Abscisic acid and assimilate partitioning during seed development

Abscisinezuur en assimilatenverdeling tijdens de ontwikkeling van zaden

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op gezag van de rector magnificus
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Stellingen

1. De bewering dat symplastische unloading (zoals in zaadhuiden van *Phaseolus*) een 'low resistance pathway' biedt en 'relatively energy conserving' is ten opzichte van apoplastische unloading (Offler en Patrick, 1986), geldt niet voor sinks waarbij de symplastische unloading uit de zeefelementen gevolgd wordt door een apoplastische stap.
   Offler, C.E. and J.W. Patrick (1986) In J. Cronshaw, W.J. Lucas, R.T. Giaquinta (eds.) Phloem Transport, Alan R. Liss, New York, USA, p. 295-306.

2. Het op basis van het mechanisme voor de unloading van assimilaten gemaakte onderscheid tussen reversibele en irreversibele sinks (Offler en Patrick, 1986; Ho, 1988) is erg kunstmatig en voldoet niet voor een aantal zogenaamde irreversibele sinks die vroeger of later weer source worden (zoals ontwikkelende bladeren, aardappelknollen en zaden).
   Ho, L.C. (1988) Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:355-378.
   Offler, C.E. and J.W. Patrick (1986) In J. Cronshaw, W.J. Lucas, R.T. Giaquinta (eds.) Phloem Transport, Alan R. Liss, New York, USA, p. 295-306.

3. Het is een ernstige tekortkoming dat Bangerth in zijn uitvoerige hypothese ter verklaring van het verschil in dominantie tussen vruchten en andere sinks wel aandacht besteedt aan hormoonconcentraties en transport van hormonen, maar geen enkel woord wijdt aan mogelijke verschillen in gevoeligheid voor hormonen tijdens de ontwikkeling van deze sinks.
   Bangerth, F. (1989) Physiol. Plant. 76:608-614.

4. De verschillen in drooggewichttoename van sojaboonzaden bij afwijkende lichtregimes, zoals gevonden door Morandi *et al.* (1990), zijn verwaarloosbaar als de data gefit worden met een niet-lineaire vergelijking (bijvoorbeeld die voor een sigmoïde) in plaats van via lineaire regressie.
   Morandi, E.M., J.R. Schussler and M.L. Brenner (1990) Ann. Bot. 66:605-611.

5. Zowel Perl *et al.* (1991) als Charles *et al.* (1993) claimen condities te hebben gevonden waarbij zich zeer synchroon knollen ontwikkelen uit aardappelexplantaten; in beide gevallen ontbreken echter de data waaruit deze synchroniteit zou moeten blijken.
   Perl, A., D. Aviv, L. Willmitzer and E. Galun (1991) Plant Sci. 73:87-95.
   Charles, G., L. Rossignol and M. Rossignol (1993) J. Plant Physiol. 142:474-479.

6. Het gebruik van de term 'seed tubers' voor moederknollen in de aardappelfysiologie is niet alleen botanisch onjuist maar ook hoogst verwarrend en dient dus vermeden te worden.

7. De door Faust *et al.* (1991) gelegde relatie tussen de mate waarin water in rustende appelknoppen gebonden is (zoals vastgesteld met behulp van magnetische resonantie imaging) en de vorm van rust waarin deze knoppen verkeren, wordt door hen op geen enkele manier fysiologisch onderbouwd.
   Faust, M., D. Liu, M.M. Millard and G.W. Stutte (1991) HortSci. 26:887-890.

8. De positieve correlatie tussen het hormoongehalte en de groeisnelheid van zaden is geen enkel bewijs dat het betreffende hormoon betrokken is bij het bepalen van de groeisnelheid van deze zaden.
   Dit proefschrift.

9. Het voorkomen van ABA in de hersenen en het centrale zenuwstelsel van zoogdieren biedt interessante aanknopingspunten voor speculaties over de fysiologische rol van ABA in dieren of mensen, zoals stressbestendigheid, regulering van de gasuitwisseling en waterhuishouding, of de aantrekkingskracht voor assimilaten.
   Le Page-Degivry, M.-Th., J.-N. Bidard, E. Rouvier, C. Bulard and M. Lazdunski (1986) Proc. Natl. Acad. Sci. USA 83:1155-1158.

10. De opvatting van de klassiek-homeopathische volgelingen van Hahnemann dat de werking van gepotentieerde en sterk verdunde homeopathische middelen berust op het spiritualiseren van materiële substantie en dat dit een van de pijlers is van de homeopathie, is strijdig met het zoeken naar een farmacokinetische verklaring voor het werkingsmechanisme door onderzoekers uit deze groep.
    Hahnemann, S.C.F. (1979) The Healing Art of Homoeopathy - The Organon of Samuel Hahnemann. Beaconsfield Publishers Ltd, Beaconsfield, Bucks., UK, § 269.
    De Bruijn, S.M. (1991) Ned. Tijdschr. Klass. Homoeopath. 2:6-13.

11. Het convenant tussen gemeentebesturen en horeca-ondernemers over de aanpak van gokverslaving is voor de gemeenten een gemiste kans en voor de gokverslaafde een verlies.

12. Subsidiëring van kinderopvang door de overheid creëert een vorm van inkomensongelijkheid ten opzichte van ouders die bewust geen gebruik willen maken van deze voorzieningen.

13. Regelmatig wordt bij discussies in confessionele kring over de problematiek van asielzoekers en dak- en thuislozen te weinig gewicht toegekend aan de bijbelse voorschriften ten aanzien van naastenliefde en barmhartigheid.

14. Zolang men niet in staat is om met een redelijke betrouwbaarheid voorspellingen te doen over de weersomstandigheden voor een periode langer dan vijf dagen, is er alle reden om zijn bedenkingen te hebben over de waarde van de sombere voorspellingen voor het klimaat over vijftig jaar.

15. Het promotiereglement van de Landbouwuniversiteit stelt slechts 5000 studiebelastingsuren als norm voor een promotie-onderzoek inclusief de afronding daarvan met een proefschrift; de echte wetenschappers onder de promovendi (van wie verwacht wordt dat ze 's avonds en in het weekend werken: Karssen, 1993) worden hiermee zwaar ondergewaardeerd, aangezien ze al na anderhalf jaar aan deze norm voldoen.
    Karssen, C.M. (1993) WUB 2/9/1993.

16. De naam 'Het Gouden Kalf' voor een filmprijs geeft een goede typering van de moderne filmcultuur.

17. Het is onbegrijpelijk dat mensen die niet in God geloven, er toch zo vaak behoefte aan hebben om Zijn naam te gebruiken, en zelfs Hem vragen allerlei onheil over hen te brengen.

Stellingen behorende bij het proefschrift 'Abscisic acid and assimilate partitioning during seed development' door S.M. de Bruijn, in het openbaar te verdedigen op donderdag 9 december 1993, te Wageningen.

The Master's touch

We see the Master's touch
in every living thing,
we see the loving evidence
of what His hand can bring.
We see the beautiful miracle
reborn in every day,
we see life bloom and grow
in God's most gracious way.

A rainbow bends, a bird takes flight,
His love is there to see,
to witness all the wonder
in each seed and budding tree.
How precious the compassion
and the joy that's felt so much
from the kind and gentle hands
of the Master's loving touch.

Voorwoord

Het is een kenmerk van vele moderne wetenschappelijke publikaties dat ze zo min mogelijk persoonlijk getinte ervaringen bevatten. Dat verhoogt weliswaar de objectiviteit van de waarnemingen, maar vaak ook de saaiheid van de geboden stof. Ook in dit proefschrift zult u vergeefs zoeken naar een omschrijving van de moeite die het kostte om vierhonderd hydrocultuurpotten met erwteplanten te beluchten, of van de hoofdpijn die je kreeg als je zo'n duizend *Arabidopsis*-zaden uit de hauwen had gepeuterd en geteld. Evenmin vindt u een beschrijving van het enthousiasme dat je treft wanneer je uiteindelijk toch verschillen vindt in vetzuur- en suikersamenstelling van deze zaden. Of dit proefschrift daardoor een saai geheel geworden is, laat ik aan het oordeel van de lezer over. Ik troost me met de gedachte dat het Voorwoord en het Curriculum vitae tot de meest gelezen onderdelen van een proefschrift behoren. Juist het Voorwoord biedt me alle ruimte om toch een aantal persoonlijk getinte opmerkingen te plaatsen. Die gelegenheid wil ik benutten om verschillende mensen te bedanken.

Allereerst wil ik mijn dank uitspreken in de richting van mijn begeleiders. Mijn promotor, prof.dr. C.M. Karssen wil ik hartelijk dankzeggen voor zijn inbreng in mijn werk, zowel bij de voortgangsrapportages als tijdens andere discussies. Cees, je hebt me bij het lezen van mijn manuscripten herhaaldelijk verweten dat ik te bescheiden was; het is echter geen kwestie van bescheidenheid wanneer ik vaststel dat je een duidelijk stempel op mijn werk en op dit proefschrift hebt gedrukt.

Mijn co-promotor, dr. D. Vreugdenhil, ben ik nog meer dank verschuldigd. Dick, in het begin zaten we op één kamer, maar de reden dat ik die kamer verlaten moest was blijkbaar niet dat je me moe was; immers, ik ben nu opnieuw aangesteld op een van je

projecten. Ik kon altijd bij je binnenlopen; de vele gesprekken die we hadden beperkten zich overigens niet tot het werk en ik vond het heel fijn dat we bijvoorbeeld tijdens onze reizen konden discussiëren over allerlei dogma's zonder dat dat tot een 'afscheiding' leidde. Ik hoop dat dat ook tijdens onze toekomstige samenwerking zo blijft. Je was een prima begeleider; het enige gebrek dat ik zou kunnen noemen is dat je misschien wat strenger zou moeten zijn voor je promovendi.

Een eervolle vermelding is op zijn plaats voor die groep mensen die vallen onder de categorie OBPers. De naam 'ondersteunend en beheerspersoneel' zegt het al: bij deze mensen kun je steun vinden. Elly Koot-Gronsveld bijvoorbeeld; zij wordt weliswaar alleen in de auteurslijst van Hoofdstuk 3 genoemd, maar dat betekent niet dat haar bijdrage beperkt is gebleven tot dit hoofdstuk, integendeel. Casper Pillen, Aart van Ommeren, Gerrit van Geerenstein en Wilbert Alkemade hebben heel wat werk gehad aan mijn plantjes: hydrocultuur is niet altijd zo simpel, en dubbelmutanten zijn dat al helemaal niet. Desondanks is het iedere keer gelukt om plantmateriaal beschikbaar te hebben. Ruth van der Laan en Ben van der Swaluw verdienen een pluim voor het ontwerpen en maken van verschillende 'staaltjes van vakmanschap': het assimilatiekamertje (Figuur 5.2) is daar een fraai voorbeeld van. Natuurlijk verdienen ook andere mensen een plaats in deze opsomming, zoals Evert Vermeer, Sybout Massalt, Paul van Snippenburg voor hun hulp op het gebied van respectievelijk de chromatografie, fotografie en het tekenwerk. Allex Haasdijk tekende de omslag van dit proefschrift, dat is wel een aparte vermelding waard.

Vele andere collega's hebben in meerdere of mindere mate bijgedragen aan dit proefschrift, het zou heel moeilijk zijn om hiervan een compleet overzicht te geven. Toch wil ik een aantal mensen met name bedanken: Jaap Ooms voor zijn hulp en inspiratie bij het werken met de *Arabidopsis*-mutanten, met jou zowel als met Hans Helder, Frans Tetteroo en Folkert Hoekstra heb ik vele leerzame discussies gehad, onder andere over de suikerbepalingen op de Dionex. Thijs Cornelussen dank ik voor het gebruik van en de hulp bij de mede door hem ontwikkelde ELISA voor ABA.

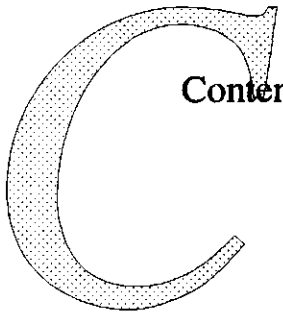
Verschillende studenten hebben een belangrijke rol gespeeld bij het tot stand komen van dit proefschrift. Ook al zijn er veel experimenten uitgevoerd die niet of nauwelijks meer in dit proefschrift terug te vinden zijn, toch heeft elk van de studenten op zijn of haar manier bijgedragen aan de beeldvorming rond de hormonale regulering van assimilatenverdeling. Bert Schnieders, Anne-Marie Kuijpers, Charles Buddendorf en Vivianne Vleeshouwers, allemaal hartelijk dank voor jullie inbreng. Ik denk nog steeds met plezier terug aan de periodes waarin ik met jullie mocht samenwerken.

Ik ben ook dank verschuldigd aan diverse mensen van buiten de vakgroep: dr. P. Wolswinkel en drs. M. van Oene (vakgroep Botanische Oecologie en Evolutiebiologie, Rijksuniversiteit Utrecht) voor de leerzame instructies met betrekking tot de 'empty seed-coat'-techniek. Het McAb-3G4 antilichaam dat gebruikt is in de ABA-ELISA was afkomstig van de groep van dr. P.M. Boonekamp (Laboratorium voor Bloembollen Onderzoek, Lisse). Prof.dr. M. Koornneef (vakgroep Erfelijkheidsleer, Landbouwuniversiteit Wageningen) heeft me de mogelijkheid geboden om met *Arabidopsis*-mutanten te werken: niet alleen het beschikbaar stellen van de mutanten, maar ook de instructies door Corrie Hanhart en het gebruik van kweekfaciliteiten op de vakgroep Erfelijkheidsleer heb ik zeer op prijs gesteld. De ABA-deficiënte erwtemutant is afkomstig van wijlen prof. G.A. Marx (New York State Agricultural Experiment Station, Geneva, VS).

Ook mijn ouders verdienen een plaats in dit Voorwoord. Niet alleen hebben ze mij de mogelijkheid geboden om te gaan studeren, maar bovendien is hun voortdurende belangstelling een goede stimulans geweest. Tenslotte wil ik Anja hartelijk bedanken. Jouw bijdrage aan dit proefschrift ligt op een heel ander vlak. Ik heb veel steun, geduld en liefde van jou ontvangen, wat me telkens weer de moed gaf om verder te gaan. Vele uren hebben jij en de kinderen moeten opofferen voor 'dat boekje'. Maak je geen zorgen: ik begin voorlopig nog niet aan het tweede. Ik hoop dat ik nu wat meer tijd krijg voor ons gezin.

Na het lezen van dit Voorwoord denkt u misschien dat het doen van onderzoek en het schrijven van een proefschrift alleen afhangt van goede contacten met collega's, studenten en familie. Dat is echter een misvatting. Wat voor mij persoonlijk een grote inspiratiebron is geweest, wordt mooi verwoord in het gedicht dat aan dit Voorwoord voorafging. Hoe dieper je inzicht krijgt in allerlei plantenfysiologische processen, hoe meer bewondering je krijgt voor de Maker van dit alles, voor 'the Master's touch'. Juist dat inzicht maakt een onderzoeker bescheiden. De Epiloog van dit proefschrift beschrijft dat op een manier waar ik van harte mee in kan stemmen.

Steef de Bruijn.



Contents

1 General introduction

Crop productivity

The factors that primarily determine the total biomass production and the harvest index (the weight ratio between the harvested organs and the total plant) are inherently associated with the route from carbon dioxide in the atmosphere to the storage carbohydrates, lipids and proteins in the yieldable products. The fixation of carbon dioxide requires a photosynthetic machinery that generates energy from intercepted light and utilizes this energy to reduce carbon dioxide to sugars (mostly sucrose); subsequently in many cases a transport mechanism is needed to carry the sugars from the source leaves *via* the vascular system to sink organs, and eventually the sugars are converted to the desired products. The total biomass production is influenced by a multitude of factors that are mainly confined to the process of photosynthesis, *e.g.* the amount of light that is intercepted, the part of photosynthetically active radiation in the received light, losses by reflection, transmission or ineffective absorption of the light, and losses of fixed carbon by respiration (Gifford and Evans, 1981; Gifford *et al.*, 1984; prerequisites like soil fertility, pest control, water availability *etc.* are left out of consideration here). The harvest index, on the other hand, depends mainly on the partitioning of assimilates over the various sinks in the plant.

The distribution of assimilates between sinks in plants is an important process, in terms of economic relevance. The harvest index, has drastically increased during the last century for most crops, although the total biomass production of these crops hardly changed (Duncan *et al.*, 1978; Austin *et al.*, 1980; Barneix, 1989; Figure 1.1). This

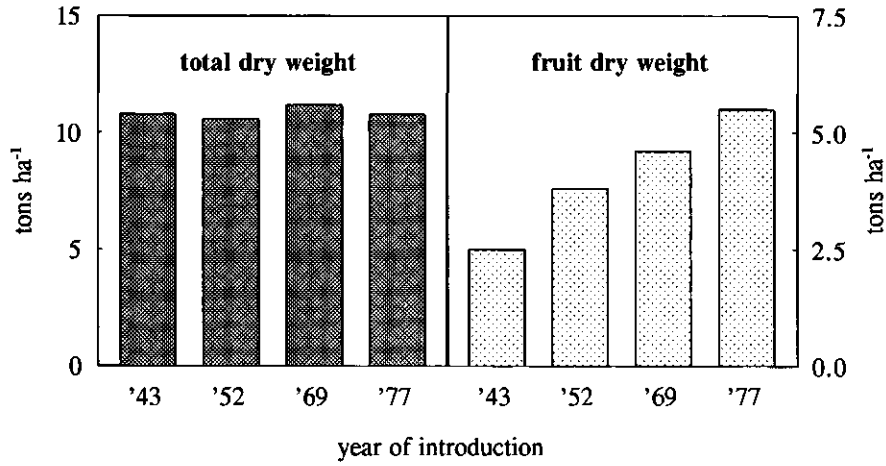


Figure 1.1 Maximum total biomass production and fruit production of four peanut (*Arachis hypogaea* L.) cultivars. All cultivars were grown in 1976, under equal conditions. Apparently, selection for higher yield in peanuts has not resulted in an increase in total dry matter production but in the development of cultivars that partition more of their assimilates to the fruits. Data from Duncan *et al.*, 1978.

illustrates clearly that the gain in crop yield was not primarily caused by an overall increase in the amount of photosynthetically fixed carbon but by a shift in the distribution of assimilates towards the harvested organs, at the expense of the non-harvestable parts of the plants. Nevertheless, the fact that *e.g.* in 1974 the average yield of six major grain crops was still four to seven times lower than the best experimental yield (Wittwer, 1975), suggests that the upper limit of the harvest index is not yet reached, and that considerable improvement may be achieved.

On the other hand, it has to be realized that very high harvest indices are naturally coupled to low investments in the non-harvestable parts (*e.g.* roots, stem and leaves, for grain crops), whereas these parts in most cases play an important role in the water supply, physical rigidity and texture of the plant, and the available photosynthetic area; these factors become more important as the weight or size of the harvestable parts increases. From that point of view, selection for maximal harvest indices for crops may lead to more vulnerable plants, such as an increased susceptibility to water stress because the investments in roots were reduced. It is likely that when a particular optimal harvest index has been reached, an increase of the total biomass production will be easier to achieve.

Assimilate partitioning

In some crops (*e.g.* spinach, lettuce and other green vegetables), the photosynthetically active tissues are the yieldable products themselves, whereas in other crops (*e.g.* potato, sugar beet, peanuts) the harvestable parts consist of non-photosynthetic tissue. In the first case the harvest index is highest when no carbohydrates are exported from the green tissues; under-ground crops, on the other hand, are completely dependent on import of assimilates from source leaves. In both situations, however, harvestable organs have to compete with other sinks on the plant, *e.g.* the roots, apices, young leaves, the stems or the reproductive tissues. *Assimilate partitioning is the process of distribution of dry matter among the various sinks, coordinated according to their changing requirements (their sink strength) throughout the life of a plant* (Hay and Walker, 1989).

Traditionally, sinks are defined as plant parts with a net import of assimilates, whereas sources export assimilates (Wareing and Patrick, 1975; Evans, 1975), although the terms sink and source are not restricted to the distribution of photoassimilates but can be applied to any particular substance and at any level - organelle, cell, tissue, organ, plant, or even between organisms in plant-parasite relationships (Warren Wilson, 1972). Derived from this definition, the transport pathway of substances from sinks to sources can be very short (*e.g.* the flux of carbon from soluble sugars in the cytosol to starch in the plastids) or very long (*e.g.* the transport of nitrogen through the vascular bundles from the roots to the shoot apex in trees), but usually the terms sink and source are used in the context of the distribution of assimilates from mature leaves to the stems, roots, apices, fruits or seeds.

The transport pathway

Transport of carbohydrates from sources to sinks can be divided in several sub-processes: efflux from the mesophyll towards the phloem, loading into the sieve elements, transport through the phloem, unloading from the sieve elements into the sink organ or tissue, and finally: storage or metabolism within the sink.

The transport of sugars in the source leaves in the direction of the phloem is supposed to occur mainly *via* a continuum of cytosoles connected with plasmodesmata (the symplast), although also some evidence exists for transport of assimilates through the intercellular free space (the apoplast) of the leaves (Delrot and Bonnemain, 1979). Two

basic models have been described for the transport of solutes into the sieve tube/companion cell complex of the phloem: the symplastic pathway supposes the presence of plasmodesmata between mesophyll cells and sieve elements, whereas the apoplastic pathway assumes active transport of solutes across cell membranes through cell walls. Several variants on these models were recently discussed by Van Bel (1992, 1993). Phloem loading is a selective process: depending on the vein type (Gamalei, 1989) sucrose or raffinose is preferentially loaded, whereas reducing sugars are almost absent in phloem sap (Ziegler, 1975). Apoplastic loading is an energy-requiring process: sucrose in the apoplast binds to a membrane carrier, together with a proton, and is released inside the symplast of the sieve element/companion cell complex, against a sucrose concentration gradient; this carrier is driven by a downhill proton gradient brought about by a membrane ATPase (sucrose-proton co-transport: Giaquinta *et al.*, 1983; Komor and Orlich, 1986). A more speculative mechanism for symplastic phloem loading against a concentration gradient, based on a trap mechanism of polymerized sugars, was proposed by Turgeon (1991).

Mass-flow transport of solutes through the phloem is driven by a pressure gradient between source and sink (pressure-flow hypothesis: Münch, 1930). Active phloem loading in sieve elements at the source end of a phloem vessel increases the osmotic pressure, water (from the xylem) enters the sieve elements and causes an increased turgor pressure. At the sink end the osmotically active solutes are released from the phloem (unloading), water leaves the system and the turgor pressure decreases, thus creating a pressure gradient throughout the phloem. An oppositely directed transport of water occurs in the xylem. Not only sugars, but also potassium ions may play a significant role in the establishment of the turgor pressure gradient in the phloem pathway (Lang, 1983). Such a source-to-sink gradient of potassium has been demonstrated in cassava and castor bean stems (Vreugdenhil, 1985). During the transport, solutes leak out from the phloem and are reabsorbed (Minchin and Thorpe, 1984) by a carrier-mediated proton co-transport (Grimm *et al.*, 1990); reloading is probably facilitated by the symplastic isolation of domains of sieve element/companion cell complexes (Van Bel and Kempers, 1991).

Phloem unloading

Solutes can leave the phloem *via* plasmodesmata (symplastic) or cross the plasmamembrane into the cell wall (apoplastic). Evidence for symplastic phloem unloading was

found in the young, expanding leaves of sugar beet (Gougler Schmalstig and Geiger, 1985) and tobacco (Turgeon, 1987), developing tubers of potato (Oparka and Prior, 1986) and root apices of corn (Giaquinta *et al.*, 1983) and pea (Dick and ap Rees, 1975). Apoplastic sieve element unloading was demonstrated in sugar cane stalks (Glasziou and Gayler, 1975), sugar beet tap roots (Wyse, 1979), in fruits of citrus (Koch *et al.*, 1986) and tomato (Damon *et al.*, 1988), and in stems of *Phaseolus* (Hayes *et al.*, 1987) and *Ricinus* (Van Bel and Kempers, 1991). However, some of these findings were recently criticized by Patrick (1990) and Oparka (1990). In some cases, apoplastic phloem unloading is associated with hydrolysis of sucrose by an acid invertase prior to uptake by the plasmamembrane of the receiving cells (corn endosperm: Porter *et al.*, 1985; tomato fruit: Damon *et al.*, 1988). It has been supposed that extracellular hydrolysis of sucrose prevents reloading of hexoses into the phloem and thus stimulates phloem unloading (Eschrich, 1986; but see Gougler Schmalstig and Hitz, 1987).

