B) Simplified model of fruit development. Two mechanisms of parthenocarpic fruit set have been proposed. The first mechanism, highlighted in yellow, involves the induction of GA biosynthesis driven by IAA. The second mechanism, highlighted in red, involves the inhibition of GA catabolism. Genes reported to induce parthenocarpy are framed in red. Genes involved in parthenocarpy under cold condition are indicated by a blue snowflake. Abbreviations: ACO, 1-aminocyclopropane-1-carboxylate oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; AGL6, AGAMOUS-LIKE 6; ARF5/7/8/19, auxin-response factors; AUX/IAA9, auxin/indole-3-acetic acid 9; CDK, cyclin-dependent kinase; CYP, cytochrome P450;



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Two hormonal mechanisms for inducing parthenocarpic fruit set have been proposed in the literature (Figure 2B). The first entails the induction of GA_4 and GA_7 biosynthesis, driven by IAA (highlighted in yellow). For instance, in tomatoes, the rise in IAA levels in the ovary prompts the expression of GA 20-oxidases (GA20ox), GA20ox1 or GA20ox2, leading to the biosynthesis of GA_4 and GA_7 . The second mechanism involves the inhibition of GA catabolism in conjunction with an induction of GA_3 biosynthesis (highlighted in red).



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).

8 Signal transduction from ovule to carpel and vascular canalization

- Fruit initiation involves coordinated intra- and interorgan signalling between the ovule and carpel.
- Numerous signalling components in the ABA, ethylene, cytokinin, GA and 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.





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.



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, *Prunus* and apple, 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. Competition also occurs between parthenocarpic fruit and seeded fruit, which are usually stronger.

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.
9 Current models of fruit initiation

- 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.
- Development of provascular strands would act as efficient drainage canals, thereby developing the observed vascular networks.
- 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.
- Prior to fertilization, ARF8 is expressed in the female gametophyte, the endothelium of the inner integument and in the chalaza/funiculus regions. At this stage, the activity of ARF8 would restrict the auxin response, possibly through self-reinforcement.



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.