Thus far, it was not possible to definitely attribute the different unloading mechanisms to specific sink functions, although it was proposed by Ho and Baker (1982) and by Offler and Patrick (1986) that symplastic phloem unloading predominantly occurs in young, developing systems since they require a low-resistance pathway where disruption of sieve element turgor should be avoided (*e.g.* in apices). It is imaginable that apoplastic phloem unloading is more important in sinks where solutes accumulate to very high levels (*e.g.* in fruits or seeds). The distinction between apoplastic and symplastic phloem unloading is not very sharp, however, since it was shown that apoplastic unloading from axial phloem along bean stems can switch to symplastic unloading, depending on the conditions (Offler and Patrick, 1986; Hayes *et al.*, 1987). A similar process is supposed to occur in developing potato tubers: phloem unloading in stolon tips is apoplastic, whereas unloading in tubers occurs *via* the apoplast (Oparka and Prior, 1986; Helder, 1994).

In developing seeds phloem unloading is generally apoplastic (according to the terminology of Oparka and Van Bel, 1992): the assimilates move symplastically from the sieve elements through several layers of maternal tissues (*e.g.* parenchyma, placental tissues, transfer cells) and subsequently enter the extracellular space or cavity surrounding the embryo and/or endosperm (Thorne, 1985; Grusak and Minchin, 1988). In legume seeds, the phloem terminus in the seed-coat is sometimes reticulated (soybean: Thorne, 1981), or restricted to a few veins (pea and broad bean: a major median strand and two lateral veins: Hardham, 1976; Offler *et al.*, 1989). Delivery of radiolabelled assimilates into pea seed-coats is not equally distributed over the seed-coat surface (Grusak and

Minchin, 1988). Symplastic connections between the maternal and embryonal tissues have never been observed: all assimilates have to pass through the apoplast separating the two generations, and must be taken up actively by the plasmamembrane of the cells in the cotyledons or the endosperm. Uptake kinetics of solutes by the cotyledons or the endosperm consist of an active, energy-requiring component and a diffusion component (soybean: Lichtner and Spanswick, 1981; Thorne, 1982; wheat: Ho and Gifford, 1984). Again a sucrose-proton co-transport seems to be involved.

Use of assimilates within sinks

Assimilates that arrive in sink organs are used for growth, maintenance or storage. Based on the fate of the imported assimilates, sinks can be distinguished in growth sinks and storage sinks (Ho and Baker, 1982). In growth or utilization sinks (mostly meristems), assimilates are primarily used for structural growth and respiration, whereas storage sinks (*e.g.* seeds, fruits, tubers, roots or stems) accumulate the largest part of the imported assimilates. The distinction between growth sinks and storage sinks is not sharp since in many cases growth sinks develop into storage sinks.

Storage sinks can contain very high levels of reserve material. Carbohydrate reserves can accumulate up to 70 or 80 % of dry weight (xyloglucan in tamarind seed: Kooijman, 1961; starch in wheat endosperm: Jennings and Morton, 1963; sucrose in sugar beet tap root: Milford, 1973), or even 61 % of fresh weight in dates (Biale, 1960). In oil-rich seeds, the imported sucrose is converted to lipid reserves: the dry matter of oilseeds may contain up to 74 % lipids (coconut endosperm: Salunkhe *et al.*, 1992). Combinations of different storage materials are also possible: cottonseed kernels contain 39 % protein and 33 % oil on a dry weight basis (Morrison, 1936). Conversion of one type of storage material into another is a common phenomena: tomato fruits initially accumulate starch but hydrolyse this to hexoses when the fruits mature (Ho *et al.*, 1982/83); rape seeds also start with the accumulation of starch, but in mature seeds all starch is converted to lipids (Norton and Harris, 1975).

Control of assimilate partitioning

Regulation of the distribution pattern of assimilates is most likely to happen at the sink end of the transport pathway: the source determines the timing and the quantity of

assimilates available for export, but not their destination (Gifford and Evans, 1981). It is also unlikely that the vascular system itself restricts or controls the allocation pattern of assimilates (Gifford and Evans, 1981), except in the case of developing primordia where the absence of a differentiated transport channel limits the supply of assimilates (Patrick, 1972). Although the transport of solutes through a sieve element is by nature unidirectional, transport in two directions has often been detected in different sieve elements or different vascular bundles in the same stem (Eschrich, 1975). Moreover, in many plants the assimilate transport from source leaves to sink organs is restricted to their own orthostichy (vertical arrangement of leaves, scales or flowers along an axis), because sources and sinks in the same orthostichy share a more or less separate network of vascular pipelines with few lateral connections to that of other orthostichies (Borchers-Zampini *et al.*, 1980; Murray *et al.*, 1982). The vascular system may also function as a kind of messenger channel for the interactions between source and sink. It has been shown that reduction of sink strength causes reduced phloem loading of sucrose and an increased sucrose content in the apoplast and symplast of the source leaves (Van Oene *et al.*, 1992a,b), probably *via* an effect on photosynthesis (Mayoral *et al.*, 1985; Plaut *et al.*, 1987).

Sinks compete at the phloem termini for the produced assimilates. Sink strength was originally defined as the product of sink size and sink activity (Warren Wilson, 1972; Wareing and Patrick, 1975), sink activity being the rate of uptake or incorporation of assimilates per unit weight of sink tissue. Which factors determine the competitive ability of a sink, its sink strength? Below, two factors are discussed: a turgor-regulation mechanism, and the influence of hormones. Several other factors, *e.g.* number of endosperm cells (Brocklehurst, 1977; Chojecki *et al.*, 1986), light or phytochrome (Mor and Halevy, 1980a,b; Eschrich and Eschrich, 1987; Beltrano *et al.*, 1991), photoperiod (Cure *et al.*, 1982; Morandi *et al.*, 1988), temperature (Grusak and Minchin, 1989; Tashiro and Wardlaw, 1989; Wolf *et al.*, 1991), stresses (Setter, 1990) and sucrose as a messenger (Farrar, 1992) are left out of consideration.

Turgor-regulation

According to the pressure-flow mechanism (Münch, 1930), the sink strength of a sink seems to be determined by its ability to remove assimilates from the phloem end, either by utilization or storage, thus steepening the turgor pressure gradient along the phloem

path. Several mechanisms have been proposed to increase the mobilizing ability of a sink. In the case of apoplastic phloem unloading, hydrolysis of sucrose by apoplastic acid invertase will decrease the apoplastic sucrose level, prevent reloading and stimulate unloading (Eschrich, 1980, 1986). Hydrolysis of sucrose within the symplast, storage in the vacuoles of receiving cells or conversion of sucrose to starch has a similar effect (Ho and Baker, 1982; Ho, 1988). On the other hand, hydrolysis of sucrose is not a pre-requisite for apoplastic unloading since it does not always occur (Gougler-Schmalstig and Hitz, 1987; Lingle, 1989).

However, the traditional concept that phloem unloading of sucrose in sinks is enhanced by relatively low apoplastic sucrose levels, has to be reviewed, at least in the case of developing seeds. Wolswinkels review (1992) has presented ample evidence that in dicotyledonous seeds phloem unloading is not inhibited but stimulated when the apoplastic concentration of solutes is rather high. According to the Münch pressure-flow hypothesis, a high concentration of osmotically active solutes in the apoplast surrounding the cotyledons will result in an osmotic efflux of water from the sieve tubes and other cells in the seed-coat, this will cause a reduction of the turgor pressure at the sink end of the phloem pathway (in the symplast of the seed-coat phloem strands) and thus steepen the turgor pressure gradient between the source end and the sink end of the phloem pathway, causing increased phloem transport towards developing seeds (Wolswinkel, 1990, 1992). The Münch hypothesis further supposes a circulating water flow from source to sink through the phloem and back through the xylem. However, in the view of the high apoplastic solute concentrations, this idea has to be abandoned. The apoplast surrounding the cotyledons of legume seeds is more or less isolated from the apoplast in source tissues (Murray, 1987): less developed or interrupted xylem connections of the seed with the pod prevent draining of apoplast solutes along the water potential gradient back to the transpiring leaves (Wolswinkel, 1992).

This turgor-regulation mechanism is not restricted to dicotyledonous seeds, but probably has a more universal validity: bathing parts of the roots of *Phaseolus* in polyethylene glycol increases the translocation to these parts (Lang and Thorpe, 1986) and a sudden increase in the rate of sugar translocation towards grape berries some weeks before ripening is correlated with increased internal sugar concentrations due to loss of compartmentalization in the berries (Lang and Düring, 1991). A similarly directed turgor-regulation mechanism was found for uptake of solutes by sink tissues, lower osmotic potentials causing enhanced uptake of solutes (legume cotyledons: Wolswinkel *et al.*,

1986; sugar beet tap root: Wyse *et al.*, 1986; potato tuber: Oparka and Wright, 1988). Thus, an increase in apoplastic solute levels will simultaneously enhance phloem unloading and stimulate uptake by the sink tissue. A consequence of this model is that a suddenly increased uptake of solutes by a strong sink would result in decreased phloem unloading. Patrick *et al.* (1986) have described a mechanism ('turgor homeostat') that protects sink tissues against the effect of decreased solute concentrations in the apoplast, by a putative turgor-sensitive porter in the plasmamembrane.

Although turgor-sensitive transport of assimilates may, at least in developing seeds, be a predominant determinant of high sink strength, the question remains how differences in sink strength arise. The turgor-regulation mechanism may explain why a sink is a strong sink, and how it remains a strong sink, but not how it became a strong sink.

Hormones

In 1937, Went and Thimann defined a hormone as 'a substance which, being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process', analogous to the definition for hormones in animal physiology (Bayliss and Starling, 1904). One year before, Dollfus described the auxin content of seeds in developing fruits of *Rosa* and *Symphoricarpus*, and he found that substitution of the seeds with a lanolin paste containing indoleacetic acid resulted in ovary growth (Dollfus, 1936). In the same period, Gustafson studied the distribution of auxins in tomato fruits and found the highest concentrations of auxin in the seeds and the surrounding tissues, and he hypothesized that 'the initiation of growth of the ovary into a fruit resulted from the auxin' (Gustafson, 1939). The experiment of Dollfus was repeated by Nitsch with strawberry fruits: replacement of the achenes by a control lanolin paste inhibited fruit growth immediately, whereas an auxin-containing lanolin paste fully restored growth of the receptacles (Nitsch, 1950).

The above-mentioned experiments clearly illustrate the traditional approach of hormone research: a correlation was found between the level of a hormone (auxin) and a response (ovary growth); removing the natural hormone source (the seed) eliminated the response, and replacement of the hormone source with a substitute (lanolin with auxin) was able to restore the response. At least one of the questions of these 'hormone-research pioneers' is still relevant: 'Some may hesitate to accept the idea of auxin playing such an important role in the growth of fruits. (..) If auxin is able to do this when added from the

outside, is there any reason why it should not play an important part under normal conditions?' (Gustafson, 1939). Below, it will be shown that there are several reasons why applied hormones may lead to artefactual responses.

Phytohormones have often been mentioned as possible candidates for a role in determining growth or sink strength (Wareing and Patrick, 1975; Ho *et al.*, 1982/83; Evenari, 1984; Wyse, 1986; Brun *et al.*, 1986; Patrick, 1987; Brenner, 1987), so often that they mostly are referred to as 'growth substances' or 'growth regulators'. In most cases the involvement of hormones was derived from a correlation between hormone level and the increase in sink size (*e.g.* cytokinin content of pea seeds: Burrows and Carr, 1970; abscisic acid content of pea seeds: Browning, 1980; Wang *et al.*, 1987; Ross and McWha, 1990) or from increased sink size or increased transport of assimilates to the sinks after hormone applications (*e.g.* Eeuwens and Schwabe, 1975; Adepipe *et al.*, 1976; Düring and Alleweldt, 1980, 1984; Berüter, 1983; Archbold, 1988; *cf.* Table 1.I).

Developing seeds and fruits generally contain rather high levels of phytohormones (Pharis and King, 1985; Brenner, 1987; Ross and McWha, 1990; Figure 1.2). The principal question has been raised (Komor, 1983; Thorne, 1986) whether phloem transport or phloem unloading could be regulated by hormones when the phloem itself also serves as a transport pathway for these molecules. Two arguments can be made to answer this question: first, sink tissues may differ from vascular tissues with respect to sensitivity to hormones (Trewavas, 1981, 1991), and second, it has to be considered that in many cases

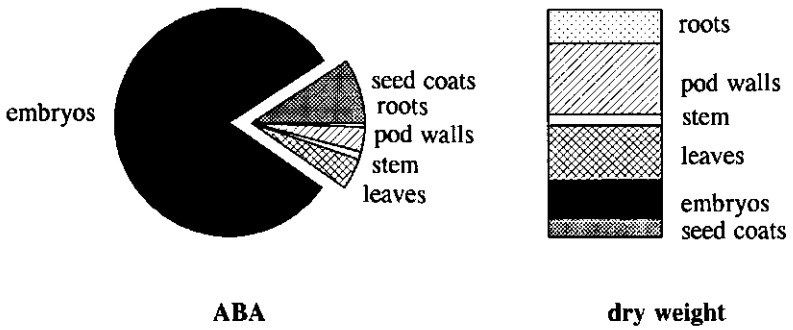


Figure 1.2 Distribution of ABA and dry weight over various tissues of *Pisum sativum* L. plants. Plants were harvested 24 days after anthesis at the lowest reproductive node. The seeds contained more than 90 % of all the ABA present in the plants, although they took up only 25 % of the dry weight. Data from Ross and McWha, 1990.

hormones are also synthesized in the sink region, without previous transport (*e.g.* synthesis of ABA in developing seeds: McGlasson and Adato, 1976; Karssen *et al.*, 1983; Groot *et al.*, 1991; synthesis of auxins in the achenes of strawberry fruits: Nitsch, 1950). Nevertheless, the high levels of hormones in developing fruits or seeds justify a second question: are these high levels the cause or the result of enhanced transport rates to developing sinks?

The difficulty to answer these questions is illustrated in the following example. Wheat kernels accumulate ABA during development (McWha, 1975; King, 1976), mainly due to biosynthesis within the grain (Dewdney and McWha, 1978). The accumulation pattern of ABA correlates positively with the increase in grain dry weight (McWha, 1975). Injection of ABA into wheat grains increased the transport of ^{14}C-labelled photosynthates towards the ear as compared to water injection (Dewdney and McWha, 1979). Both the correlation between ABA level and increase in grain weight, and the enhanced transport of assimilates after ABA injection, indicate a role for ABA in determining the sink strength of developing wheat kernels. However, these experiments were repeated by King and Patrick (1982) who found that injection of water or ABA injured the seeds and drastically reduced the overall transport of assimilates to the seeds. When wheat ears were detached and placed on a sucrose-containing culture solution with ABA, the grain ABA content increased 3- to 14-fold, but grain growth was not promoted in these kernels (King and Patrick, 1982).

In this example, the mode of hormone application was decisive for the obtained result, it should be as non-invasive as possible. Several other factors are important: penetration of the hormone in the tissue of study, metabolism or compartmentalization of the hormone, and responsiveness to the hormone. Despite all these pitfalls, a lot of claims have been made in literature for stimulating or inhibiting roles of phytohormones with respect to seed development. Table 1.I gives a survey of these data, and clearly illustrates the controversies in literature on this subject.

Outline of the thesis

During the last five years, I have been working at the interface of two sections of the Department of Plant Physiology (Wageningen Agricultural University): 'Survival strategies' and 'Differentiation *in vivo* and *in vitro*'. The 'Survival strategies'-section studies processes related to desiccation tolerance, viability, longevity and germination of

Table 1.I Literature survey of reports on the correlation between hormone levels and a specific response concerned with the sink strength of developing seeds or fruits, and reports on effects of increased hormone levels on such responses. Effects were mostly found after application of a hormone, but in some cases different hormone levels were achieved by inhibitors, culture conditions, stress, etc. Effects on accumulation of storage proteins and other reserves were left out of consideration. +, a positive correlation or a larger response; 0, an unclear correlation or no response; -, a negative correlation or a smaller or negative response compared to control treatments.

species	response	correlation	effect	reference
Abscisic acid				
Citrus sinensis	fruit growth rate	+	0	Hofman, 1990
Fragaria x ananassa	^{14}C-sucrose import into fruits		+	Archbold, 1988
Glycine max	cotyledon growth, embryo growth	0		Hein *et al.*, 1984
	embryo growth during early embryogenesis		+	Ackerson, 1984
	seed growth rate	0		Schussler *et al.*, 1991
	seed growth rate	+		Ackerson, 1984; Schussler *et al.*, 1984; Morandi *et al.*, 1990; Lopez *et al.*, 1989
	sucrose accumulation in seeds	0		Schussler *et al.*, 1991
	sucrose accumulation in seeds	+		Morandi *et al.*, 1990
	sucrose release from 'empty' seedcoats		+	Gifford and Thorne, 1986
	sucrose uptake into embryos	+	+	Schussler *et al.*, 1984
	sucrose uptake into cotyledons		+	Brenner *et al.*, 1982, 1986
Hordeum vulgare	^{14}C-import into ear		-	Wagner, 1974
	^{14}C-import into ear		-/+	Dörffling *et al.*, 1984
	^{14}C-transport to ear		+	Tietz *et al.*, 1981
	grain growth rate	0		Quarrie *et al.*, 1988
	grain growth rate	+		Naumann and Dörffling, 1982
	grain growth rate	+	0/+	Dörffling *et al.*, 1984
	thousand kernel weight		0	Dörffling *et al.*, 1984
	thousand kernel weight		+	Tietz *et al.*, 1981
Lupinus luteus	^{14}C-transport to flower head		-	Porter, 1981
Lycopersicon esculentum	fruit growth rate	0		Ho *et al.*, 1982/83
	fruit growth rate	0/+		McGlasson and Adato, 1976
	seed dry weight	+	0	Groot *et al.*, 1991
Phaseolus coccineus	embryo growth rate	+		Perata *et al.*, 1990
Phaseolus vulgaris	^{14}C-release from excised seed-coats		+	Clifford *et al.*, 1986

Table 1.I (continued)

species	response	correlation	effect	response
Phaseolus vulgaris	embryo growth rate	+		Hsu, 1979
	embryo growth rate		-	Sussex and Dale, 1979
	seed growth rate		+	Clifford *et al.*, 1987
	sucrose release from excised seed-coats		+	Offler and Patrick, 1986
	sucrose uptake into cotyledons		0	Offler and Patrick, 1986
Pisum sativum	^{11}C-release from 'empty' seed-coats		+	Ross *et al.*, 1987
	^{14}C-release from 'empty' seed-coats		0/+	Clifford *et al.*, 1990
	embryo growth rate		-/0	Davies and Bedford, 1982
	pod dry weight		-/0	Schroeder, 1984
	pod elongation		0	Schroeder, 1984
	pod elongation	-	-/0	Eeuwens and Schwabe, 1975
	seed dry weight		0	Schroeder, 1984
	seed growth rate	-	-/0	Eeuwens and Schwabe, 1975
	seed growth rate	0		Wang *et al.*, 1984
	seed growth rate	+		Browning, 1980; Ross and McWha, 1990
Prunus persica	embryo/integuments growth rate	0/+		Piaggesi *et al.*, 1991
Pyrus malus	fruit growth rate	-/+		Berüter, 1983
	sorbitol uptake into fruit		+	Berüter, 1983
Triticum aestivum	^{14}C-import into grains		-	Borkovec and Procházka, 1992
	^{14}C-import into grains		0	King and Patrick, 1982
	^{14}C-import into grains		+	Dewdney and McWha, 1979
	^{14}C-import into grains	+		Dewdney and McWha, 1978
	grain dry weight	-/0		Quarrie *et al.*, 1988; Koshkin and Tararina, 1990
	grain growth rate	-		Rademacher and Graebe, 1984; Lee *et al.*, 1989
	grain growth rate	0		Quarrie *et al.*, 1988
	grain growth rate	0	0	King, 1976
	grain growth rate	+		McWha, 1975
	grain set		0	Dembinska *et al.*, 1992
	grain set	-		Lee *et al.*, 1988
	grain size	-		Rademacher and Graebe, 1984
Vicia faba	seed growth rate	+		Gräbner *et al.*, 1980
Vitis vinifera	sugar content of berries	+	+	Düring and Alleweldt, 1980
Zea mays	endosperm growth rate		0	Ober and Setter, 1990
	kernel growth rate	+		Jones and Brenner, 1987

Table 1.I (continued)

species	response	correlation	effect	reference
Zea mays	sugar accumulation	+		Jones and Brenner, 1987
	endosperm cell number, endosperm dry weight		-	Myers *et al.*, 1990
Auxins				
Fragaria sp.	fruit growth rate	+	+	Nitsch, 1950
Glycine max	cotyledon growth, embryo growth	0		Hein *et al.*, 1984
	sucrose release from 'empty' seed-coats		+	Gifford and Thorne, 1986
Hordeum vulgare	^{14}C-import into ear		+	Wagner, 1974
Lycopersicon esculentum	fruit growth rate	0		Ho *et al.*, 1982/3
	fruit growth rate, fruit set		+	Starck *et al.*, 1987
Phaseolus vulgaris	^{14}C-import into pod and seed		+	Patrick, 1987
	embryo growth rate		-/0	Sussex and Dale, 1979
Pisum sativum	^{14}C-import into fruit		+	Achhireddy *et al.*, 1984
	embryo growth rate		0	Corke *et al.*, 1990
	pod dry weight		0/+	Schroeder, 1984
	pod elongation		0	Schroeder, 1984; Barratt, 1986a
	pod elongation	+	+	Eeuwens and Schwabe, 1975
	seed dry weight		0	Schroeder, 1984; Barratt, 1986a
	seed growth rate	+	+	Eeuwens and Schwabe, 1975
Pyrus malus	sorbitol uptake into fruit		-	Berüter, 1983
Triticum aestivum	^{14}C-transport to ear		+	Wardlaw and Moncur, 1976
	grain growth rate	+		Rademacher and Graebe, 1984
	grain size	+		Rademacher and Graebe, 1984
Vigna radiata	seed dry weight		-	Clifford, 1981
Vitis vinifera	berry dry weight		+	Weaver *et al.*, 1969
	^{14}C-import into berry		+	Weaver *et al.*, 1969
Cytokinins				
Lycopersicon esculentum	fruit growth rate	0		Ho, 1984
Phaseolus vulgaris	^{14}C-release from excised seed-coats		+	Clifford *et al.*, 1986

Table 1.1 (continued)

species	response	correlation	effect	reference
Phaseolus vulgaris	embryo growth rate		0	Sussex and Dale, 1979
	seed growth rate		+	Clifford *et al.*, 1987; Patrick, 1987
	sucrose release from excised seed-coats		+	Offler and Patrick, 1986
	sucrose uptake into cotyledons		0	Offler and Patrick, 1986
Pisum sativum	^{14}C-import into fruit		+	Achhireddy *et al.*, 1984
	pod dry weight		+	Schroeder, 1984
	pod elongation		0	Eeuwens and Schwabe, 1975
	pod elongation		+	Schroeder, 1984; Barratt, 1986a
	seed dry weight		0	Schroeder, 1984
	seed dry weight		-	Barratt, 1986a
	seed growth rate	+		Burrows and Carr, 1970
Triticum aestivum	^{14}C-import into grains		+	Borkovec and Prochazka, 1990
	cell division in endosperm	+		Jameson *et al.*, 1982
Vigna radiata	seed dry weight		+	Clifford, 1981
Vigna unguiculata	^{14}C-import into fruit, fruit weight		+	Adepipe *et al.*, 1976
Vitis vinifera	^{14}C-sugar import into berry		+	Weaver *et al.*, 1969
	berry dry weight		+	Weaver *et al.*, 1969
Ethylene				
Ficus carica	nutrient flow to fruits		+	Maxie and Crane, 1968
Glycine max	seed weight		0	Stutte and Rudolph, 1971
Pisum sativum	^{14}C-import into fruit		+	Achhireddy *et al.*, 1984
Prunus persica	^{14}C-import into fruit		+	Chalmers *et al.*, 1976
Zea mays	kernel dry weight		-	Cliquet *et al.*, 1991
Gibberellins				
Citrus sinensis	fruit growth rate	+	0	Hofman, 1990
Hordeum vulgare	^{14}C-import into ear		+	Wagner, 1974
	grain growth rate	+		Mounla and Michael, 1973
Lycopersicon esculentum	fruit growth rate	0		Ho, 1984
	seed dry weight		+	Groot *et al.*, 1987

Table 1.1 (continued)

species	response	correlation	effect	reference
Phaseolus vulgaris	embryo growth rate		0	Sussex and Dale, 1979
Pisum sativum	^{11}C-import into young ovaries		+	Jahnke *et al.*, 1989
	^{14}C-import into fruit		+	Achhireddy *et al.*, 1984
	^{14}C-import into young ovaries		+	Peretó and Beltrán, 1987
	pod dry weight		0/+	Schroeder, 1984
	pod elongation		+	Schroeder, 1984; Barratt, 1986a; Brenner and Ozga, 1991
	pod elongation	+	+	Eeuwens and Schwabe, 1975
	pod growth rate	+		Ingram and Browning, 1979
	seed dry weight		0	Schroeder, 1984
	seed dry weight		-	Barratt, 1986a
	seed growth rate	0		Frydman *et al.*, 1974
	seed growth rate	+		Ingram and Browning, 1979
	seed growth rate	+	+	Eeuwens and Schwabe, 1975
Prunus persica	^{14}C-import into fruit		+	Chalmers *et al.*, 1976
Triticum aestivum	grain growth rate	+		Mounla and Michael, 1973
Vigna radiata	seed dry weight		+	Clifford, 1981
Vitis vinifera	^{14}C-sugar import in berry		+	Weaver *et al.*, 1969
	berry dry weight		+	Weaver *et al.*, 1969

seeds, embryoids and pollen, whereas the 'Differentiation *in vivo* and *in vitro*'-section works on aspects of propagation and differentiation of cells, tissues or organs, transport of assimilates, tuber induction and secondary metabolites. Both groups pay much attention to the role of phytohormones in these processes.

Hypothesis

This thesis deals with the effects of ABA on assimilate partitioning to seeds and the allocation of these assimilates over the various types of storage reserves in the seeds. The hypothesis was two-fold:

- ABA enhances the sink strength of developing seeds and thus stimulates the transport of assimilates to seeds, especially under conditions of a limited supply of

assimilates, and
- ABA influences the storage of reserve material in oil-seeds: it increases the amount of lipids and concomitantly decreases the amount of starch in those seeds.

Evidence for a possible influence of ABA on sink strength of developing seeds was based on:
- correlations found between ABA levels in developing seeds and the accumulation of dry matter (Düring and Alleweldt, 1980; Schussler *et al.*, 1984; Table 1.I),
- effects of ABA applications on transport of ^{14}C-assimilates (Schussler *et al.*, 1984; Saftner and Wyse, 1984; Table 1.I),
- effects of ABA on the synthesis of reserve material (storage proteins, lipids) in seeds (Finkelstein *et al.*, 1985; A.S. Basra, unpublished observations)

Approach

Since application of hormones by injection, lanolin pastes, dipping or spraying may lead to artefacts or results that are difficult to interpret, we have chosen for a different approach, *viz.* the use of hormone mutants. During the last two decades, several hormone-deficient or hormone-insensitive mutants have been isolated (reviews: Koornneef, 1986; Reid, 1986; Quarrie, 1987). Hormone mutants have several advantages: they enable non-invasive manipulation of hormone levels or hormone sensitivity and are ideal blanks in experiments with hormone applications. With respect to seed development, hormone mutants make it possible to study the influence of ABA on storage reserve deposition *in vivo*, whereas this type of research is generally done with *in vitro* cultured embryos (Finkelstein *et al.*, 1985; Barratt, 1986b). Furthermore, in the case of recessive mutations, cross-pollination of hormone mutants with wild-type plants affords the possibility to generate wild-type phenotype sinks on a mutant mother plant (Figure 1.3). The sink strength of these hormone-containing and/or hormone-sensitive sinks can be compared with that of self-pollinated, hormone-deficient and/or hormone-insensitive sinks, on the same mother plant.

Based on the evidence in literature (Table 1.I), effects on sink strength of seeds or fruits have been reported for all classes of hormones, although data on the correlation between hormone levels and growth rate of sink tissues are scarce for cytokinins and ethylene. Since at the beginning of this project only few mutants were available that are impaired in the synthesis of auxins, cytokinins or ethylene, we had to chose between GA

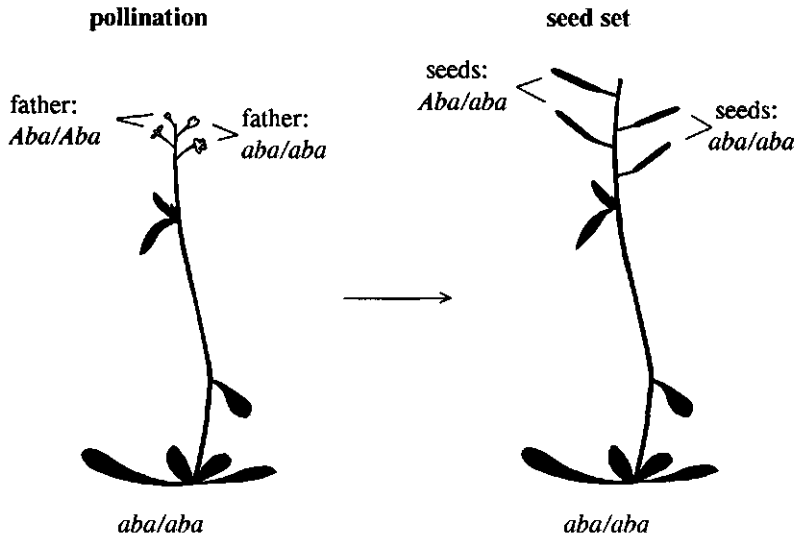


Figure 1.3 Scheme describing the procedure to obtain genetically different seeds on the same mother plant (*e.g. Arabidopsis thaliana*). Flowers of a hormone-deficient (*aba/aba*) mother plant are pollinated with pollen from either wild-type (*Aba/Aba*) or deficient (*aba/aba*) plants. These crosses result in hormone-deficient (*aba/aba*) or hormone-containing heterozygous (*Aba/aba*) seeds; both types of seed compete for the same assimilates.

and ABA mutants. Although several gibberellin mutants are known (Reid, 1986), most of the GA-deficient mutations are tissue-specific, in such a way that the seeds have wild-type levels of endogenous GAs (Potts and Reid, 1983; Pharis and King, 1985). Sensitivity to GAs in GA-responsiveness mutants may also be tissue-specific (Lenton *et al.*, 1987). Moreover, studies with mutants that have reduced levels of GAs in their seeds, revealed that GAs do not influence the ultimate seed weight (*Arabidopsis*: Barendse *et al.*, 1986) or the seed growth rate (tomato: Groot *et al.*, 1987), although fruit growth rate was stimulated by GAs. For these reasons, we confined our study to ABA mutants.

Two species were used for this study: *Arabidopsis thaliana* and *Pisum sativum*. *Arabidopsis* was used because several ABA-mutants have been isolated in this species (Koornneef *et al.*, 1982, 1984, 1989), and because this plant is a fast-growing species which produces a lot of seeds, and has a relatively short life cycle. Since the pea mutant was only partly described (Marx, 1976; Donkin *et al.*, 1983a,b; Wang *et al.*, 1984; Jackson and Hall, 1987), a number of characteristics of this mutant had to be studied first

(Chapter 2). The mutant is compared with the non-wilty isogenic line that was obtained after backcrossing with a wild-type pea. Data on the ABA content of developing seeds of both lines are also presented.

Chapters 3 to 5 deal with the influence of ABA on long-distance transport of assimilates to seeds. The use of the pea mutant made it possible to study phloem unloading in and sugar release from seed-coats by means of the empty-seed-coat technique (Wolswinkel and Ammerlaan, 1983; Thorne and Rainbird, 1983). Effects of ABA on sugar release from these seed-coats are presented in Chapter 3. In Chapters 4 and 5 the growth rate of pea seeds and the transport of radiolabelled photosynthates to *Arabidopsis* seeds is studied in a situation with seeds of different genotypes competing for the same source.

Chapter 6 describes the effect of ABA-deficiency and ABA-insensitivity in *Arabidopsis* plants on the deposition of storage material in the seeds. Lipid and carbohydrate content and composition during seed development were determined and related to the genotypes.

In Chapter 7, the results are discussed, placed in a wider context, and compared with other studies concerning ABA and assimilate partitioning into or within seeds.

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2 Characterization of the ABA-deficient *Pisum sativum* *'wilty'* mutant

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Summary

In this Chapter, an ABA-deficient *Pisum sativum* mutant is physiologically characterized. A wilty pea line (*wil*) was backcrossed six times with a non-wilty line, and then selfed to obtain near-isogenic lines. These lines were used for further studies. The plants were grown hydroponically and at high relative humidity. The ABA-deficient mutant grew more slowly in mass and height and invested less dry matter in its roots. Mutant leaves wilted rapidly under drought conditions, and had a lower water content than wild-type leaves. ABA-deficient plants had fewer and smaller seeds than wild-type plants, but the weight ratio of reproductive to vegetative parts was similar in both lines. Seeds of mutant plants contained about five times less ABA than wild-type seeds. It is suggested that the lower growth rate of both vegetative and reproductive parts is not directly caused by the lower ABA content, but by disturbed water relations.

Introduction

During the last two decades a series of mutants that are either ABA-deficient or altered in their sensitivity to ABA have become available (Koornneef, 1986). Although only a few of them are well-characterized (Zeevaart and Creelman, 1988), it is clear that these mu-

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tants play a significant role in physiological research. Comparisons of mutants with their wild-types have successfully answered questions on the role of ABA in water relations (*e.g.* in tomato: Tal and Nevo, 1973) and the biosynthetic pathway of ABA (*e.g.* in *Arabidopsis*: Rock *et al.*, 1992). Also several aspects of the role of ABA in seed development have been elucidated with the aid of hormone mutants. For example, the development of seed desiccation tolerance was found to be absent in an ABA biosynthesis and responsiveness double mutant of *Arabidopsis* (Koornneef *et al.*, 1989), and the accumulation of long-chain fatty acids was reduced by an ABA-desensitizing mutation (Finkelstein and Somerville, 1990). The role of mutants in research on ABA and seed dormancy and germination was recently reviewed by Hilhorst and Karssen (1992).

It is obvious, however, that not all the differences between ABA mutants and their wild-types can be directly attributed to the absence of, or insensitivity to, ABA. Several pleiotropic effects are described (Koornneef, 1986), and even in monogenic mutants, cross-resistance to other hormones has been reported (Blonstein *et al.*, 1991). Marx (1976) described a recessive gene mutation ('*wilty*', *wil*) in pea, that caused early and dramatic wilting during drought stress. Leaves of this mutant appear to contain considerably less ABA compared to other pea lines (Wang *et al.*, 1984; Jackson and Hall, 1987). Unfortunately, the original breeding line is not available (Donkin *et al.*, 1983). In the present study, the mutant is compared with a non-wilty line that has been backcrossed with the wilty parent line to obtain near-isogenic lines. Since we are primarily interested in the role of ABA on assimilate partitioning, special attention is paid to the dry matter distribution in the mutant, with respect to both vegetative and reproductive organs. To determine whether this mutation is organ-specific (for example, some GA-deficient mutants have wild-type GA levels in their seeds: Potts and Reid, 1983), ABA concentrations in seeds have been determined during their development.

Materials and methods

Plant material

Seeds of ABA-deficient pea plants (*Pisum sativum* L., *wil* mutant, line B78-918) were kindly provided by prof. G.A. Marx (NYSAES, Geneva, NY, USA). The original non-wilty wild-type line (B1777-155) that was used for the backcrosses was not an isogenic line but closely approximated the phenotype of the wilty lines (G.A. Marx, personal com-

munication). The heterozygote F_1 that resulted from a cross between the wilty line and the wild-type line was backcrossed with the wilty parent line. To discern in the offspring between heterozygous plants and homozygous recessive ones, some leaflets were detached from each plant, and the decrease in fresh weight was monitored. Leaves of ABA-deficient plants transpire at a higher rate (Wang *et al.*, 1984; *cf.* Figure 2.1). The heterozygotes were backcrossed again with the wilty parent line, and this procedure was repeated four times. Heterozygotes that resulted from the sixth backcross were selfed, and the progeny were selfed again to obtain reasonably homozygous lines for the characterization experiments. According to Marx (1976), the gene symbol *wil* is used to designate this mutant.

About 250 seeds of each line were disinfected with a fungicide (thiram) and germinated in a glasshouse at ambient conditions, in a large Petri dish with glass beads and half-strength Hoagland medium (Hoagland and Arnon, 1950), covered with wetted filter paper. Seven to ten days after germination each seedling was transferred to a 2-litre jar with well aerated half-strength Hoagland medium, supplemented with a fungicide (propamocarb, 5 mg l^{-1}). During the growth period, the medium in the jars was replaced three times, with due care to prevent damage to the roots. Iron deficiency was prevented by adding a few droplets of a 0.1 M Fe,Na-EDTA solution to the nutrient solution each week. Plants were raised in a glasshouse with minimum day temperatures of 19 °C and night temperatures of 16 °C, under natural light, supplemented with artificial illumination from high-pressure sodium lamps (Philips, SON/T), to give a 16-h day length. Plants were protected from direct sunlight. The floors of the greenhouse were regularly sprinkled with water and care was taken to keep the relative humidity in the glasshouse above 60 %.

Growth characteristics

At eight different stages after sowing, samples of randomly selected plants of both lines were harvested. The number of plants in wild-type samples varied from 8 to 15, in mutant samples from 11 to 15. For sake of security, the genotype of each plant was checked by determining the rate of decrease in fresh weight of detached leaflets (Wang *et al.*, 1984, *cf.* Figure 2.1) prior to the harvest.

Measurements were made of fresh and dry (after 24 h at 105 °C) weights of roots, stems, leafs, pods, seeds and lateral shoots, as well as the number of internodes, leaves, pods and seeds. Leaf areas were determined with a LI-COR model 3100 Area Meter.

Flowers were tagged at the day of flowering, and seeds were only harvested separately from the fruit when the fruit length exceeded 5 cm; at earlier times the seeds were too small to weigh, *i.e.*, fresh weight 1 mg or less.

ABA determinations

Flowers were tagged on plants of both lines and 80 fruits were harvested at various ages, ranging from 11 to 35 days after flowering. Directly after harvest, the seeds were separated in half. One half of the harvested material was weighed and immediately frozen in liquid nitrogen and stored at -70 °C for later determination of ABA levels. The other half was used for both fresh and dry weight determinations. The dry matter content was used to select a series of 34 representative fruits. The harvested material of individual fruits was homogenized in 10 ml 70 % (v/v) methanol and 0.2 g purified seasand, for about 10 min in a Pulverisette (Fritsch, Germany) (since older seeds could not be homogenized in a mortar), at 4 °C. The homogenate was centrifuged (400 *g*, 5 min), and the volume of the supernatant was reduced to 0.5-1 ml under a flow of nitrogen.

A series of six dilutions of the residue was subsequently analysed in triplicate using an indirect enzyme-linked immuno sorbent assay (ELISA) as described by Ross *et al.* (1987). In short: microtitre plates were coated with 50 mg l^{-1} KLH-ABA conjugate (ABA C_1-linked to keyhole limpets haemocyanin) dissolved in a coating buffer (34.8 mM $NaHCO_3$, 16.0 mM Na_2CO_3, 6.5 mM NaN_3 and 0.5 mM $MgCl_2.H_2O$; pH 9.6). The plates were incubated for at least 24 h. After rinsing with water and PBST (phosphate buffered saline with Tween: 8.1 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 6.5 mM NaN_3, 0.05 % (v/v) Tween-20; pH 7.4), 100 μl samples of ABA (standard curve) or pea seed homogenate dilutions were added to the wells, followed by 100 μl of a mouse monoclonal antibody raised against ABA (McAb-3G4, Boonekamp *et al.*, 1990). The plates were sealed, covered with a lid, wrapped in aluminium foil and incubated overnight at 4 °C. After washing the plates with PBST, 200 μl aliquots of a goat anti-mouse IgG (whole molecule) conjugated to alkaline phosphatase (Sigma), diluted in TTBST (TRIS and TES buffered saline with Tween and BSA: 20 mM TRIS, 20 mM TES, 137 mM NaCl, 2.7 mM KCl, 6.5 mM NaN_3, 0.5 % (w/v) BSA, 0.05 % (v/v) Tween-20; pH 7.4) were added. After sealing and wrapping the plates were incubated for three hours at 35 °C. Then, after washing, 200 μl *p*-nitrophenyl phosphate (1 g l^{-1}) in coating buffer were added. After an hour's incubation at 35 °C the plates were read at 405 nm with a

MIOS (Merck) reader.

The antibodies used in this ELISA were very sensitive (range: 1 to 100 pmol) and highly specific to *cis*-(+)-ABA (cross-reactivity of *cis*-(±)-ABA: 50 %; of *trans*-(-)-ABA: 0.9 %) (Boonekamp *et al.*, 1990). Cross-reactivity to the glucose-ester of ABA was rather high, but levels of these conjugates are low and constant during seed development in pea (Ross and McWha, 1990). Since the antibodies were raised against protein conjugates using the C-1 moiety of ABA, cross-reactivity to phaseic acid and interference with organic acids (as reported for C-4´-conjugated ABA: Belefant and Fong, 1989) is expected to be low (M.H.M. Cornelussen, personal communication).

Results and discussion

Water relations

Originally, the *wil* mutant was described as 'wilty', since its wilting behaviour is one of the most obvious characteristics. In general, the wilting response of intact plants was only observed at conditions of relatively severe water stress. Wang *et al.* (1984) described special treatments to induce wilting symptoms. We experienced that these conditions were indeed necessary when the plants were cultured in soil. The experiments described here were performed on plants cultured on hydroponics, and these plants had a remarkably different wilting behaviour. Figure 2.1 shows the loss of water from detached leaflets over time, for both lines. Within 45 min, the mutant leaves had lost half of their initial fresh weight, whereas wild-type leaves lost less than 20 % during the same period. For comparison, the mutant plants grown in soil used by Wang *et al.* (1984) needed three hours to lose 30 % of their initial fresh weight; the conditions during the measurement were identical to our conditions. It seems likely that the plants cultured in soil became to some extent acclimatized to water shortage and needed more time and more extreme conditions to show wilting symptoms, whereas the optimal availability of water in hydroponic culture prevented the plants from stress accommodation.

The role of ABA in maintaining the water balance in plant leaves has been much studied (*cf.* Davies and Mansfield, 1983; MacRobbie, 1991). It seems self-evident that the wilting symptoms in ABA-deficient mutants are caused by an inability, relative to wild-type plants, to close their stomata. Although stomata of the wilty pea mutant and non-wilty lines respond in similar ways to ABA, KCl and CO_2 (Donkin *et al.*, 1983), the

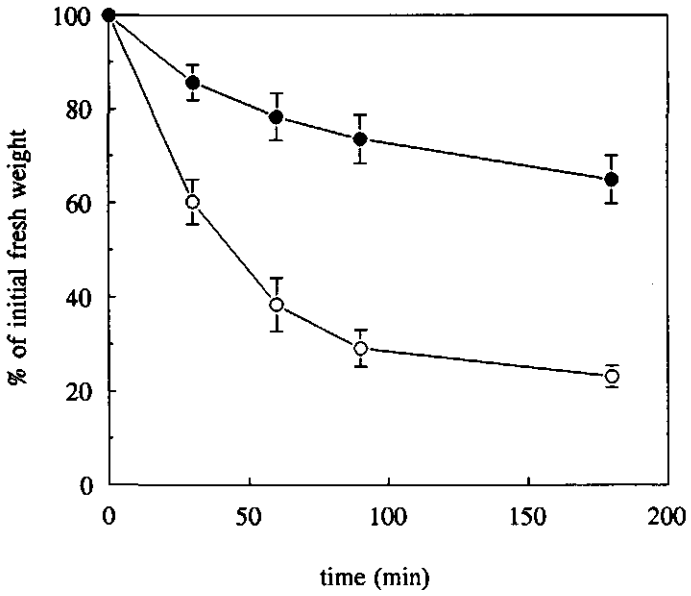

Figure 2.1 Time-course of the loss of fresh weight (mean ± SD, n=12) from detached leaflets of wild-type (●) and ABA-deficient (○) *Pisum sativum* plants over three hours.

mutant leaves fail to accumulate ABA and consequently maintain a higher stomatal conductance during water deficiency (Wang *et al.*, 1984; Jackson and Hall, 1987), which leads to rapid wilting. In unstressed conditions, mutant leaves possess a more negative water potential and larger stomatal apertures than those of the wild type (Donkin *et al.*, 1983). The resulting lower leaf dry matter content of wild-type plants (Donkin *et al.*, 1983) has been partially confirmed by the present study (Table 2.I). However, the percentage dry matter content in our plants was considerably lower than that reported by Donkin *et al.* (1983) (14.3 % compared to 21.3 % for mutant plants). This difference may be related to the hydroponics system we used.

Vegetative parts

Because mutant plants wilted so rapidly, the fresh weight determinations on harvested mutant plants were done quickly (within 10 min). Directly after harvesting, the roots were separated from the shoot, fruits and flowers were detached and seeds were isolated from

9. Het voorkomen van ABA in de hersenen en het centrale zenuwstelsel van zoogdieren biedt interessante aanknopingspunten voor speculaties over de fysiologische rol van ABA in dieren of mensen, zoals stressbestendigheid, regulering van de gasuitwisseling en waterhuishouding, of de aantrekkingskracht voor assimilaten.

   Le Page-Degivry, M.-Th., J.-N. Bidard, E. Rouvier, C. Bulard and M. Lazdunski (1986) Proc. Natl. Acad. Sci. USA 83:1155-1158.

10. De opvatting van de klassiek-homeopathische volgelingen van Hahnemann dat de werking van gepotentieerde en sterk verdunde homeopathische middelen berust op het spiritualiseren van materiële substantie en dat dit een van de pijlers is van de homeopathie, is strijdig met het zoeken naar een farmacokinetische verklaring voor het werkingsmechanisme door onderzoekers uit deze groep.

    Hahnemann, S.C.F. (1979) The Healing Art of Homoeopathy - The Organon of Samuel Hahnemann. Beaconsfield Publishers Ltd, Beaconsfield, Bucks., UK, § 269.

    De Bruijn, S.M. (1991) Ned. Tijdschr. Klass. Homoeopath. 2:6-13.

11. Het convenant tussen gemeentebesturen en horeca-ondernemers over de aanpak van gokverslaving is voor de gemeenten een gemiste kans en voor de gokverslaafde een verlies.

12. Subsidiëring van kinderopvang door de overheid creëert een vorm van inkomensongelijkheid ten opzichte van ouders die bewust geen gebruik willen maken van deze voorzieningen.

13. Regelmatig wordt bij discussies in confessionele kring over de problematiek van asielzoekers en dak- en thuislozen te weinig gewicht toegekend aan de bijbelse voorschriften ten aanzien van naastenliefde en barmhartigheid.

14. Zolang men niet in staat is om met een redelijke betrouwbaarheid voorspellingen te doen over de weersomstandigheden voor een periode langer dan vijf dagen, is er alle reden om zijn bedenkingen te hebben over de waarde van de sombere voorspellingen voor het klimaat over vijftig jaar.

15. Het promotiereglement van de Landbouwuniversiteit stelt slechts 5000 studiebelastingsuren als norm voor een promotie-onderzoek inclusief de afronding daarvan met een proefschrift; de echte wetenschappers onder de promovendi (van wie verwacht wordt dat ze 's avonds en in het weekend werken: Karssen, 1993) worden hiermee zwaar ondergewaardeerd, aangezien ze al na anderhalf jaar aan deze norm voldoen.

    Karssen, C.M. (1993) WUB 2/9/1993.

16. De naam 'Het Gouden Kalf' voor een filmprijs geeft een goede typering van de moderne filmcultuur.

17. Het is onbegrijpelijk dat mensen die niet in God geloven, er toch zo vaak behoefte aan hebben om Zijn naam te gebruiken, en zelfs Hem vragen allerlei onheil over hen te brengen.

Stellingen behorende bij het proefschrift 'Abscisic acid and assimilate partitioning during seed development' door S.M. de Bruijn, in het openbaar te verdedigen op donderdag 9 december 1993, te Wageningen.

Stellingen

1. De bewering dat symplastische unloading (zoals in zaadhuiden van *Phaseolus*) een 'low resistance pathway' biedt en 'relatively energy conserving' is ten opzichte van apoplastische unloading (Offler en Patrick, 1986), geldt niet voor sinks waarbij de symplastische unloading uit de zeefelementen gevolgd wordt door een apoplastische stap.
   Offler, C.E. and J.W. Patrick (1986) In J. Cronshaw, W.J. Lucas, R.T. Giaquinta (eds.) Phloem Transport, Alan R. Liss, New York, USA, p. 295-306.

2. Het op basis van het mechanisme voor de unloading van assimilaten gemaakte onderscheid tussen reversibele en irreversibele sinks (Offler en Patrick, 1986; Ho, 1988) is erg kunstmatig en voldoet niet voor een aantal zogenaamde irreversibele sinks die vroeger of later weer source worden (zoals ontwikkelende bladeren, aardappelknollen en zaden).
   Ho, L.C. (1988) Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:355-378.
   Offler, C.E. and J.W. Patrick (1986) In J. Cronshaw, W.J. Lucas, R.T. Giaquinta (eds.) Phloem Transport, Alan R. Liss, New York, USA, p. 295-306.

3. Het is een ernstige tekortkoming dat Bangerth in zijn uitvoerige hypothese ter verklaring van het verschil in dominantie tussen vruchten en andere sinks wel aandacht besteedt aan hormoonconcentraties en transport van hormonen, maar geen enkel woord wijdt aan mogelijke verschillen in gevoeligheid voor hormonen tijdens de ontwikkeling van deze sinks.
   Bangerth, F. (1989) Physiol. Plant. 76:608-614.

4. De verschillen in drooggewichttoename van sojaboonzaden bij afwijkende lichtregimes, zoals gevonden door Morandi *et al.* (1990), zijn verwaarloosbaar als de data gefit worden met een niet-lineaire vergelijking (bijvoorbeeld die voor een sigmoïde) in plaats van via lineaire regressie.
   Morandi, E.M., J.R. Schussler and M.L. Brenner (1990) Ann. Bot. 66:605-611.

5. Zowel Perl *et al.* (1991) als Charles *et al.* (1993) claimen condities te hebben gevonden waarbij zich zeer synchroon knollen ontwikkelen uit aardappelexplantaten; in beide gevallen ontbreken echter de data waaruit deze synchroniteit zou moeten blijken.
   Perl, A., D. Aviv, L. Willmitzer and E. Galun (1991) Plant Sci. 73:87-95.
   Charles, G., L. Rossignol and M. Rossignol (1993) J. Plant Physiol. 142:474-479.

6. Het gebruik van de term 'seed tubers' voor moederknollen in de aardappelfysiologie is niet alleen botanisch onjuist maar ook hoogst verwarrend en dient dus vermeden te worden.

7. De door Faust *et al.* (1991) gelegde relatie tussen de mate waarin water in rustende appelknoppen gebonden is (zoals vastgesteld met behulp van magnetische resonantie imaging) en de vorm van rust waarin deze knoppen verkeren, wordt door hen op geen enkele manier fysiologisch onderbouwd.
   Faust, M., D. Liu, M.M. Millard and G.W. Stutte (1991) HortSci. 26:887-890.

8. De positieve correlatie tussen het hormoongehalte en de groeisnelheid van zaden is geen enkel bewijs dat het betreffende hormoon betrokken is bij het bepalen van de groeisnelheid van deze zaden.
   Dit proefschrift.

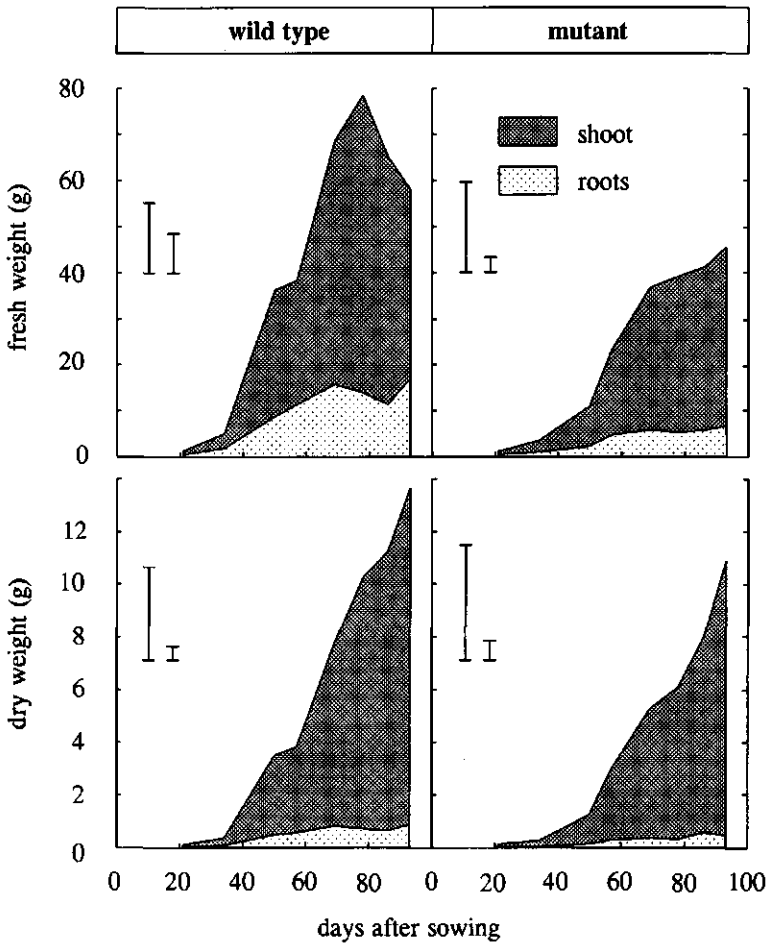

Figure 2.2 Growth patterns of shoots and roots of wild-type (left panels) and ABA-deficient (right panels) *Pisum sativum* plants, both on a fresh weight (upper panels) and a dry weight (lower panels) basis. The error bars in each panel indicate the largest standard deviation of the measurements on shoots (left bar) and roots (right bar), respectively.

the fruits. All parts were weighed separately.

Figure 2.2 illustrates the most important differences between the lines. Clearly, wild-type plants grew faster than ABA-deficient plants, both on a fresh and on a dry weight basis. Wild-type plants always had smaller shoot/root ratios than mutant plants (Table 2.I). Similar effects of ABA on shoot/root ratios were found in several other systems

Table 2.I Comparison of both vegetative and reproductive parts of wild-type and ABA-deficient *Pisum sativum* plants (mean ± SD (n)). Unless otherwise indicated, calculations are based on dry weights of organs. The letters Y or N in the last column indicate whether or not significant differences exist at the P=0.05 level, respectively (Student's *t*-test).

parameter	age (days)	wild type	mutant	s
Vegetative parts				
dry matter content of leaves (%)*		11.2 ± 3.7 (55)	14.3 ± 3.4 (82)	Y
shoot/root ratio	50	6.3 ± 0.8 (10)	7.9 ± 1.0 (14)	Y
	57	6.1 ± 1.1 (9)	9.9 ± 2.0 (12)	Y
	69	8.8 ± 1.8 (9)	14.2 ± 2.4 (13)	Y
number of lateral shoots*		2.1 ± 2.0 (57)	1.9 ± 1.8 (91)	N
lateral shoot fresh weight (g)*		0.6 ± 0.9 (122)	2.4 ± 4.3 (171)	Y
fresh weight ratio of lateral shoots to whole shoot*		0.02 ± 0.03 (57)	0.15 ± 0.26 (91)	Y
number of leaves per plant	50	88 ± 6 (10)	71 ± 12 (14)	Y
	57	92 ± 10 (9)	96 ± 27 (12)	N
	69	134 ± 34 (9)	150 ± 57 (13)	N
	86	121 ± 14 (9)	180 ± 72 (13)	Y
leaf area per leaf (cm^2)*		9.36 ± 1.85 (46)	5.01 ± 1.25 (16)	Y
leaf area ratio ($cm^2\ g^{-1}$)	50	226 ± 19 (10)	245 ± 19 (14)	Y
	57	223 ± 37 (9)	205 ± 17 (12)	N
	69	165 ± 28 (9)	160 ± 23 (13)	N
specific leaf area ($cm^2\ g^{-1}$)	50	519 ± 33 (10)	502 ± 32 (14)	N
	57	489 ± 59 (9)	450 ± 31 (12)	N
	69	468 ± 29 (9)	413 ± 38 (13)	Y
leaf weight ratio	50	0.44 ± 0.02 (10)	0.49 ± 0.02 (14)	Y
	57	0.45 ± 0.03 (9)	0.45 ± 0.02 (12)	N
	69	0.35 ± 0.05 (9)	0.39 ± 0.04 (13)	N
Reproductive parts				
flowering date (days after sowing)		57.1 ± 4.4 (45)	57.2 ± 3.6 (59)	N
first flowering node (counted from basis upwards)		20.9 ± 1.4 (54)	21.5 ± 1.8 (72)	N

Table 2.I (continued)

parameter	age (days)	wild type	mutant	s
weight ratio of	69	0.16 ± 0.08 (9)	0.16 ± 0.08 (13)	N
reproductive to	78	0.41 ± 0.07 (9)	0.32 ± 0.12 (13)	Y
vegetative parts	86	0.54 ± 0.08 (9)	0.39 ± 0.13 (13)	Y
	93	0.62 ± 0.04 (8)	0.59 ± 0.06 (11)	N
number of fruits per plant*		5.4 ± 3.3 (44)	5.0 ± 3.0 (62)	N
number of seeds per plant*		36.9 ± 12.7 (35)	18.4 ± 10.9 (50)	Y
seed weight (mg)	69	6.3 ± 8.5 (273)	5.9 ± 10.2 (245)	N
	78	42.6 ± 41.8 (397)	24.2 ± 36.0 (372)	Y
	86	99.5 ± 75.2 (366)	61.7 ± 74.8 (340)	Y
	93	135.8 ± 79.4 (393)	94.5 ± 84.3 (464)	Y

*values based on several harvests

(Creelman *et al.*, 1990; Saab *et al.*, 1990) and in ABA-deficient mutants of potato (*droopy*: Waggoner and Simmonds, 1966) and tomato (*flacca*: Bradford, 1983). These findings favour the hypothesis that ABA promotes the growth of roots as a measure to increase the volume of soil explored under dry conditions, and to prevent an undesirable water status of the leaves (Davies and Mansfield, 1983). The relatively high values for the shoot/root ratio in both genotypes in our system are most likely caused by the hydroponic culture and indicative for a low degree of water deficiency (Setter, 1990).

In the present study, ABA-deficient plants showed more outgrowth of the axillary buds, especially at the base of the plant. The number of lateral shoots per plant was not significantly different, but both the absolute lateral shoot weight per plant and the ratio of lateral shoot weight to total shoot weight were considerably higher in mutant plants (Table 2.I). This reduced degree of apical dominance has not been reported for other ABA-deficient mutants. On the contrary, ABA-deficient mutants of *Arabidopsis thaliana* had smaller numbers of side-shoots than wild-type plants (Koornneef *et al.*, 1982). Information on the effect of ABA on apical dominance is still somewhat inconsistent since both stimulation and inhibition of lateral bud growth by ABA application have been reported (*e.g.* Hartung and Fünfer, 1981; Everat-Bourbouloux and Charnay, 1982; Cline, 1991). It cannot be excluded, however, that loss of apical dominance in mutant pea plants was partly due to incidental wilting of the apex, or to accelerated senescence of the basal leaves observed in mutant plants. The reduced apical dominance in mutant plants also finds expression in a larger number of leaves and a smaller leaf area per leaf (Table 2.I),

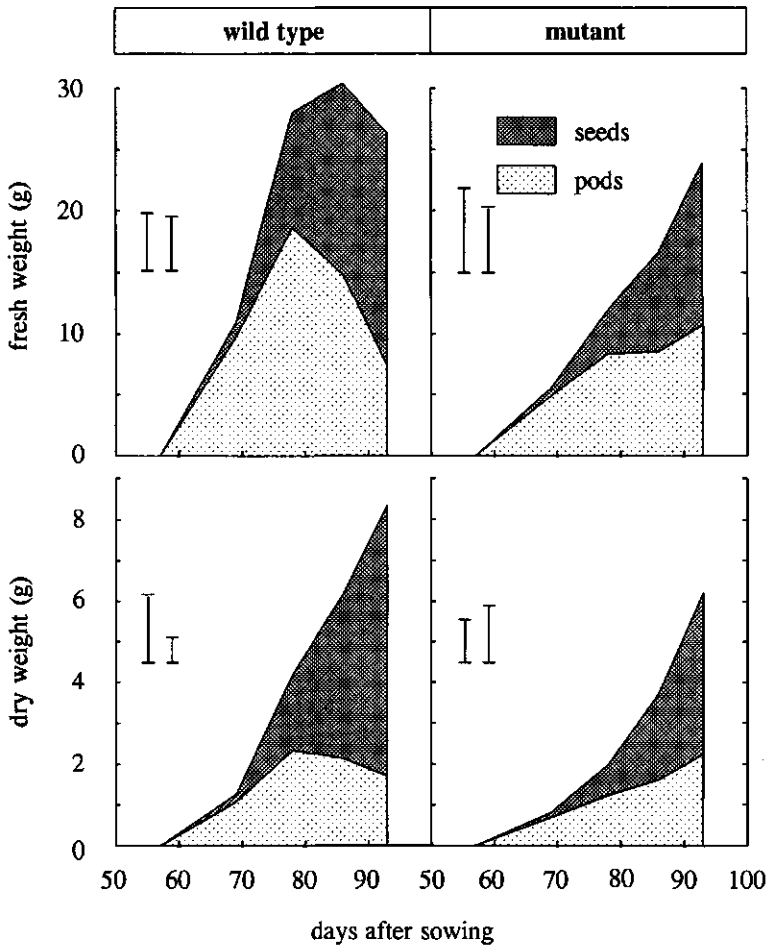

Figure 2.3 Growth patterns of pods and seeds on wild-type (left panels) and ABA-deficient (right panels) *Pisum sativum* plants, both on a fresh weight (upper panels) and a dry weight (lower panels) basis. The experiment was finished just before full maturation of the mutant seeds. The error bars in each panel indicate the largest standard deviation of the measurements on seeds (left bar) and pods (right bar), respectively.

since the lateral shoots had many small leaves. ABA-deficient tomato mutants also have a larger number of leaves than wild-type plants (Quarrie, 1987).

Other parameters, *viz.* the leaf area ratio (leaf area/plant weight), the specific leaf area (leaf area/leaf weight) and the leaf weight ratio (leaf weight/plant weight) showed

only minor differences between the genotypes (Table 2.I).

Reproductive parts

Both wild-type and mutant plants started to flower at the same age, and in both lines the first flowers appeared in the same node (*i.e.*, 21-22 nodes above the cotyledons, Table 2.I).

ABA-deficient plants invested less dry matter in their pods and seeds. The final seed dry weight per plant was 65 % higher in wild-type plants (Figure 2.3), whereas the ratio of reproductive to vegetative parts was also higher in this genotype (Table 2.I). However, at the end of development this difference was less pronounced: it seemed that mutant plants were slower but finally reached similar ratios as wild-type plants. Since seed number per plant was smaller in mutant plants, the average weight per seed was only slightly (but significantly) lower for mutant seeds. The number of fruits was not significantly different between the lines (Table 2.I). In general, ABA-deficient plants carried fewer seeds per fruit. Figure 2.4 displays the frequency distribution of the number of seeds per fruit for both lines.

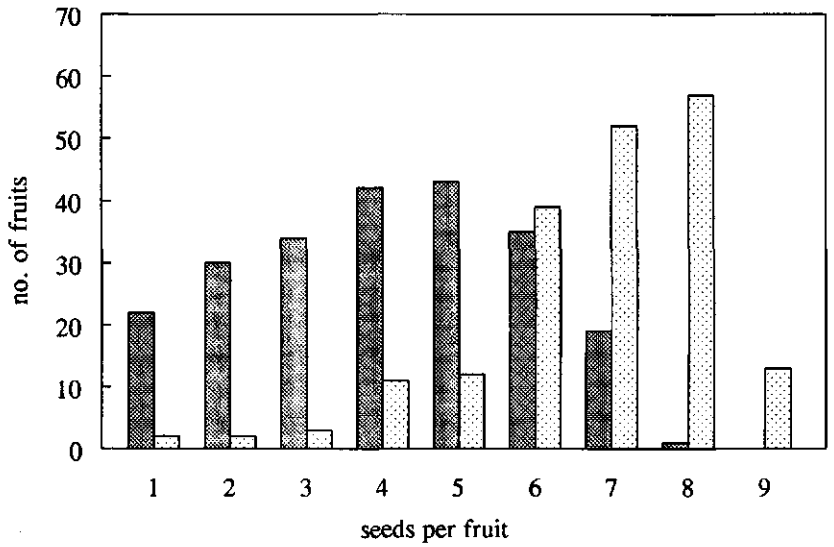

Figure 2.4 Frequency distribution of the number of seeds per fruit in wild-type (hatched bars) and ABA-deficient (open bars) *Pisum sativum* plants.

Previous studies on tomato and *Arabidopsis* ABA-deficient mutants revealed no effects of the reduced ABA levels on seed fresh and dry weight (Karssen *et al.*, 1983; Groot *et al.*, 1991). Seeds of *Arabidopsis* mutants that were both ABA-deficient and ABA-insensitive were impaired in several developmental processes such as ripening and desiccation tolerance (Koornneef *et al.*, 1989), but still attained fresh and dry weights similar to those of wild-type seeds (*cf.* Figure 6.1). Apparently, in these species, ABA does not determine the sink strength of the seeds.

Although the pea mutant in the present study has a somewhat lower seed weight and a reduced mass of reproductive organs per plant, it remains to be seen whether this is a direct effect of the lower ABA level in the seeds. Since by the end of development, the weight ratio of reproductive to vegetative plant parts is similar in both lines (Table 2.I), the lower seed weight seems to be the indirect consequence of the disturbed water relations, and not to be a primary ABA effect. This conclusion is supported by the finding that pea wild-type seeds developing in an ABA-deficient pod have the same growth rate as ABA-deficient seeds in the same pod (Chapter 4).

ABA levels in developing seeds

From plants of both genotypes, 34 fruits were selected. The fresh weight of their seeds and their concentration of ABA measured by ELISA are presented in Figure 2.5. Wild-type seeds showed two distinct peaks, one during the initial rapid-growth phase and the other just preceding the maturation phase. The ABA concentration of mutant seeds was much more constant and typically a fifth of wild-type values. The increase in ABA at the end of development in both genotypes when expressed on a fresh weight basis (Figure 2.5B) coincided with a decrease of fresh weight during maturation, due to water loss of the seeds. The oldest seeds have dry matter contents of about 85 %. This increase in ABA was much less apparent when the data are expressed on a dry weight basis (Figure 2.5C).

Previous studies of endogenous ABA levels in developing seeds have shown two peaks (Hsu, 1979; Browning, 1980; Wang *et al.*, 1987; Groot *et al.*, 1991). There is evidence in *Arabidopsis* and tomato that the first one mainly originates from maternal tissues, and the second from the embryo itself (Karssen *et al.*, 1983; Groot *et al.*, 1991). In those studies, the first ABA peak was shown to be considerably reduced in heterozygous seeds from an ABA-deficient mother plant, while the second ABA peak was retained (Karssen *et al.*, 1983; Groot *et al.*, 1991). This mechanism will probably also

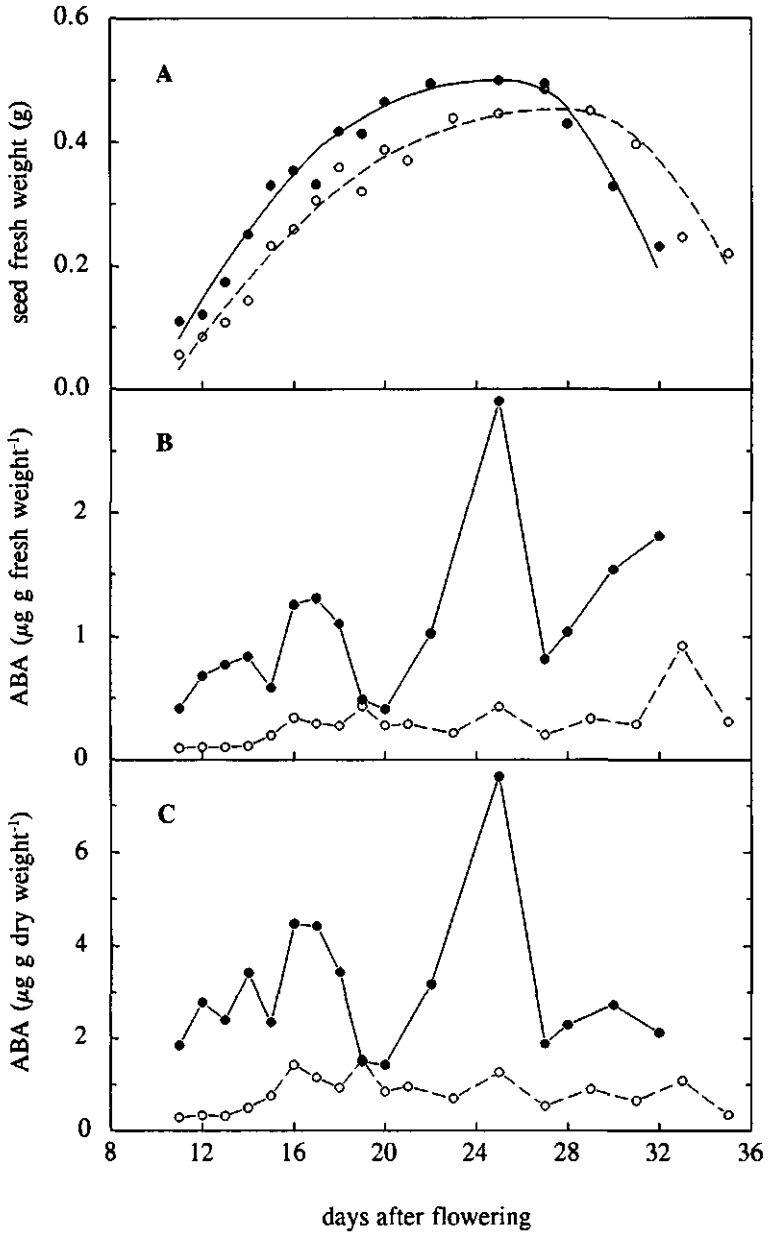

Figure 2.5 Fresh weight (A), ABA content expressed on a fresh weight basis (B) and on a dry weight basis (C) of developing wild-type (●) and ABA-deficient (○) seeds.

hold for pea seeds, since Wang *et al.* (1987) demonstrated that at the time of the first ABA peak, the hormone was also present in large amounts in the pod and the testa, whereas at the end of development both these tissues contained little ABA. Similar results were obtained from *Phaseolus* (Hsu, 1979).

The ABA levels presented in this study deviate to some extent from literature data. The main differences with previous reports are the relatively high ABA concentrations in wild-type seeds at the end of development. Data from ripe pea seeds are absent from the literature, since most authors did not collect seeds with dry matter contents exceeding 50 % (Eeuwens and Schwabe, 1975; Browning, 1980; Wang *et al.*, 1987; Ross and McWha, 1990). Ripe *Phaseolus* and *Vicia* seeds, however, contain almost no ABA (Hsu, 1979; Gräbner *et al.*, 1980). Nevertheless, several species have been described that maintain considerable amounts of ABA in ripe seeds (*e.g.* Piaggesi *et al.*, 1991), although in these species the presence of ABA is generally coupled to dormancy (Black, 1983). Whether the observed differences in ABA level between mature seeds of ABA-deficient and wild-type plants in the present study have led to deeper dormancy for wild-type seeds could not be detected, since pea seeds of both lines usually germinated immediately. In some cases, however, mutant seeds germinated viviparously in the fruits, which was never seen in wild-type seeds.

The fivefold difference between the ABA content of ABA-deficient and wild-type pea seeds corresponds with previous reports of 9-15 or 2-3 times less ABA (Wang *et al.*, 1984, and Jackson and Hall, 1987, respectively) in leaves of this mutant as compared to those of wild-type. A remarkable difference between the pea mutant and other ABA-deficient plants is that pea mutant leaves will accumulate at least some ABA after prolonged exposure to water deficiency or flooding (Wang *et al.*, 1984; Jackson and Hall, 1987), whereas other mutants do not (Quarrie, 1982; Neill and Horgan, 1985; Rock *et al.*, 1992). A slight increase in ABA level upon stress was reported in a wilty tobacco mutant (Parry *et al.*, 1991).

In general, ABA levels in the seeds of ABA-deficient mutants are very variable, and range from half of that of the wild type level in embryos of a viviparous maize mutant (Brenner *et al.*, 1977; Smith *et al.*, 1989) to less than 5 % in the *Arabidopsis aba-1* mutant and 3 % in the tomato *sit*^{w} mutant (Karssen *et al.*, 1983; Groot *et al.*, 1991). The extent to which various ABA mutants are 'leaky' will partly depend on how the mutations interfere with the biosynthetic pathway of ABA. This has been demonstrated in a series of tomato mutants (Taylor and Tarr, 1984) but, so far, the step in ABA biosynthesis

where the pea mutation acts has not been identified. Duckham *et al.* (1989) have shown that the biochemical basis of the *wilty* pea mutant is not the same as in the *flacca* and *sitiens* tomato mutants and the *droopy* potato mutant, which are impaired in the last step of ABA biosynthesis, the conversion of ABA-aldehyde to ABA. Moreover, leaves of the pea mutant have wild-type xanthophyll levels, in contrast to *Arabidopsis aba*-1 mutants in which accumulated zeaxanthin levels indicate a block early in the ABA biosynthetic pathway (Duckham *et al.*, 1991).

Conclusion

The most striking characteristics of the ABA-deficient pea mutant in comparison with its wild type can be summarized as follows: the mutant wilts much faster, grows more slowly, has less apical dominance, has larger shoot/root ratios, invests less dry matter in its roots and seeds, and has five times less ABA in its seeds.

Does ABA influence assimilate partitioning? Several growth parameters differed significantly between the genotypes, but the question remains still unanswered whether these differences were directly caused by the lack of ABA in the mutant. In a similar study, the 25 % slower relative growth rate of the ABA-deficient tomato *sitiens* mutant was primarily attributed to altered water relations, and not to an effect of ABA on sink strength of different plant organs (Nagel *et al.*, 1991). Although the experimental conditions in our study were made as favourable as possible, the higher dry matter content in mutant leaves indicate a disturbed water balance in ABA-deficient plants, which may have caused the retarded development of both vegetative and reproductive parts. Because of the water deficiency, dry matter production and its partitioning into the pods and seeds may be slower. However, at the end of plant development the weight ratio of reproductive to vegetative parts in wild-type plants is equalled by mutant plants (Table 2.I). This finding does not support the idea that ABA influences the sink strength of reproductive organs.

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3 Abscisic acid and assimilate partitioning to developing seeds. I. Does abscisic acid influence sugar efflux from 'empty' seed-coats in an ABA-deficient *Pisum sativum* mutant?

S.M. de Bruijn, E.A.M. Koot-Gronsveld and D. Vreugdenhil

Summary

The effect of abscisic acid on the release of sucrose from surgically modified *Pisum sativum* ovules was studied. Since several reports in literature claimed a significant but small and variable effect, we tried to confirm these results with the use of a wilty pea mutant and variations in the amount of available assimilates. However, phloem import, as measured by the rate of sucrose release from attached ovules, appeared not to be influenced by applied ABA, independent of the endogenous ABA level and the use of source-limited conditions.

Introduction

The involvement of plant hormones in assimilate partitioning towards developing seeds has been subject of research since a number of decades. Several arguments plead for a role of abscisic acid (ABA) in determining or at least influencing the sink strength of developing seeds: seeds generally contain high levels of ABA (King, 1982), and these levels correlate rather well with dry matter accumulation (*e.g.* Düring and Alleweldt, 1980; Schussler *et al.*, 1984). Moreover, stimulating effects of ABA application on seed growth were reported (*e.g.* Düring and Alleweldt, 1980; Schussler *et al.*, 1984; Clifford *et al.*, 1987). However, recent evidence has seriously questioned the influence of ABA on the growth rate of seeds (Quarrie *et al.*, 1988; Schussler *et al.*, 1991).

It has to be stressed that studies on ABA and the growth rate of seeds will only reveal

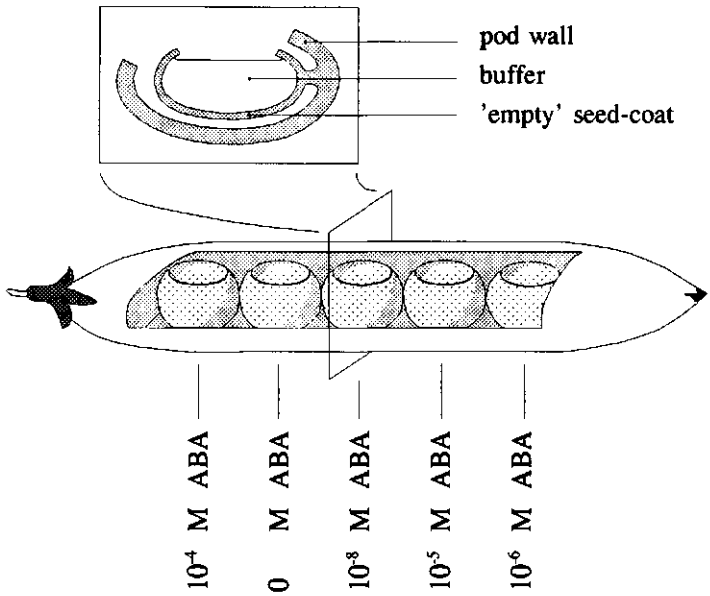

Figure 3.1 Scheme describing the empty-seed-coat technique (see text for explanation).

effects if ABA is the limiting factor. Even if no overall-effect of ABA is found, it cannot be excluded that ABA affects one of the sub-processes of assimilate partitioning, like phloem unloading, transport of assimilates from the seed-coat to the embryo or endosperm, or storage of the assimilates.

About ten years ago, a method to study one of these sub-processes was developed. Several research groups independently introduced the use of the empty-seed-coat technique to study the transport of assimilates from the seed-coat to developing embryos of legume seeds (Patrick, 1983; Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983). By means of a surgical treatment the embryo is removed from the seed-coat and replaced by a solution or agar trap, while leaving most of the maternal tissue intact (Figure 3.1). The medium imitates the apoplastic fluid surrounding the embryo, and receives assimilates from the seed-coat.

Initially, most attention was paid to the effect of the osmolality of the medium on the transport rate; it turned out that, at least in dicotyledons, assimilate release from the seed-coats is a turgor-regulated process, in that sense that relatively high solute concentrations

in the seed apoplast are needed to maintain a high sink strength (reviews: Wolswinkel, 1990, 1992). Also considerable attention has been paid to the metabolic regulation of unloading into the seed-coat apoplast. Evidence was found for energy-dependent unloading since treatments with low temperature, uncouplers or sulfhydryl-group modifiers like *p*-chloromercuribenzenesulfonic acid caused a decrease in assimilate efflux from the seed-coat (Thorne and Rainbird, 1983; Patrick, 1983; Wolswinkel and Ammerlaan, 1983; Gifford and Thorne, 1986).

The empty-seed-coat technique was also exploited to study the possible influence of growth regulators on the efflux of solutes. In a slightly modified experimental system, Van Bel and Patrick (1984, 1985) reported an inhibition of the fusicoccin-stimulated proton extrusion from detached *Phaseolus vulgaris* seed-coats by ABA and interpreted these data as a stimulation of the sucrose unloading by ABA. Clifford *et al.* (1986) demonstrated promotion of photosynthate unloading from these seed-coats by ABA and 6-benzylamino-purine, whereas auxins, gibberellins and another cytokinin were not effective. Gifford and Thorne (1986) found stimulation of sucrose efflux from soybean seed-coats by both ABA and indole acetic acid. Ross *et al.* (1987) described a system that enabled continuous monitoring of ^{11}C-assimilate efflux into a surgically altered seed-coat and reported a rapid effect of ABA on the efflux rate.

However, some of these studies suffer from considerable experiment-to-experiment variability in the observed responses (Clifford *et al.*, 1990), possibly due to variations in endogenous ABA concentrations in the seeds, depending on the photosynthate supply to the seeds (Brenner *et al.*, 1982; Clifford *et al.*, 1987, 1990). In the present study, we used an ABA-deficient pea mutant which has a tenfold lower ABA level in its leaves as compared to the wild type (Wang *et al.*, 1984), in order to compare plants with different endogenous ABA levels. Both source-limited and unrestricted conditions for seed growth were tested, for both genotypes, since we hypothesized that the effect of exogenously applied ABA might be more pronounced at conditions of reduced availability of assimilates (Clifford *et al.*, 1987).

Materials and methods

Plant material

Seeds of ABA-deficient pea plants (*Pisum sativum* L., *wil* mutant, line B78-918) were

kindly provided by prof. G.A. Marx. The non-wilty wild-type line (B1777-155) that was used was not an isoline (because the original parent line was not available: Marx, 1976) but closely approximated the phenotype of the wilty lines (G.A. Marx, personal communication), also with respect to seed development.

Pea plants were cultured hydroponically on well aerated half-strength Hoagland medium in 2-litre jars. Plants were raised in a greenhouse at day temperatures of 17 - 24 °C and night temperatures of 10 - 15 °C, under natural light, supplemented with artificial illumination from high-pressure sodium lamps (SON/T), to give a 16-h day length. Relative humidity in the greenhouse was kept above 60 %.

About one week before the experiments, pea plants with developing pods were pruned to one pod per plant. In some experiments the plants were defoliated (except for the stipules nearest to the pod) to obtain source-limited conditions, four or seven days before the efflux experiments.

Preparation of seed-coat cups

The surgical treatment of the ovules was performed mainly as described by Wolswinkel and Ammerlaan (1983). Fourteen to 20 days after flowering (relative water content of the seeds: 85-77 %), the pods were fixed on a small platter, with some moistened paper tissues underneath the pod and an aluminium-foil covering the pod to prevent evaporation. A rectangular part of the pod wall was removed to expose the developing ovules, without damaging the vascular bundles of the pod wall (Fig 3.1). A 3-mm wide hole was made in the seed-coat and the embryo was carefully removed with a spatula. The seed-coats were not detached from the pod. Four to six ovules per pod were treated, seeds at the end positions of the pod were not used. Three experiments were performed on defoliated ABA-deficient plants, three on ABA-deficient plants at non-limited conditions, and three on wild-type plants.

After the removal of the embryos, the seed-coats were filled with a buffer (2 mM MES, 0.5 mM $CaSO_4$ and 400 mM mannitol; pH 5.5) with or without ABA ((±)-abscisic acid, Fluka). Each pod contained at least one control seed-coat (surgically treated, filled with a buffer without ABA) while the other seed-coats were filled with various ABA concentrations (ranging from 10^{-8} to 10^{-4} M). The seed-coats were filled to the edge, the volume of the seed-coats ranged from 80 to 150 μl. After an initial wash-out of the seed-coats for *ca.* 15 minutes, the contents of the seed-coats were replaced with the same

solution, every two hours, over a period of 12 to 14 hours. The solutions collected from the seed-coats were stored at -20 °C until analysis.

Carbohydrate analysis

The solutions collected from the seed-coats were monitored for sucrose and occasionally also for glucose. Both sugars were determined enzymatically according to the procedures as prescribed by Boehringer (Mannheim, Germany), (Bergmeyer and Bernt, 1974). The sucrose content was calculated from the difference in glucose content before and after treatment of the sample with invertase.

Results

The primary aim of this study was to demonstrate an unequivocal effect of ABA on the release of sucrose from surgically altered seed-coats of *Pisum sativum*. Optimal osmolality of the medium (400 mM) was used, according to Wolswinkel and Ammerlaan (1984, 1986) and Ross *et al.* (1987). The kinetics of sucrose efflux from the seed-coats were similar to the patterns observed by Gifford and Thorne (1986): the initial high rate of unloading was followed by a six to eight hours long period of a more or less constant or slowly declining rate of sucrose release. Even after 24 hours small amounts of sucrose were released. Since the ratio of released glucose to released sucrose appeared to be constant and low (about 5 %: Wolswinkel and Ammerlaan, 1983), in most experiments only sucrose levels were analysed.

In all experiments, each pod contained at least one 'control treatment': a surgically altered seed without added ABA, while the other seed-coats were filled with various concentrations of ABA. In a first experiment the rate of leakage of applied ABA between seed-coats was determined. ^{3}H-ABA (10^{-4} M) was applied to a seed-coat and the amount of radiolabel that appeared in the medium of the neighbouring seed-coats was monitored. It was found that less than 0.1 % of the applied radioactivity was transported to the medium in neighbouring seed-coats. Although it was not checked whether the radiolabel that arrived in a neighbouring seed-coat was still ABA, these data show that the leakage of ABA from the seed-coats was negligible. However, in order to exclude possible interference between adjacent seed-coats, large differences in ABA-levels between neighbouring seed-coats were avoided in the further experiments.

From the same experiment convincing evidence was derived that applied ABA indeed entered the seed-coat and elevated the tissue level of ABA. The seed-coats were filled with 10^{-4} M ^{3}H-ABA, and the solutions were replaced every two hours. At the end of the experiment, the concentration of radiolabel in the seed-coat represented about $1.5 \cdot 10^{-4}$ M ABA (40 μg g^{-1} fresh weight, about 60 times higher than the original endogenous level in wild-type seed-coats). It was concluded that applied ABA easily penetrated the seed-coat and that the endogenous ABA level approximately increased to the applied concentration.

Subsequently, the effect of ABA on the efflux of sucrose from seed-coats was studied. The cumulative amount of sucrose released from individual ABA-treated seed-coats was expressed as the percentage of the amount of sucrose released from the control seed-coat in the same pod. The first sample of each seed (the 15-minutes wash-out of the seed-coat) was discarded since the sucrose efflux in this period varied considerably between seeds (*cf.* Thorne, 1986). The steady-state sucrose efflux corresponded well with the dry matter gain of the seeds at this developmental stage (about 5-7 mg day^{-1}: Wolswinkel and

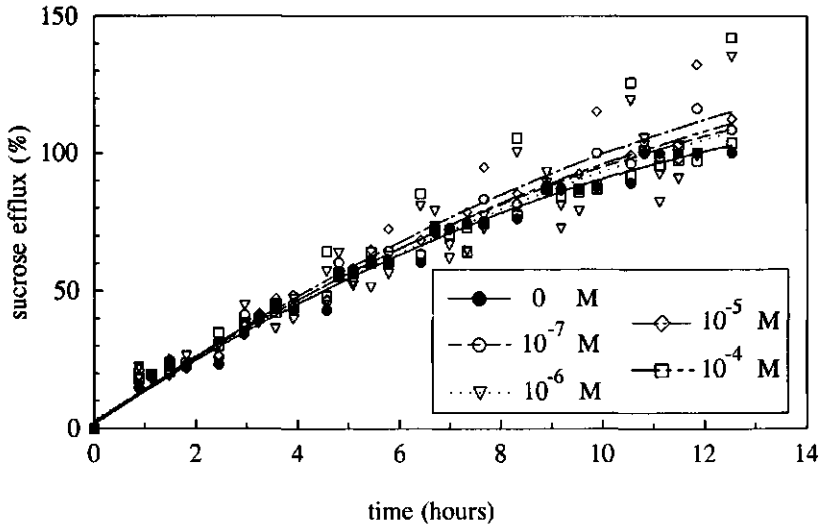

Figure 3.2 Cumulative efflux of sucrose from surgically modified seed-coats of wild-type *Pisum sativum* plants at various ABA concentrations. No restrictions on available assimilates were exerted. All values are expressed relative to the control treatment (without ABA). Data of 30 seeds from 6 pods. The data belonging to the same ABA concentration were taken together and fitted with a third-order polynomial.

Ammerlaan, 1985).

Initially, the experiments were performed on wild-type plants. The release data of 30 seeds from six pods were combined in Figure 3.2. Since no influence of applied ABA was found, the same experiments were carried out with ABA-deficient plants; however, the results were essentially similar to the data of wild-type plants (data not shown, but *cf.* Table 3.I).

Subsequently, the plants were subjected to source-limited conditions by defoliating them four days before the experiment. However, the rates of sucrose release from seed-coats on those plants were similar to the rates at unrestricted conditions, indicating that source-limitation had not been attained. In this experiment no significant differences between treatments were observed (data not shown, but *cf.* Table 3.I). When plants were defoliated seven days before the experiment, they had a 25 % lower release rate of sucrose as compared to control plants (58 ng s^{-1} in stead of 79 ng s^{-1}). But also at these source-limited conditions no effect of ABA on sucrose release was discerned (Figure 3.3, 44 seeds from 11 pods). Further reduction of the amount of available assimilates by

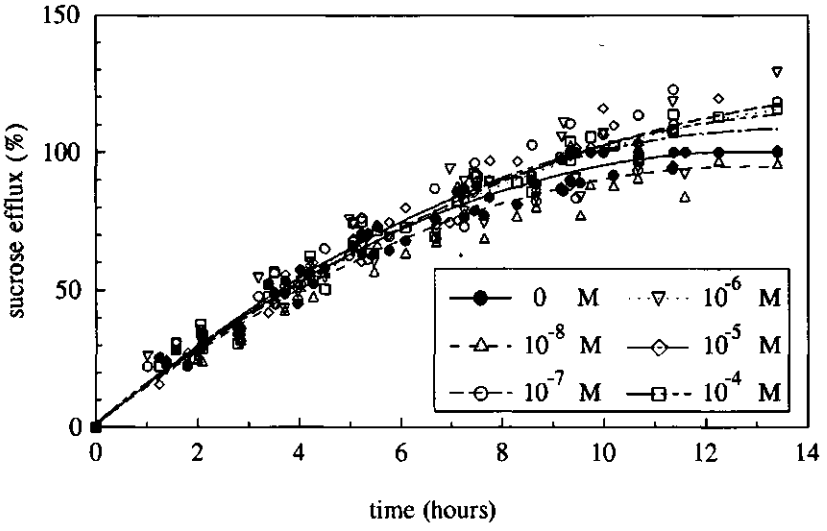

Figure 3.3 Cumulative efflux of sucrose from surgically modified seed-coats of ABA-deficient *Pisum sativum* plants at various ABA concentrations. The plants were defoliated seven days before the experiment. All values are expressed relative to the control treatment (without ABA). Data of 44 seeds from 11 pods. The data belonging to the same ABA concentration were taken together and fitted with a third-order polynomial.

defoliating the plants earlier during development caused abortion of the seeds.

Although the data presented in Figures 3.2 and 3.3 show some variation, it is obvious that the presence of ABA in the medium had little or no effect on the release of sucrose in those seeds. To get more insight in the release rate during the period of near-constant sucrose unloading, the data of the sucrose release in each seed (relative to the control seeds) were individually fitted (third-order polynomial, r^2 in all cases higher than 0.99) and the rate of sucrose release at six and ten hours after the start of the experiment was calculated. Subsequently, the data belonging to the same ABA concentration were averaged (Table 3.I). No significant differences between genotypes and treatments were observed (Student's *t*-test, $P = 0.10$).

Discussion

The kinetics of sucrose release from seed-coats in our study closely resembled the data presented by Gifford and Thorne (1986). The first phase of rapid sucrose release was interpreted as apoplastic purging of the seed-coat and equilibration of symplastic sucrose with the medium; this period was followed by a phase of constant or slowly declining sucrose release, supposed to reflect the import of sucrose *via* the phloem connection with the mother plant (Wolswinkel and Ammerlaan, 1985; Gifford and Thorne, 1986; Ellis and Spanswick, 1987). Although the use of measurements of assimilate release from surgically modified ovules over time-spans greater than two or three hours has been criticized by Minchin and Thorpe (1989), Wolswinkel and Woerselman-Kooij (1992) have shown that the rate of sucrose transport into and unloading from 'empty' seed-coats can be almost constant during considerably longer periods, in experiments with stagnant solutions that are replaced not too frequently. Experiments on perfused seed-coats (Minchin and Thorpe, 1989) obviously have different release kinetics.

Comparison of data from attached and detached seed-coats revealed that after eight hours by far the largest part of released sucrose originated from phloem import (Wolswinkel and Ammerlaan, 1985; Gifford and Thorne, 1986), in contrast to released amino acids and phosphate which at that time still mainly originate from reserves in seed-coat tissues (Wolswinkel and Ammerlaan, 1985; Lanfermeijer *et al.*, 1992). Pea seed-coats also have carbohydrate reserves (Boyer, 1981) that seem to be mobilized during seed development (Minchin and Thorpe, 1990), but it is still uncertain whether and in what way these starch reserves interfere with the sucrose release in 'empty' seed-coats. Thus,

Table 3.I Relative efflux rates of sucrose (mean ± SD (n)) from surgically modified seed-coats of both wild-type and ABA-deficient *Pisum sativum* plants at various ABA concentrations, at six and ten hours after the start of the experiment. Both source-limiting and unrestricted conditions were used. Efflux rates were calculated from the tangent of the curves fitted through the data of individual seed-coats.

ABA-concentration (M)	wild type	ABA-deficient		
	unrestricted	unrestricted	defoliated four days before	defoliated seven days before
t = 6 h				
0	7.0 ± 1.2 (9)	7.4 ± 1.1 (11)	6.7 ± 0.5 (4)	7.9 ± 1.0 (10)
10^{-4}	7.2 ± 1.7 (10)	7.4 ± 1.6 (8)	7.2 ± 2.3 (3)	8.5 ± 0.8 (9)
10^{-5}	7.6 ± 2.6 (11)	7.0 ± 1.6 (7)	n.d.	8.5 ± 1.5 (8)
10^{-6}	6.8 ± 2.0 (7)	7.9 ± 2.1 (7)	n.d.	8.7 ± 1.0 (7)
10^{-7}	7.0 ± 1.8 (8)	7.7 ± 1.1 (7)	7.4 ± 1.2 (3)	8.5 ± 2.3 (7)
10^{-8}	n.d.*	7.0 ± 1.4 (4)	5.9 ± 1.4 (3)	7.5 ± 1.3 (6)
t = 10 h				
0	5.2 ± 1.4 (9)	5.4 ± 0.8 (11)	5.1 ± 0.8 (4)	5.2 ± 1.4 (10)
10^{-4}	5.9 ± 2.1 (10)	5.1 ± 1.1 (8)	4.8 ± 2.4 (3)	6.0 ± 1.6 (6)
10^{-5}	5.9 ± 2.2 (11)	4.8 ± 1.0 (7)	n.d.	5.5 ± 1.8 (8)
10^{-6}	5.5 ± 2.1 (7)	5.1 ± 1.1 (7)	n.d.	6.0 ± 2.1 (7)
10^{-7}	5.5 ± 2.0 (8)	5.4 ± 1.1 (7)	5.4 ± 1.5 (3)	5.8 ± 1.5 (7)
10^{-8}	n.d.	4.9 ± 0.7 (4)	4.4 ± 1.7 (3)	5.2 ± 1.5 (6)

* n.d.: not enough data

it has to be kept in mind that the sucrose that is released into the medium is the net result of import (unloading) from the phloem and metabolism or compartmentalization in the seed-coat symplast. However, it seems reasonable to conclude from the above-mentioned evidence that the observed net sucrose release between four and ten hours after the start of the experiment reflects at least for the largest part the import of photoassimilates from source tissues to the seed-coat by the phloem.

In the first instance, the presented data were fitted according to the equation proposed by Lanfermeijer *et al.* (1992). This equation consisted of two exponential components describing the release from the vacuolar and the cytoplasmic compartment in the seed-coat cells, and a linear term that represents the phloem import through the funiculus. After fitting, however, the reliability of the linear component appeared to be very low, for the

same reason as mentioned by Lanfermeijer *et al.* (1992). Therefore, we decided to fit the data as polynomial curves and to derive the sucrose import rate from the tangent of these curves.

Does ABA influence the sucrose release from 'empty' seed-coats of pea? The reports in literature concerning the effect of applied ABA can be divided into two categories: short-term studies particularly describing effects of ABA on the release of assimilates in the testa apoplast or symplast, and long-term studies on the effects of ABA on phloem import. Ross *et al.* (1987) found variable results in a system for short-term monitoring of ^{11}C-assimilate efflux into attached pea seed-coats: stimulation of tracer unloading at 10^{-8} M ABA, slight but variable enhancement at 10^{-6} M ABA and conflicting results at 10^{-4} M ABA. In studies on excised seed-coats, also a rapid but variable stimulation of sucrose release was reported by Clifford *et al.* (1986, 1990). ABA was able to enhance the release of sucrose by 30-40 %, within 10-20 minutes. In a long-term experiment with soybean, Gifford and Thorne (1986) have claimed a small (10-15 %) but significant stimulating effect of $4 \cdot 10^{-6}$ M ABA on the steady state efflux of sucrose from attached seed-coats.

Variation in seed ABA concentrations was supposed to cause the variability in the responses to exogenous ABA observed in some of the above-mentioned studies (Clifford *et al.*, 1990). For this reason, in the present study an ABA-deficient pea mutant with a tenfold lower ABA content in its leaves (Wang *et al.*, 1984) and a fivefold lower ABA content in its seeds (Figure 2.5) was compared with its wild type. Since a limited amount of available assimilates might increase (Clifford *et al.*, 1987) or decrease (Clifford *et al.*, 1990) the effect of applied ABA on photosynthate unloading towards the seed, experiments were performed both at source-limited and unrestricted conditions. We hypothesized that the effect of exogenously applied ABA might be more pronounced at conditions of reduced availability of assimilates and on plants with low levels of endogenous ABA.

It was somewhat puzzling that defoliation of the plants initially hardly influenced the rate of sucrose release from the seed-coats. From other experiments with the same plants and conditions we know that seeds of defoliated plants have final dry weights of about 40 % of the weight of control seeds (119 $\pm$ 48 mg and 297 $\pm$ 61 mg, respectively), so there is no doubt that the conditions are restricting enough. Initially the experiments were performed four days after defoliation, but apparently at that time the plant still managed to mobilize enough reserves (from the stem, stipules and fruit wall) to keep the amount of released sucrose as high as in the control situation. At seven days after defoliation the

amount of released sugars was about 25 % lower, but even at those conditions no effect of ABA was detected.

Reduction of available assimilates will also decrease the supply of maternal ABA to the seeds (Brenner *et al.*, 1982). In the present study, this effect will be enhanced in ABA-deficient plants. However, no influence of applied ABA was observed in any of the four experiments, even not in seed-coats of mutant plants at source-limited conditions. The absence of influence by ABA on assimilate transport fits well with other findings from our group that pea seeds in the same pod with a different ABA content have similar growth rates (Chapter 4). In the present study, no attention was paid to the short-term release of assimilates from seed-coats, but only to the influence of ABA on phloem import. Therefore, an effect of ABA on the release of assimilates during the first phase, the purging period, cannot be excluded by our data. Another experimental setup is necessary to answer that question.

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4 Abscisic acid and assimilate partitioning to developing seeds. II. Does abscisic acid influence the growth rate of pea seeds?

S.M. de Bruijn and D. Vreugdenhil

Summary

A wilting mutant (*wil*, line B78-918) of pea (*Pisum sativum* L.) with a reduced ABA content was used to determine the effect of ABA on the growth rate of seeds. This mutant was backcrossed with a non-wilty line to achieve isogenic lines. Crosses were performed to obtain pods with both ABA-containing and ABA-deficient seeds. After opening the pods, the diameters of the seeds were measured daily with a pair of callipers. Normal growth patterns of the seeds were not disturbed by these manipulations. No effect of the genotype on the overall growth pattern of the seeds was detected. We conclude that ABA has not a major influence on the growth rate of pea seeds.

Introduction

The involvement of plant growth regulators in the transport of assimilates towards developing seeds or fruits has been the subject of study for many years. Traditional techniques to study the influence of growth regulators on different physiological processes imply application of the compound in study or the inhibition of its synthesis or activity by chemical means. Main disadvantage of these techniques is that exogenous application of a hormone generally leads to availability at the inappropriate place, time or concentration. Moreover, inhibitors of hormone synthesis or activity might cause undesirable side-effects, and the techniques that are used to apply the

hormones or their inhibitors sometimes cause severe injury to the tissues.

Of all classes of hormones, ABA seems to be a suitable candidate for a role in the regulation of assimilate partitioning (Brenner, 1987; Patrick, 1987). The numerous reports on the correlation between the level of ABA and the growth rate of fruits or seeds (*e.g.* Eeuwens and Schwabe, 1975; Düring and Alleweldt, 1980; Browning, 1980; Berüter, 1983; Schussler *et al.*, 1984; Wang *et al.*, 1987; Lopez *et al.*, 1989; Ross and McWha, 1990) have led to extensive research to establish a causal relationship between these two phenomena.

However, the data on the involvement of ABA in the regulation of assimilate partitioning towards developing seeds are controversial up to now. Initially, inhibition of assimilate transport to barley seeds by applied ABA was reported (Wagner, 1974), but later on several researchers found stimulating effects of ABA application (Dewdney and McWha, 1979; Düring and Alleweldt, 1980; Berüter, 1983; Schussler *et al.*, 1984; Gifford and Thorne, 1986; Clifford *et al.*, 1986, 1987, 1990; Archbold, 1988). Several other authors did not find any effect of ABA on processes associated with growth of fruits or seeds (Davies and Bedford, 1982; King and Patrick, 1982; Barratt *et al.*, 1989; Ober and Setter, 1990).

All of those data, however, suffer from the above-mentioned problems. Although hormone levels were always increased by application of ABA, several authors did not consider the possibility of transport, metabolism or reduced penetration (Wagner, 1974; Düring and Alleweldt, 1980; Berüter, 1983; Schussler *et al.*, 1984; Clifford *et al.*, 1986; Gifford and Thorne, 1986), while these problems may seriously influence the results (Dewdney and McWha, 1978; Brenner *et al.*, 1982; Clifford *et al.*, 1987). Decrease of hormone levels, on the other hand, was achieved with fluridone (Barratt *et al.*, 1989), but this causes undesired bleaching of the plants because of the concomitant inhibition of carotenoid biosynthesis.

Since recently several mutants became available with a reduced abscisic acid content, the possibility arose to use these mutants as a tool to study the role of growth regulators in assimilate partitioning. Hormone mutants allow non-invasive manipulation of hormone levels or sensitivity to hormones. A special advantage with respect to transport of assimilates to seeds is that the competition between genetically different seeds can be studied.

In this chapter, we describe the use of an ABA mutant of pea, *Pisum sativum*, to obtain a system with both ABA-containing and ABA-deficient seeds within the same

pod. To determine the growth rates of individual seeds, a new non-destructive technique was developed: we opened the pod walls at an early stage and regularly measured the dimensions of the seeds with a pair of callipers. The experiments were performed at source-limited conditions, since (Clifford *et al.*, 1987) found that effects of both ABA and cytokinin could only be detected after severe defoliation of the plants.

Materials and methods

Plant material

Seeds of ABA-deficient pea plants (*Pisum sativum* L., *wil* mutant, line B78-918) were kindly provided by prof. G.A. Marx. The original parent line was not available (Marx, 1976) and hence we obtained an isogenic line after six successive backcrosses (*cf.* Chapter 2) with a normal, non-wilty line, closely approximating the phenotype of the wilty lines (B1777-155) (G.A. Marx, personal communication).

Pea plants were cultured hydroponically on well aerated half-strength Hoagland medium, either separately in 2-litre jars or on containers with 50 plants per 50 litres. Plants were raised in a glasshouse at day temperatures of 17 - 22 °C and night temperatures of 10 - 15 °C, under natural light, supplemented with artificial illumination from high-pressure sodium lamps (SON/T), to give a 16-h day length. Relative humidity in the glasshouse was kept above 60 %.

Effect of pod opening on seed development

Young pods on 200 plants grown on containers were tagged at a length of 30 mm (about five days after flowering). Seven days after tagging 116 pods were opened whereas 112 pods remained undisturbed as a control. Opening of the pods was done by making an incision along the dorsal side, across from the seeds (Figure 4.1). Seed diameter at the time of opening ranged from 2 to 4 mm, fresh weights from 30 to 80 mg. Opened pods were closed again with two small strips of parafilm. Both opened and control pods were harvested at various intervals thereafter, and fresh and dry weights (after 24 h 105 °C) of the seeds were determined.

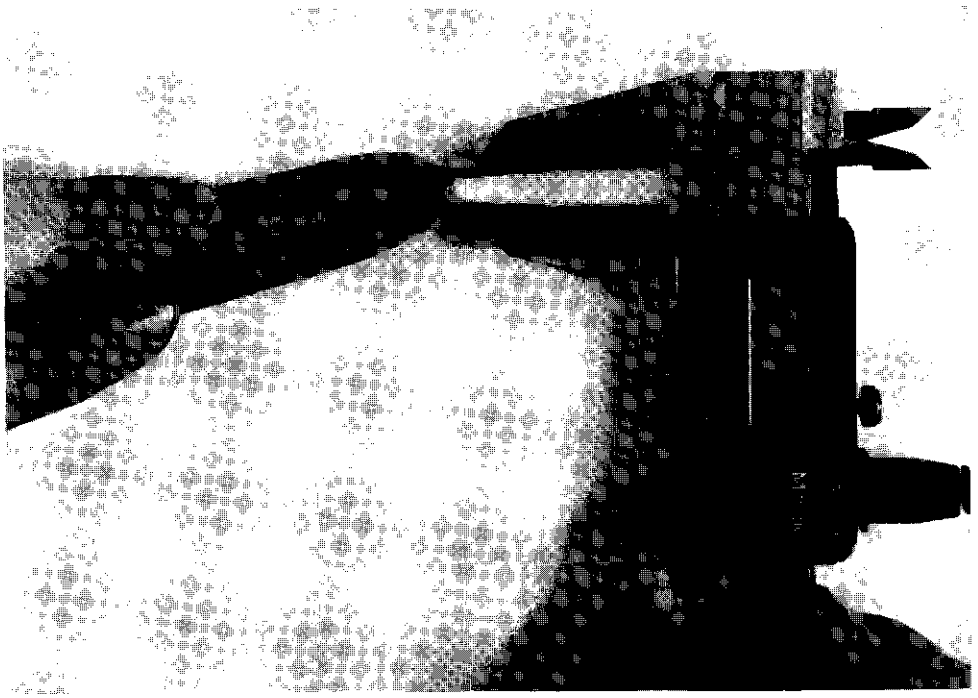

Figure 4.1 Opened pod of *Pisum sativum* and pair of callipers during measurement.

Influence of daily measurements on seed development

Pods of individually grown plants were opened at the same stage as mentioned above. In each pod, some of the seeds were measured daily with a pair of callipers, whereas the other seeds were not. The callipers were equipped with long and thin measuring faces to enable measurements within the pod; data were collected with a miniprocessor. Only the diameter in transversal direction was measured. Data were recorded when the measuring faces just visibly touched the seeds, without exerting any pressure.

Dimensions of the non-measured seeds were calculated from photographs made at the day of opening. After seven to ten days the experiment was terminated and all seeds were measured.

Influence of ABA on the growth rate of seeds

Emasculated flowers of ABA-deficient (*wil/wil*) mother-plants were pollinated with pollen from heterozygous (*Wil/wil*) plants. This caused segregation in the progeny for this trait: *Wil/wil* and *wil/wil* seeds occurred in a 1:1 ratio in the same pod. In general only one flower per plant was used. Just prior to the measurements the plants were defoliated except for the stipules nearest to the pod, to reach source-limited conditions. Growth rates of the seeds were measured as described above. The genotypes of the seeds were determined after sowing the seeds: the rate of decrease in fresh weight of detached leaflets of the seedlings was monitored (Wang *et al.*, 1984). The rate of evaporation of the ABA-deficient leaves was approximately three times higher as compared to wild-type leaves (Figure 2.1). Before sowing, the weights of the ripe seeds were determined.

Results

Influence of pod opening

Fresh and dry weights of the harvested seeds were determined and averaged per pod. Since seeds at the end positions of pods frequently fail to complete their development (a commonly observed phenomenon already mentioned by Linck, 1961), we omitted seeds when their fresh weight did not reach up to 60 % of the heaviest seed in that pod. The number of seeds that had to be omitted was the same for both control and opened pods. Figure 4.2 shows the average seed weight per pod during development.

Seed growth rate was not noticeably influenced by the opening of the pods. Only during the last week of development a small difference in fresh weight appeared due to earlier maturation of seeds in opened pods, manifested in faster yellowing and earlier water loss as compared to control seeds. This was probably due to the less humid conditions within the opened pods. This phenomenon was most obvious for seeds at the end positions of the pod.

Effect of measuring the seeds

Seed dimensions were determined in opened pods and compared with the dimensions

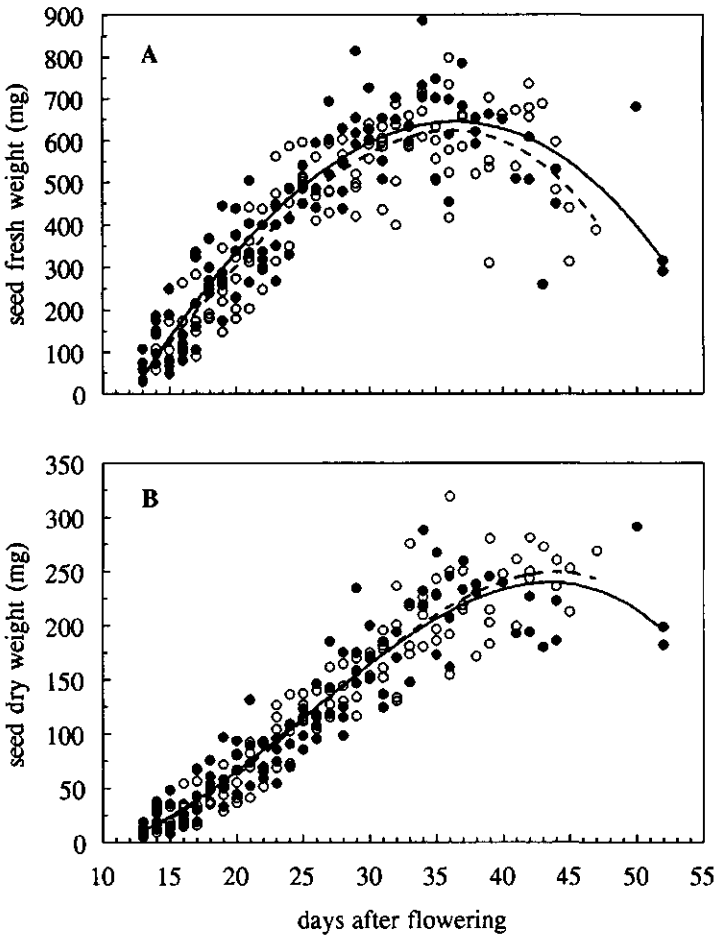

Figure 4.2 Average fresh (A) and dry (B) weights of seeds of *Pisum sativum* from control and opened pods during development. Each data point represents the average weight of the seeds within one pod, seeds were omitted when their fresh weight did not reach up to 60 % of the heaviest seed in that pod. Closed symbols and solid curve: undisturbed pods; open symbols and dotted curve: opened pods.

of undisturbed seeds in the same pod. Figure 4.3 shows two representative examples of the development of the seeds within these pods. Obviously, seed diameter was not influenced by the daily recurring measurements. The lower graph shows an example of two seeds (at the distal end of the pod) that stopped growing early during development.

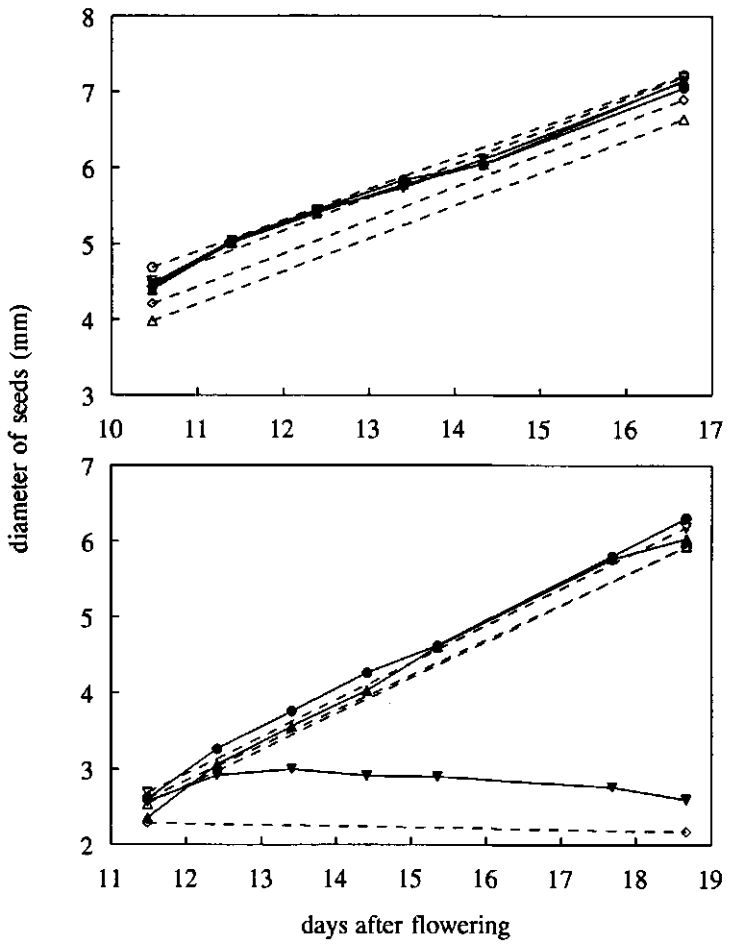

Figure 4.3 Two representative examples of the growth curves of *Pisum sativum* seeds. Each panel shows the growth pattern of all seeds of a single pod. Each curve represents the growth of an individual seed. Solid lines connect data of daily measured seeds (three in each graph); dotted lines connect the diameters of control seeds calculated from a photograph at the start of the experiment and the diameters measured at the end of the experiment (four in each graph).

Since the diameter of the seeds correlated very well with their fresh weights (Figure 4.4) this method is an appropriate technique to study growth rates of pea seeds, from about ten days after pollination. Differences in diameter of 0.1 mm could easily be determined.

Effect of ABA on the growth rate of seeds

To study the influence of ABA on the growth rate of seeds, we pollinated an ABA-deficient mother-plant with pollen from a heterozygous plant. Pods containing at least four seeds were selected for further experiments.

Measurements of the seeds, segregating for the *wil* trait, were started when the seeds had achieved a diameter between 2 and 3 mm. When the measurements were finished and the seeds were dehydrated, the seeds were sown and the genotype of the seedlings was determined by the wilting behaviour of detached leaflets.

Figure 4.5 shows two typical examples of the growth pattern of all seeds of a single pod. It is obvious from these graphs that the genotype of the seeds did not influence their sink strength.

We calculated the maximum growth rates of the seeds by fitting the curves as logistic dose-response curves, as proposed by Morgan *et al.* (1975). Twenty-seven ABA-deficient seeds were compared with 32 ABA-containing seeds (from 13 pods), and the slopes of the corresponding growth curves turned out to be normally

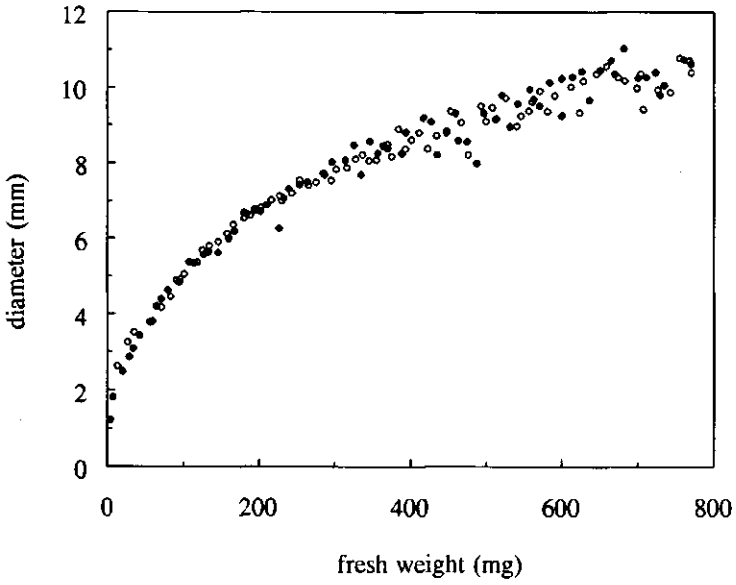

Figure 4.4 Relationship between diameter and fresh weight of *Pisum sativum* seeds. Closed symbols: seeds grown in undisturbed pods; open symbols: seeds grown in opened pods.

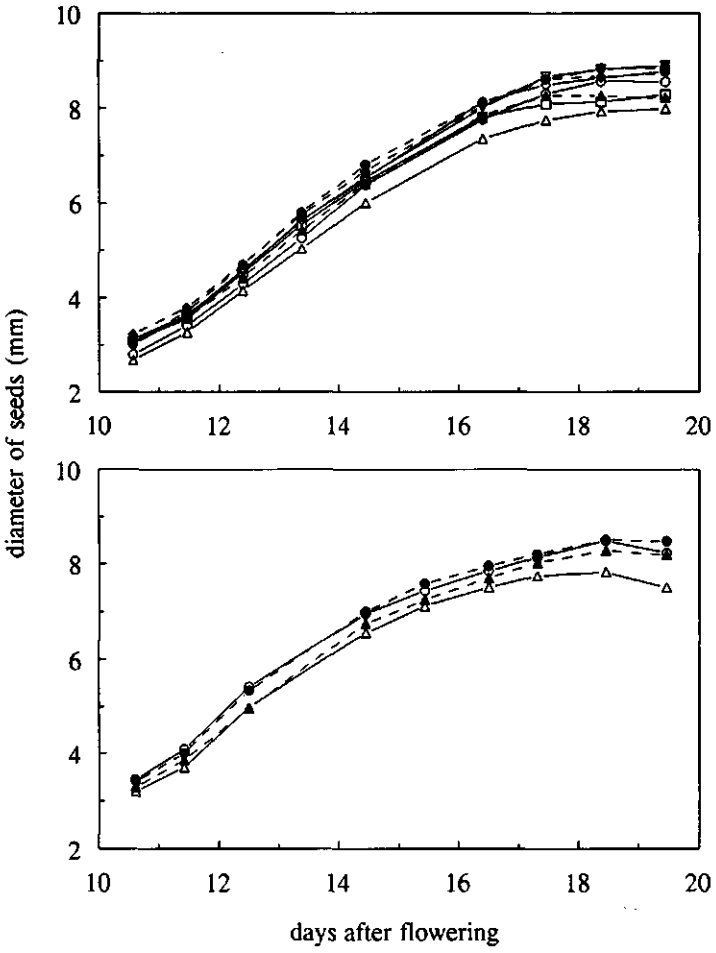

Figure 4.5 Two representative examples of the growth patterns of genetically different *Pisum sativum* seeds. Each panel shows the growth pattern of all seeds of a single pod. Each curve represents the growth of an individual seed. Dotted lines and closed symbols: ABA-containing seeds; solid lines and open symbols: ABA-deficient seeds.

distributed (rankit analysis, data not shown). ANOVA analysis of the results revealed no significant difference between the growth rates of the two genotypes. This analysis, however, was sensitive enough to detect a 5 % difference between the average growth rates.

The fresh weights of the mature seeds that were used for this experiment were also analysed by ANOVA. Again, a very low *F*-ratio was found, indicating no significant difference between the genotypes.

Discussion

The use of hormone mutants makes non-invasive manipulation of hormone levels possible, in contrast to traditional techniques. The pea mutant used in the present study not only has a tenfold lower ABA content in its leaves than the corresponding wild type (Wang *et al.*, 1984), but also its seeds are ABA deficient (Figure 2.5).

In our experiments, this difference in ABA content in the seeds was exploited. After crossing an ABA-deficient mother-plant with a heterozygous plant, we got both ABA-containing and ABA-deficient seeds in the same fruit. Since seeds in the same pod normally have very similar growth rates, the possible influence of the ABA content of the seed on its growth rate could be determined without any interference from the application of exogenous ABA.

Most studies on growth rates of seeds are based on destructive methods: flowers are tagged, at certain intervals fruits are harvested and the weight of the seeds is determined (Flinn and Pate, 1968; Schussler *et al.*, 1991). The variation in the population samples introduced by this experimental setup can be reduced by comparing seeds that developed in paired fruits (Clifford *et al.*, 1987), or by culturing detached fruits or excised embryos on media with or without ABA (Davies and Bedford, 1982; King and Patrick, 1982; Barratt, 1986; Barratt *et al.*, 1989).

To avoid such problems, a non-destructive method to determine the growth rate of individual pea seeds was developed. Opening of the pea pods at about 12 days after anthesis had no influence on the growth pattern of the seeds (Figure 4.3). A quite similar method was presented by Brenner and Ozga (1991). In this so-called split-pericarp technique the pods were also opened along the suture of the pericarp, allowing access to the seeds while maintaining pericarp growth. This technique was used to determine the role of gibberellins in the elongation of pea pods. For this aim, the pods were opened as early as a few days after anthesis. That is probably the reason why the growth of both the seeds and the pods was slightly inhibited, in contrast to our results.

Due to the above-mentioned improvements, we are now able to conclude that, at

least in pea seeds, ABA does not exert a major influence on the development of the seeds. Since Clifford *et al.* (1987) have found that ABA only increased dry weight gain by seeds when the plants were defoliated beforehand, we applied these source-limited conditions in the experiments on the effect of genotype on seed growth. Source-limitation should not only increase the difference in ABA concentration between homozygous-recessive and heterozygous seeds, since the maternal supply of ABA is reduced (Brenner *et al.*, 1982), but we also hypothesized that effects of ABA might be more pronounced when the sinks have to compete for a limited supply of assimilates. Nevertheless, no significant difference in growth rate between the genetically different seeds appeared.

Our conclusion that ABA does not play a major role as a regulator of dry matter transfer to seeds, is supported by some recent findings. Earlier, Schussler *et al.* (1984) reported that ABA concentrations in developing soybean seeds correlated well with their *in situ* seed growth rates and their *in vitro* sucrose uptake rates, and that this *in vitro* sucrose uptake was stimulated by exogenous ABA. However, in a more extended study they now state that the correlation between ABA and dry matter accumulation was absent or even negative, and that no cause-and-effect relationship between ABA levels and the rate of sucrose uptake by isolated embryos may be inferred from their data (Schussler *et al.*, 1991). Also in the study of Quarrie *et al.* (1988) no relationship was found between the levels of ABA and the growth rate of wheat and oat seeds in genetically related lines. Two wheat lines that differed 50 % in seed growth rate had a comparable ABA content in their seeds, while grains of two oat lines that differed 70 % in ABA content had the same growth rate.

Although we realize that it is difficult to disregard all the evidence that pleads for a role of ABA as a regulator of assimilate transport, we suggest that these experiments should be reconsidered or extended with the use of non-invasive methods. On the other hand, the present findings do not rule out that ABA might affect some of the (sub-)-processes associated with assimilate partitioning, *e.g.* phloem unloading or distribution of the assimilates among the various types of reserve material in the sinks.

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5 Abscisic acid and assimilate partitioning to developing seeds. III. Does abscisic acid influence the sink strength of *Arabidopsis* seeds?

S.M. de Bruijn and D. Vreugdenhil

Summary

The role of ABA in the regulation of transport of assimilates to seeds was investigated with the aid of *Arabidopsis thaliana* mutants that were ABA-deficient and/or insensitive to ABA. Subsequent flowers of mutant mother plants were alternately pollinated with pollen from either wild-type or mutant plants, and the transport of radiolabelled photoassimilates to the genetically different seeds was studied. The experiments were performed under conditions of reduced availability of source material, achieved either by reduced light quantity or by combining the ABA-deficient mutant with a starchless mutant. No effect of the genotype on the import rate of assimilates was detected, indicating that endogenous ABA does not influence the sink strength of *Arabidopsis* seeds. Reports describing contrary results are discussed.

Introduction

In general, seeds contain relatively high concentrations of hormones (Brenner, 1987). The high amounts of ABA that are present in developing seeds, and the parallel between ABA content and the rate of dry matter accumulation (King, 1982) have frequently raised the question whether the presence of high ABA levels is either the cause or the result of high assimilate unloading rates.

Unfortunately, available data on the role of ABA in seed development are quite contradictory. Both inhibition (Wagner, 1974) and stimulation (Düring and Alleweldt,

1980; Tietz *et al.* 1981; Clifford *et al.*, 1986, 1987, 1990) of assimilate partitioning to seeds due to ABA have been reported, whereas in other cases no effect of ABA on seed growth was found (King and Patrick, 1982; Quarrie *et al.*, 1988; Groot *et al.*, 1991).

The traditional approach to study the effect of hormones involves application of regulators. This technique, however, has several drawbacks. The first problem is that processes like penetration, metabolism and transport of the applied substance may considerably influence its actual concentration at the supposed site of action. Furthermore, the method of application itself often induces artefacts (e.g. Dewdney and McWha, 1979; cf. King and Patrick, 1982). Another complicating factor is the unknown endogenous hormone level; decreasing this level by inhibitors like fluridone or growth retardants may have undesirable side-effects. The endogenous level at the site of action may be over-estimated by factors like compartmentalization or inactivation, e.g. conjugation.

On the other hand, as has been outlined by Trewavas (1981, 1982), tissue sensitivity to growth substances may be far more important (and probably represents the limiting factor in plant development) as compared to amounts of growth substances. The physiological meaning of correlative studies that point to a relationship between hormone levels and responses like seed growth, may be questioned when no attention is paid to the sensitivity of the tissue under investigation.

The use of hormone mutants provides an alternative tool to obtain evidence on the role of ABA in plant development, circumventing the above-mentioned problems. The first advantage is the possibility to increase or decrease hormone levels non-invasively. Since recently several response-mutants became available, sensitivity to hormones cannot only be assessed but also manipulated.

Another advantage of the use of hormone mutants as a tool to study the role of growth regulators in transport of assimilates to seeds is the unique possibility to alter the genotype of the seeds developing on the same mother plant, and to study their competitive strength for photosynthates as compared to unchanged seeds, developing on the same mother plant.

In this Chapter, we describe the partitioning of assimilates using *Arabidopsis* mutants that were mutated in their capacity to synthesize ABA. This trait was combined with insensitivity to ABA or with a deficiency in starch synthesis. We included the starchless mutant since Clifford *et al.* (1987) found that effects of both ABA and cytokinin could only be detected at source-limited conditions.

Materials and methods

Plant material

All *Arabidopsis thaliana* (L.) Heynh. mutants that were used were either derived from or backcrossed with the pure line Landsberg 'erecta' (wild type). The isolation and characterization of the ABA-deficient (*aba*-1, isolation number A26) and the ABA-insensitive (*abi3*, isolation number CIV, presently denoted as *abi3*-1: Nambara *et al.*, 1992) mutant were described before (Koornneef *et al.*, 1982, 1984). The recombinant *aba,abi3* was originally isolated from a population segregating for the *aba* gene (Koornneef *et al.*, 1989) and recognised by the dark green colour of the mature seeds. This recombinant was maintained by transferring near-mature seeds from the siliques (the fruits) directly to moist filter paper.

The starchless (*pgmP*) mutant was derived from line TC35 (Caspar *et al.*, 1985). This mutant was crossed with the *aba* mutant to obtain recombinants that were both ABA-deficient and starchless. The *aba,pgmP* recombinants were recognised by the slightly different leaf colour and absence of dormancy that are characteristic for the *aba* mutants, and staining of the ethanol-decolourized leaves with iodine solution (Caspar *et al.*, 1985).

Culture conditions

Seeds were sown in 9-cm Petri dishes on moist filter paper (Schleicher & Schüll nr. 595), and allowed to germinate in a climate room at 24 °C, under continuous fluorescent light. After three or four days, the seedlings were planted out in 5.5 cm pots with a mixture of sand and humus. The plants were grown in a greenhouse at day temperatures of 17-22 °C and night temperatures of 15-17 °C, under natural light, supplemented with artificial illumination from high-pressure sodium lamps (SON/T, irradiance 150-200 μmol m^{-2} s^{-1}), to give a 16-h day-length. Starchless mutants were grown under continuous light conditions. Relative humidity was kept above 75 %. Three or four days before the labelling experiments, the plants were transferred to a climate room with conditions comparable to those in the greenhouse: 19/15 °C and a relative humidity of 80 %. The light conditions in the climate room were relatively low: the irradiance was 50 μmol m^{-2} s^{-1} (coolwhite fluorescent illumination) for the starchless mutants and 100 μmol m^{-2} s^{-1} for the other genotypes.

Crosses

Crosses were performed after emasculating the female parent just prior to opening of its flowers. Pollination was carried out immediately, with fresh pollen, and repeated several times on the same flower, to obtain maximum seed set. To obtain fruits with genetically different seeds on the same mother plant, subsequent flowers were alternately pollinated with pollen from plants with either the same or a dissimilar genotype (Figure 1.3). After crossing, the flowers were tagged with a small thread, the colour of the thread corresponding to the genotype of the male parent. Not more than five fruits were allowed to develop; crosses were performed on two subsequent days. Unfortunately, a lot of crosses failed, most likely due to the stress imposed on these fragile mutants during crossing.

The scheme in Figure 5.1 illustrates the crosses that were performed. In the first experiment, an *aba,pgmP* recombinant was pollinated with pollen from either a *pgmP* single mutant or an *aba,pgmP* recombinant. In the second experiment, an *aba,abi3* double mutant was crossed with either wild-type plants or identical double mutants.

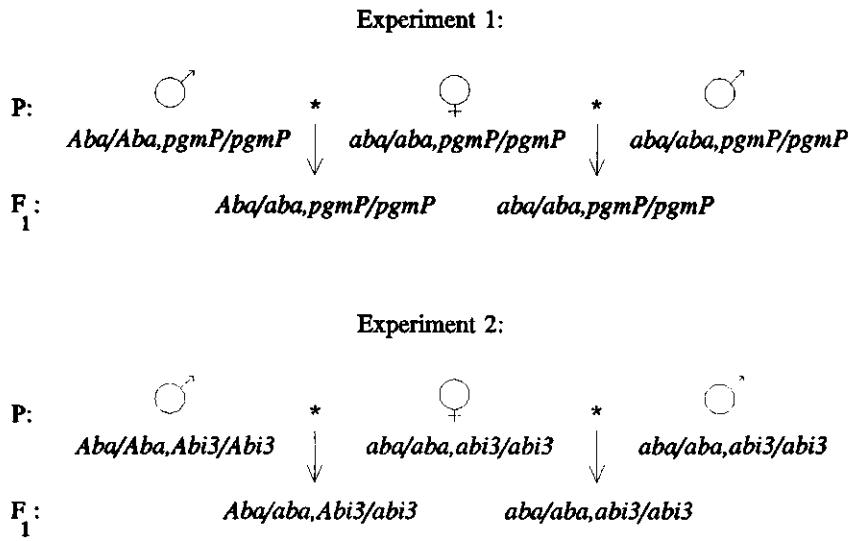

Figure 5.1 Scheme describing the crosses performed in the two experiments.

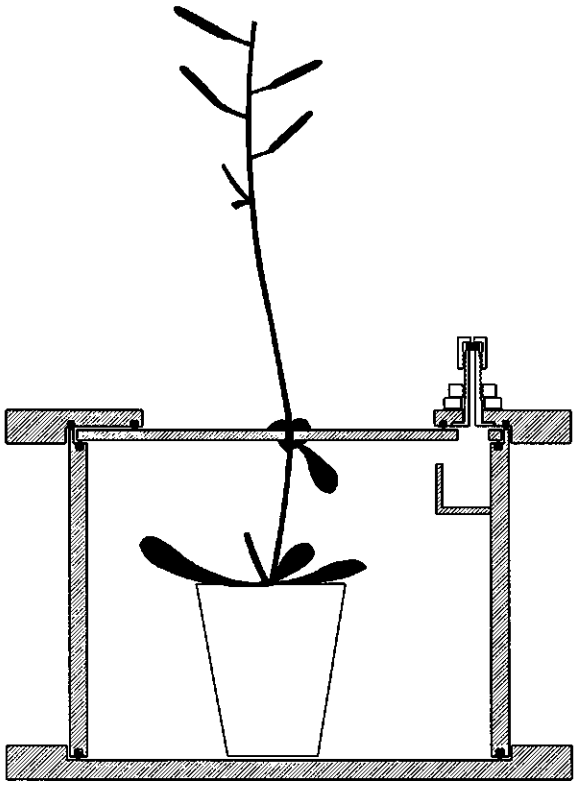

Figure 5.2 Schematic illustration of the perspex chamber used for the labelling experiments.

Pulse C-14 labelling

Plants with uniformly developed siliques were exposed to $^{14}CO_2$, 12 to 15 days after flowering. At this stage *Arabidopsis* seeds showed a maximal increase in seed dry weight (*cf.* Figure 6.1). Seed development to maturity takes 20 to 22 days.

The plants were put in a small air-tight perspex chamber, provided with a septum through which $Na_2{}^{14}CO_3$ (0.2 - 0.4 MBq) and malic acid were injected (Figure 5.2). The inflorescence with the siliques remained outside the chamber. Starchless mutants were illuminated for only 6 h from the start of the labelling, to obtain source-limited conditions; other genotypes were kept under continuous light. After 24 h of exposure, an aliquot of KOH was added to the chamber, to trap excess $^{14}CO_2$ (since only a small fraction of the

available $^{14}CO_2$ was assimilated), and 10 to 25 seeds were isolated from each silique. Radioactivity imported into the individual seeds was determined by liquid scintillation counting of either the 80 %-ethanol-soluble material (starchless mutants) or trapped carbon after combustion of the seeds in a sample oxidizer (other genotypes). Prior to combustion, the dry weight of the individual seeds was determined on a high-precision balance (Mettler UM3), with an accuracy of 0.5 μg.

Results

In the first series of experiments, the crosses resulted in plants with two types of siliques, containing seeds that were either homozygous recessive or heterozygous for *aba*, but both starchless (Figure 5.1). These plants were exposed to $^{14}CO_2$. Since *pgmP* mutants accumulate their reserves almost exclusively as hexose sugars (Caspar *et al.*, 1985), we quantified the assimilate transport to the seeds as the amount of ethanol-extractable radioactivity.

In the second series of experiments, again two types of siliques were compared, containing seeds that were either homozygous recessive or heterozygous for both *aba* and *abi3*. Since these seeds may store their photosynthates partly as starch, the amount of radioactivity was determined after combustion of the seeds.

Figures 5.3 and 5.4 show a summary of the results of both types of experiments. No significant difference between genotypes was observed with respect to the number of seeds per silique. In the first series of experiments, the average seed number was 42, the siliques of the more severely impaired *aba,abi3* plants of the second experiment contained around 23 seeds. Because in the second experiment the dry weights of the seeds were determined, we expressed the results in Figure 5.4 per μg seed, though expression as amount of radioactivity per seed yielded the same conclusion. Both figures show a relatively small variation in import per seed, in contrast to the considerable variation between siliques. The data of plant 2 in Figure 5.4 show a significant difference (Student's *t*-test, $P = 0.05$) between the first and second silique, and between the third and fourth silique, respectively. Nevertheless, expressing the import in mutant seeds relative to wild-type phenotype seeds and averaging these normalized data resulted in values very close to 100 % (100.9 and 99.5 for the two experiments respectively). Thus, despite this variation, we conclude that neither of the genotypes is particularly favoured with regard to the amount of imported radioactivity.

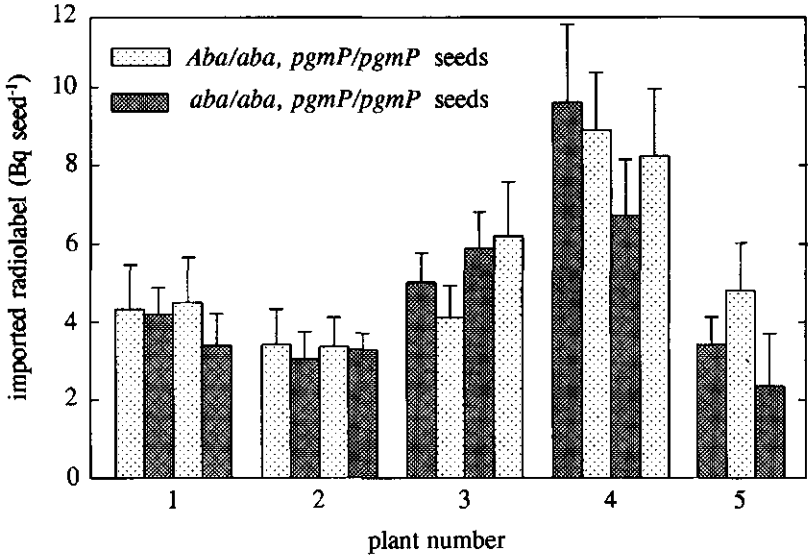

Figure 5.3 Amount of radioactivity transported to genetically different *Arabidopsis* seeds. Each group of bars represents a single plant; each bar represents the average (± SD) amount of radiolabel imported into the individual seeds of a single silique. For each plant, the bars in the graph are arranged from old to young siliques, the latter being about two days younger. Open bars represent ABA-containing seeds (*Aba/aba,pgmP/pgmP*) whereas hatched bars stand for double mutant seeds *(aba/aba,pgmP/pgmP*). The figure is based on 745 seeds.

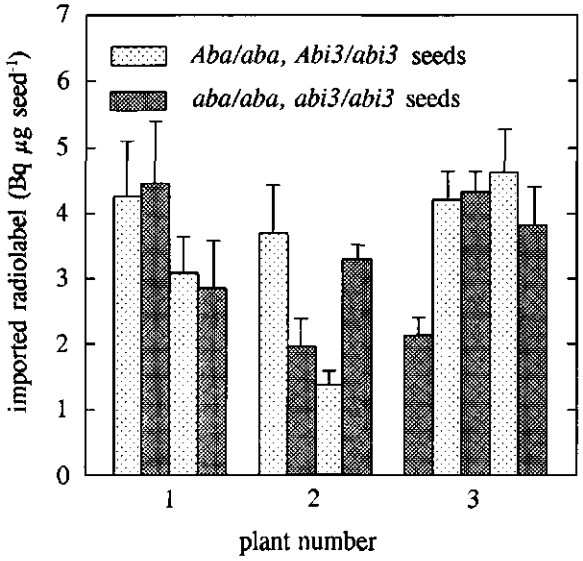

Figure 5.4 Amount of radioactivity transported to genetically different *Arabidopsis* seeds. Other details as in Figure 5.3. Open bars represent wild-type phenotype seeds (*Aba/aba, Abi3/abi3*) whereas hatched bars stand for double mutant seeds (*aba/aba, abi3/abi3*). 122 seeds were used in this experiment.

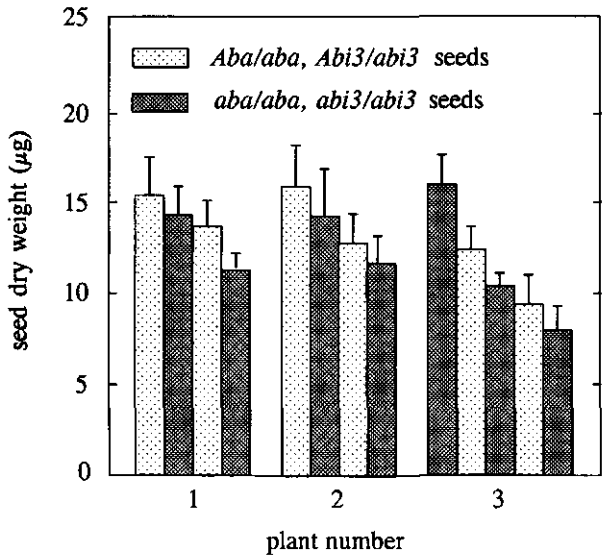

Figure 5.5 Average dry weight of genetically different *Arabidopsis* seeds. Other details as in Figure 5.4, except that the bars represent the mean (± SD) dry weight of the seeds from a specific silique.

In the second series of experiments, the dry weights of the seeds were determined. If the presence of ABA and the sensitivity to ABA in the wild-type phenotype seeds would have caused an increased accumulation rate of assimilates to those seeds, one would expect that they achieved a higher dry weight as compared to the homozygous recessive seeds. Not surprisingly, seeds from younger siliques had accumulated less dry weight, but no evident deviation in the height of the bars occurred that would have pointed to a consistent effect of the genotype on the dry weight of the seeds (Figure 5.5). It is obvious that the genotype of the seeds had no major influence on sink strength. In a parallel experiment, we determined the weights of developing seeds of the parent lines of these crosses, and these also achieved almost identical maximum fresh and dry weights (*cf.* Figure 6.1).

Discussion

The use of hormone mutants makes non-invasive manipulation of both hormone levels and sensitivity to hormones possible, in contrast to traditional techniques. The *aba* mutant used

in the present study has a twentyfold lower ABA content and a thirtyfold lower ABA-biosynthetic capacity than the corresponding wild type (Karssen *et al.*, 1983; Rock and Zeevaart, 1991), whereas the *abi3* mutant is 5 to 20 times less sensitive to the addition of exogenous ABA with respect to inhibition of germination and seedling growth (Koornneef *et al.*, 1984).

In our experiments, these differences in ABA content and sensitivity to ABA were exploited. On the same mother plant, two types of fruits were compared, containing seeds that were either heterozygous or homozygous recessive for the *aba* and *abi3* mutations. We assumed that the heterozygous seeds had wild-type phenotypes, irrespective of the maternal genotype, since heterozygous seeds behave like wild-type seeds with respect to ABA synthesis, dormancy and desiccation tolerance, in contrast to mutant or recombinant seeds (Koornneef, 1986; Koornneef *et al.*, 1989).

Moreover, we added the concept of source-limited conditions, since Clifford *et al.* (1987) have shown that effects of ABA on transport of assimilates to bean seeds were only detectable after severe defoliation of the plants. We hypothesized that potential stimulating effects of growth regulators on assimilate partitioning are more pronounced during competition for a limited quantity of photoassimilates. *Arabidopsis* plants accumulate considerable amounts of reserve material (mainly starch: Caspar *et al.*, 1985) in their flower stalks, since seeds on detached and defoliated stems still reached final weights comparable to control seeds (data not shown). Because it seemed unlikely that defoliation of the plants was sufficient to shorten the available supply of assimilates, a genetic approach was chosen: the *aba* mutant was combined with the *pgmP* mutation which blocks starch accumulation (Caspar *et al.*, 1985). The plants were grown under continuous light, but prior to the labelling experiments the light quantity (and consequently the level of soluble sugars) was reduced, and six hours after the start of the labelling, the lights were switched off (which should cause an immediate drop in the carbohydrate content, Caspar *et al.*, 1985). Unfortunately, crosses of *pgmP* with the recombinant *aba,abi3* yielded no triple mutants, probably because this combination is (conditionally) lethal.

In the comparison between transport of radiolabel to wild-type phenotype seeds and to ABA-deficient/insensitive seeds, no consistent effect of ABA was found. The differences found in plant 2 in Figure 5.4 might suggest that in older siliques ABA attracts assimilates, whereas in younger siliques the opposite happens. However, this seems unlikely, since no such differences were found in the other plants. Moreover, siliques one and two are only one day older than siliques three and four. We conclude that the

genotype of the seeds did not influence their sink strength.

Although these findings conflict with some data from the literature (Düring and Alleweldt, 1980; Tietz *et al.*, 1981; Berüter, 1983; Clifford *et al.*, 1986, 1987, 1990), several arguments support our results. First, any undesirable side-effect of hormone application was excluded since we used mutants to lower the ABA levels. Next, effects of plant treatment were excluded because in each experiment siliques from the same inflorescence were compared. Thirdly, absence of a stimulating effect by ABA is not due to abundant availability of assimilates, because at source-limited conditions comparable results were obtained. Fourthly, effects of 'applied' ABA on water relations of the silique are excluded, since only the seed genotypes were altered, and the fruit is a maternal tissue. Finally, the results from short-term experiments (labelling) agree well with the data from long-term observations (dry weight determinations).

One might argue that even very small amounts of ABA may be enough to regulate or influence assimilate partitioning. Indeed, this possibility cannot be excluded since Koornneef *et al.* (1989) found that impaired seed development (lack of desiccation tolerance, reduced accumulation of storage proteins, and reduced water loss) only occurred in the recombinant *aba,abi3* and not in the single mutants. They suggested that the threshold ABA level for seed development is much lower than for induction of dormancy which was already strongly reduced in the leaky *aba*-3 mutant that still contained some ABA (up to 50 % of wild-type levels: Rock and Zeevaart, 1991). Theoretically, it is still possible that a very small amount of ABA, even at reduced sensitivity, is sufficient to determine the sink strength of the seeds, although this seems highly unlikely.

On the other hand, we cannot exclude that the decrease in sensitivity to ABA that is achieved in the *abi3* mutant does not influence a hypothetical ABA-regulated or ABA-induced mechanism that attracts assimilates to the seeds. Finkelstein *et al.* (1990) have shown that the *abi1* and *abi2* mutations mainly affect responses during vegetative growth (like stomatal closure and seedling growth), while aberrations in the *abi3* mutant are primarily confined to reproductive stages. Sensitivity to hormones is not only tissue-specific or dependent on a developmental stage, but also response-specific, as was illustrated in the above-mentioned difference in effects on seed development and dormancy induction (Koornneef *et al.*, 1989). More severe *abi3* mutants that will be available shortly, may shed new light on this problem.

The conclusion that ABA does not influence transport of assimilates to seeds agrees

well with other findings of our group that pea seeds in the same pod with a different ABA content have comparable growth rates (Chapter 4). Also in the study of Quarrie *et al.* (1988) no differences were found in kernel growth rate between two oat lines that differed by 70 % in ABA content.

How can the results in the literature that suggest a role for ABA in assimilate partitioning be explained? Dewdney and McWha (1979) found an increase of the movement of photosynthetic assimilates towards wheat ears after injection of ABA into the kernels, but this work has been severely criticized by King and Patrick (1982). Düring and Alleweldt (1980) claimed not only a good correlation between ABA content and sugar content in grape berries, but also an enhanced movement of ^{14}C-assimilates to grape berries after treatment with ABA. The observed increase in sugar content however, was much lower than expected from the increase in ABA content. Tietz *et al.* (1981) demonstrated an increase of up to 70 % in the ^{14}C-transport from flag leaf to ears in barley after ABA application to the surface of the grains. However, the presented curves suggest that this effect may be due to hastened maturation of the grains treated with high concentrations of ABA. The observed increase in kernel weight after application of ABA was not reproducible (Dörffling *et al.*, 1984). Their results are also conflicting with the data of King and Patrick (1982) and Quarrie *et al.* (1988). Schussler *et al.* (1984) suggested that ABA was involved in photosynthate accumulation in storage organs by stimulating the unloading of sucrose in the seed apoplast and the uptake of sucrose by the cotyledons. Notwithstanding this, they recently stated that in these data the level of endogenous ABA was generally not correlated with the level of sucrose and the rate of dry matter accumulation in the seeds, and that endogenous ABA levels might not be the limiting factor in determining the seed growth rate (Schussler *et al.*, 1991).

In conclusion, despite the reports in favour of a stimulating role of ABA in the partitioning of assimilates to reproductive sinks (Berüter, 1983; Gifford and Thorne, 1986; Clifford *et al.*, 1986, 1987, 1990; Archbold, 1988), in view of the present findings, we are very doubtful about its role. Nevertheless, it is still quite possible that ABA exerts its influence on some of the processes associated with the distribution of assimilates within the seed. Several groups reported effects of ABA on the synthesis of storage proteins (Crouch and Sussex, 1981; Ackerson, 1984; Barratt, 1986). Since Finkelstein *et al.* (1990) found that the *abi3* mutation had a detectable effect on the quality or composition of storage material in the seeds, it may be more useful to pay attention to the role of ABA on the partitioning of assimilates among different types of reserve material.

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6 Effects of abscisic acid on reserve deposition in developing *Arabidopsis* seeds

S.M. de Bruijn, J.J.J. Ooms[1], D. Vreugdenhil and C.M. Karssen

Summary

Arabidopsis thaliana mutants that were either ABA-deficient (*aba*) or ABA-insensitive (*abi3*) and their recombinant (*aba,abi3*) were used to determine the role of ABA in the regulation of deposition of reserve material during seed development. The total net import of assimilates into seeds of these genotypes was unaffected as compared to wild-type seeds, but the distribution of these assimilates over the various types of storage materials depended on the genotype. All mutants were to the same extent impaired in the synthesis of long-chain fatty acids: their seeds contained three times less eicosenoic acid (20:1) in the triacylglycerol fraction as compared to wild-type seeds. Moreover, recombinant (*aba,abi3*) seeds accumulated considerably less neutral lipids than wild-type and single-mutant seeds, and simultaneously the levels of soluble carbohydrates and starch were increased. It is concluded that ABA plays a role in the distribution of assimilates over the various types of storage materials: absence of and insensitivity to ABA causes inhibition of acyl-chain elongation and of lipid accumulation, and as a result a higher proportion of the imported assimilates is stored as carbohydrates.

Introduction

In most plant tissues photosynthates are stored as carbohydrates. However, seeds of a

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majority of species store mainly triacylglycerols, as a way to concentrate energy in a small volume (Slack and Browse, 1984). Although assimilates usually arrive as sucrose, and oil-rich seeds are also capable to store starch (Norton and Harris, 1975; Hara *et al.*, 1985), in general carbohydrates are only temporarily present during seed development. Apparently the cells in these oil-rich seeds differ from vegetative cells with respect to the mechanism that determines the deposition of storage material. Up to now, relatively few attention has been paid to the hormonal regulation of the distribution of assimilates among different types of storage material. Although the effect of abscisic acid (ABA) on the accumulation of storage proteins in developing seeds is rather well-documented (Black, 1991; Thomas *et al.*, 1991), only few studies described the influence of ABA on the conversion of carbohydrates to fatty acids. Ackerson (1984) found an effect of exogenous ABA on the sucrose partitioning in soybean embryos cultured *in vitro*: embryos cultured in the presence of ABA accumulated more protein, sugars and starch, at the expense of lipid accumulation. Finkelstein and Somerville (1990) studied the accumulation of both storage protein and triacylglycerols in seeds of a series of ABA-insensitive mutants of *Arabidopsis thaliana*. Seeds of one of these mutants (*abi3*) had a threefold reduced level of 20:1 fatty acids. This result is in close agreement with a promoting effect of applied ABA on the accumulation of long-chain fatty acids in cultured embryos of *Brassica napus* (Finkelstein and Somerville, 1989). Recently, Holbrook *et al.* (1992) reported that applied ABA stimulated both the total fatty acid accumulation and the elongation of 18:1 and 20:1 fatty acids in microspore-derived embryoids of *Brassica napus*.

The latter studies, however, have two drawbacks: the use of embryos cultured *in vitro*, and the use of applied ABA. It is still an open question whether the effects of applied ABA on embryos cultured *in vitro* simulate the effects of endogenous ABA during *in planta* development of seeds. *In vitro*, ABA may act primarily as a germination suppressor, resulting in continued development of the embryoids. An important difference between the two systems is that the fatty acid composition of *in vitro* cultured zygotic embryos may deviate considerably from that of *in planta* developed embryos (Finkelstein and Somerville, 1989; Dutta and Appelqvist, 1991; Kim and Janick, 1991), although Taylor *et al.* (1990) achieved almost similar fatty acid profiles in embryoids as compared to mature seed. In general, the use of applied ABA has several disadvantages (Trewavas and Jones, 1991): uptake, transport and metabolism of ABA have to be checked, and control treatments may have an endogenous ABA level above the threshold level for the response in study. The use of ABA mutants offers the possibility to study both the role

of endogenous ABA in *in planta* development of embryos, and the effects of lower ABA levels or insensitivity to ABA.

The main object of the present study was to get insight into the influence of ABA on the deposition of reserve material in seeds during development. For that purpose, the fatty acid and carbohydrate composition of seeds of several *Arabidopsis* hormone mutants during development was compared with that of wild-type seeds. Both an ABA-deficient (*aba*) and an ABA-insensitive (*abi3*) mutant, and their recombinant (*aba,abi3*) were included.

Materials and methods

Plant material

All *Arabidopsis thaliana* (L.) Heynh. mutants were either derived from or back-crossed with the pure line Landsberg 'erecta' (wild type). The isolation and characterization of the ABA-deficient (*aba*-1, isolation number A26) and the ABA-insensitive (*abi3*, isolation number CIV, presently denoted as *abi3*-1: Nambara *et al.*, 1992) mutant were described by Koornneef *et al.* (1982, 1984). The recombinant *aba,abi3* was originally isolated from a population segregating for the *aba* gene (Koornneef *et al.*, 1989) and recognised by the dark green colour of the mature seeds. All plants were self-fertilized.

Culture conditions

Dry stored seeds of wild-type, *aba* and *abi3* plants were sown in 9 cm Petri dishes on moist filter paper. Since *aba,abi3* plants yielded no viable mature seeds due to lack of desiccation tolerance, this mutant was maintained by transferring near-mature seeds from the siliques directly to moist filter paper. The seeds were allowed to germinate in a climate room at 24 °C, under continuous fluorescent light. After three or four days, the seedlings were planted out in 5.5-cm pots with a mixture of sand and humus. The plants were grown in a greenhouse at day temperatures of 17 to 22 °C and night temperatures of 15 to 17 °C, under natural light, supplemented with artificial illumination from high-pressure sodium lamps (SON/T), to give a 16-h day length. Because the plants are very sensitive to wilting, within the greenhouse a mist bench was created to maintain a very high relative humidity (85 to 100 %).

Harvest of seeds

In the first series of experiments (March 1991), both weight and carbohydrate content of wild-type and *aba,abi3* seeds were determined. In the second series (December 1991), seeds of wild-type and the single mutants (*aba* and *abi3*) were used for determination of both carbohydrate and lipid composition. In a third series (April 1992), the lipid composition of wild-type and *aba,abi3* seeds was investigated.

Individual flowers were tagged at the day of anthesis and siliques were harvested at various stages after flowering (ranging from 6 to 22 days after flowering). Prior to opening, the siliques were transferred to a glove box with a high relative humidity, since young seeds readily lose water. Seeds were carefully removed from the siliques, counted and immediately transferred to vials containing either a mixture of chloroform and methanol (1:1, v/v; for lipid determinations) or 80 % (v/v) methanol (for carbohydrate determinations). These vials were stored at -70 °C, until analysis. In all series of experiments, 50 to 80 seeds were harvested of each genotype, in triplicate.

Weight determinations

Individual seeds of both wild-type and *aba,abi3* plants were harvested at various stages ranging from 7 to 22 days after flowering, as described above, but after removal from the siliques individual seeds were immediately enclosed in small, pre-weighed tin containers (app. 7 mg). The weight of these containers was determined on a high-precision balance (Mettler UM3). After drying for 16 h at 105 °C the containers were cooled down above silica and weighed again, to determine the dry weight of the seeds. Per stage and per genotype four siliques were harvested, and four representative seeds were chosen from each silique.

Neutral lipid determinations

The contents of vials containing 50 to 80 seeds and 0.3 mg triheptadecanoin (as an internal standard) were transferred to a hand-operated 1 ml-Potter tube and homogenized. The lipid content of the seeds was analysed essentially as described by Hoekstra and Van Roekel (1988). The homogenate was washed and dried, polar lipids were removed by passage over a SEP-PAK silica cartridge, and neutral lipids were trans-methylated in

0.3 N KOH in methanol at 70 °C for 15 min. Methylated fatty acids were collected by phase separation in hexane and injected in a GC (Shimadzu GC-8A, equipped with a J&W Megabore column, DB225 [J&W Scientific, Folsom, CA, USA], 30 m, operated at 210 °C; flame ionization detector). *Arabidopsis* seed lipids consist mainly of triacylglycerols (± 90 %: Kunst *et al.*, 1992).

Carbohydrate analysis

Before homogenizing the seeds, 25 μg melezitose was added to the methanol, as an internal standard. The homogenate was heated for 15 min at 75 °C and centrifuged (5 min, 10,000*g*). The pellet was stored for analysis of polysaccharides; the supernatant was vacuum-evaporated and its residue was taken up in 0.5 ml purified water (Milli-Q purification system, Millipore, Molsheim, France) and injected into a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA). The HPLC was equipped with a CarboPac PA100 (4 x 250 mm) column with appropriate guard column and a pulsed electrochemical detector (PED) with an Au working electrode and an Ag/AgCl reference electrode. Usually, mono-, di- and trisaccharides were separated by isocratic elution in 0.1 N NaOH for 30 min, at a flow rate of 1 ml min^{-1}, at ambient temperature. Peaks were identified by comparing their retention times with the retention times of a mixture of standard sugars. Both samples and standards were run at various NaOH concentrations to confirm the identification.

Polysaccharides (the carbohydrate fraction that was not extractable in 80 % methanol, as described above), were hydrolysed according to Fry (1988). Pellets were resuspended in 50 μl 58 % (v/v; *ca.* 11 M) H_2SO_4 and incubated at room temperature for 1 h. Subsequently, an anti-bumping granule and 0.5 ml water were added and the sample was stirred and heated at 120 °C for 1 h. After cooling, 0.5 ml water was added and the sample was injected directly into the HPLC (see above) without further dilution. No substantial breakdown of monosaccharides was observed when storage of samples before injection was limited to two days. The monosaccharides were separated by a modified elution program: the column was pre-equilibrated with 0.1 N NaOH and 1.1 M sodium acetate for 5 min and with 0.1 N NaOH for 15 min. The sample was injected at the end of a 10 min gradient from 0.1 N NaOH to 0.01 N NaOH and eluted isocratically at 0.01 N NaOH for 35 min. For every analysis, the column equilibration procedure was repeated.

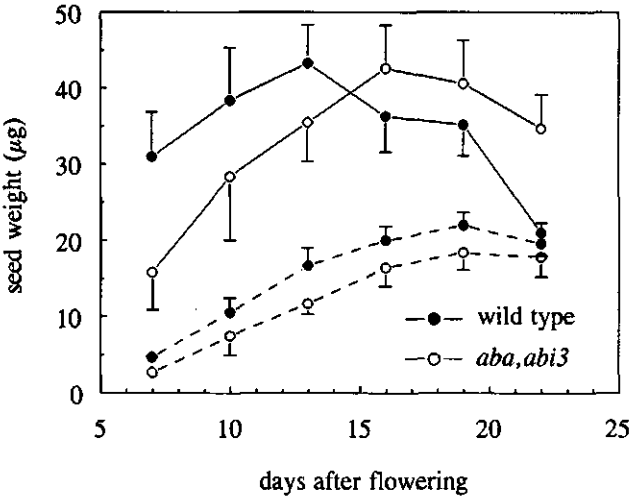

Figure 6.1 Fresh (solid lines) and dry (dotted lines) weight of wild-type and *aba,abi3* *Arabidopsis* seeds during time. Data are mean ± SD for 16 seeds from four siliques.

Results

Fresh and dry weight of seeds during development

In a preliminary experiment it was investigated whether ABA influenced the import of assimilates into the seeds. As a rough measure, the gain in fresh and dry weights of seeds of wild-type plants and of *aba,abi3* plants was compared. The latter mutant was chosen since it could be expected that ABA effects are more pronounced in this recombinant as compared to the *aba* or *abi3* single mutants (Koornneef *et al.*, 1989). Moreover, analysis of the weights of individual developing seeds would hopefully provide a 'calibration curve' to be used in further experiments, thus avoiding the laborious and time-consuming work of weighing such small seeds. Figure 6.1 shows the developmental time course of fresh and dry weight accumulation of seeds of wild-type and *aba,abi3* plants. It was evident that *aba,abi3* seeds were somewhat retarded in their development with respect to the accumulation of both fresh and dry weight. Nevertheless, the maximum fresh and dry weights of both lines were not significantly different. At day 22, siliques of *aba,abi3* plants contained both viviparously germinated seeds (since these seeds lack ABA and have

no dormancy: Koornneef *et al.*, 1989) and dried, dead seeds (since these seeds have no desiccation tolerance: Koornneef *et al.*, 1989). The latter seeds were omitted from the weight determinations.

In further experiments, it turned out that the rate of seed development varied considerably between different series. Therefore, the seed weight determined in one series can not be transferred to other series for calculations of assimilate deposition on dry weight basis.

Neutral lipid content

Figure 6.2 presents the total neutral lipid content of the seeds during development. Apparently, the single mutants did not differ from the wild type, whereas seeds of the *aba,abi3* recombinant were strongly inhibited in their lipid accumulation. Another evident feature of this graph is the difference between the series of December 1991 and April 1992: seeds of plants grown during the winter season (Figure 6.2A) started much later with the accumulation of lipids than seeds produced during spring (Figure 6.2B).

Another obvious difference among the genotypes lies in the fatty acid composition of the neutral lipids. The seeds of all three mutants contained about three times less 20:1 than wild-type seeds (Figure 6.3, Table 6.I). The level of 20:1 in *aba* seeds during development displayed a peak at 11 to 13 days after flowering but decreased later during development (Figure 6.3). This peak was not significant, however, when the data were

Table 6.I Fatty acid composition of *Arabidopsis* seeds of several genotypes. Total acyl lipids, extracted from mature seeds, were transmethylated and the acyl composition was determined by gas-chromatography of the methylesters. Data for the *aba* seeds are mean of triplicate samples.

genotype	fatty acid (% of total)								
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3
wild type	6.1	2.7	17.3	29.3	17.8	1.8	23.3	1.4	0.3
abi3	9.2	3.2	25.1	37.0	18.4	0.5	6.3	0.5	n.d.*
aba	8.5	3.3	25.0	35.4	19.2	0.7	7.3	0.5	0.3
wild type	6.5	3.3	22.2	27.4	16.7	1.7	21.1	1.0	0.1
aba,abi3	11.6	3.0	18.6	41.6	16.7	1.0	7.6	n.d.	n.d.

* n.d.: not detectable

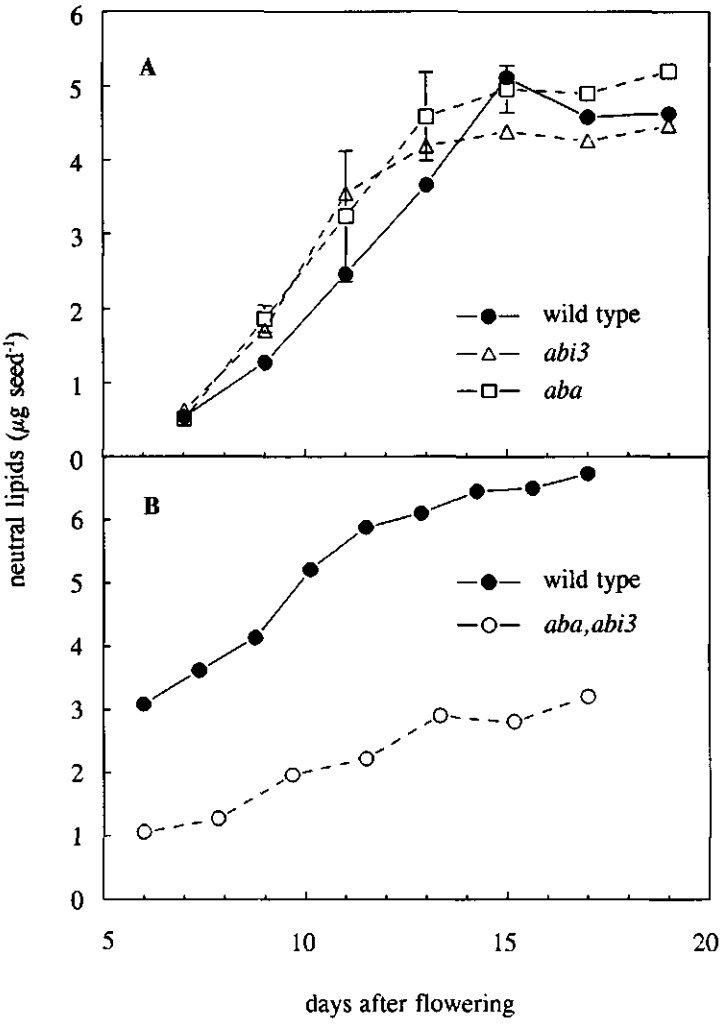

Figure 6.2 Accumulation of triacylglycerols during seed development in seeds of wild-type, *abi3* and *aba* (A) or wild-type and *aba,abi3* (B) *Arabidopsis* plants. Data for the *aba* seeds are mean ± SD of triplicate samples.

expressed as absolute amounts of 20:1 fatty acid per seed (data not shown).

The lower levels of 20:1 in the single mutants, were counterbalanced by higher levels of 16:0, 18:1 and 18:2. However, in the double mutant *aba,abi3* the 18:1 level was not

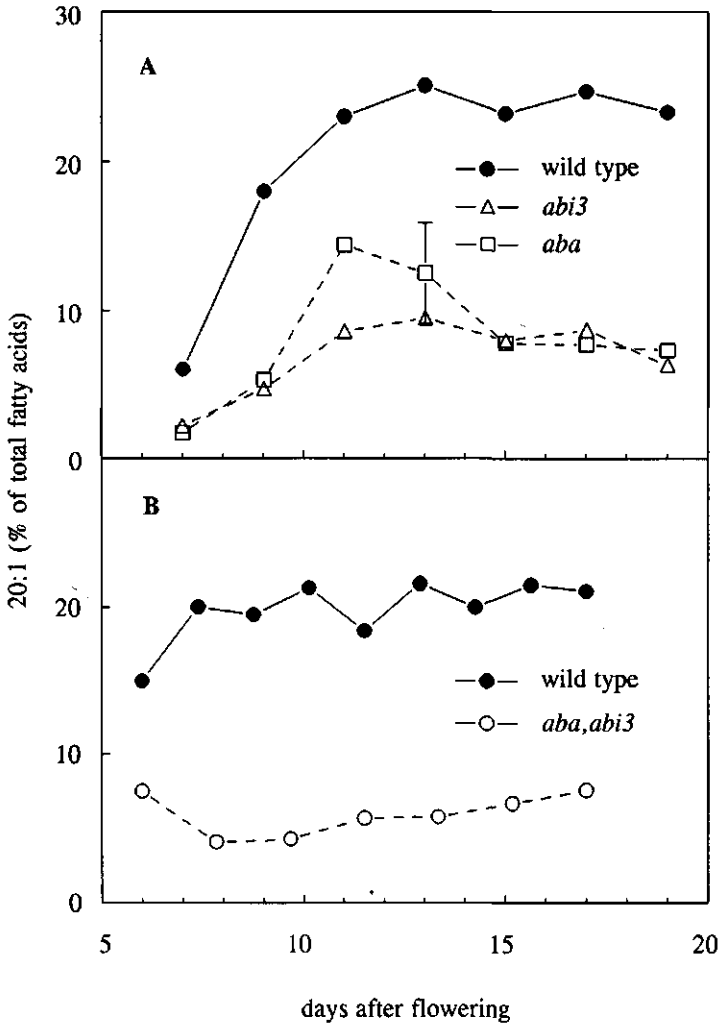

Figure 6.3 Accumulation of 20:1 fatty acid during seed development in seeds of wild-type, *abi3* and *aba* (A) or wild-type and *aba,abi3* (B) *Arabidopsis* plants, relative to the total fatty acid content. Data for the *aba* seeds are mean ± SD of triplicate samples; SD is indicated when it exceeded the size of the symbols.

elevated whereas the 16:0 and 18:2 levels were higher than in the single mutants and in the wild type (Table I; De Bruijn *et al.*, 1993). This difference was not only visible in mature seeds, but also throughout development (data not shown).

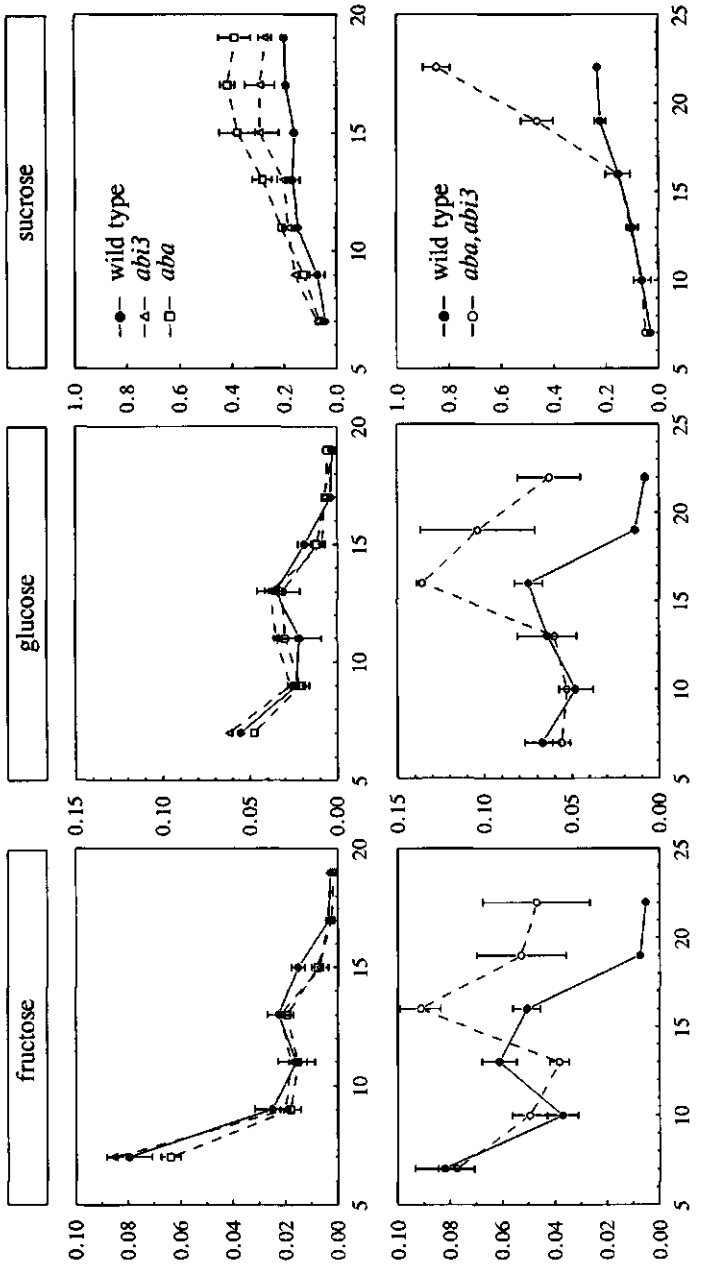

Figure 6.4 Levels of fructose, glucose and sucrose during seed development in seeds of wild-type, *abi3* and *aba* (upper panels) or wild-type and *aba,abi3* (lower panels) *Arabidopsis* plants. Data are mean ± SD of triplicate samples.

Carbohydrate content

Soluble carbohydrates

Figure 6.4 summarizes the results for fructose, glucose and sucrose (*ca.* 80 - 85 % of total soluble carbohydrates). In general, fructose and glucose levels decreased initially, had a peak later during development, and dropped again at maturity. In contrast, sucrose levels gradually increased during development. The levels of glucose and fructose in seeds of *aba* and *abi3* plants were similar to wild-type levels. In the seeds of the recombinant *aba,abi3*, glucose and fructose remained relatively high throughout development. As a result, at maturity the levels of glucose and fructose were eight times higher as compared to the levels in wild-type seeds.

The levels of sucrose in *aba* and *abi3* seeds, were up to two times higher than in wild-type seeds. The increase in sucrose level near maturity was even more pronounced in the recombinant *aba,abi3*, showing a fourfold increase as compared to wild-type seeds.

Insoluble carbohydrates

The non-soluble fractions that remained after extraction with methanol were hydrolysed with sulfuric acid, at conditions that yield monosaccharides from all types of polysaccharides (including cellulose and other cell wall polymers). A variety of monosaccharides was found, the most prominent ones were: glucose, arabinose, galactose, xylose, rhamnose and ribose (Figure 6.5). Fructose was hardly detectable. The accumulation pattern of arabinose, galactose, rhamnose and xylose was similar in all genotypes: the levels of these sugars in the polysaccharide fraction gradually increased during development. Contrary to this, glucose and ribose levels generally declined towards maturation. In wild-type seeds ribose levels decreased almost to zero; in *abi3* and *aba* (data not shown) and in *aba,abi3* seeds (Figure 6.5) passable levels were still left at the end of development.

It is very likely that the glucose found after acid-catalysed hydrolysis predominantly originated from starch, since data on starch content of seeds from the same series of experiments hydrolysed by amyloglucosidase (De Bruijn *et al*, 1993) fit exactly in these graphs. In Figure 6.6, starch contents (as derived from glucose levels) of the seeds of all genotypes are displayed: apparently the single mutants initially had slightly higher starch levels as compared to wild-type seeds; at the end of development starch had disappeared in all genotypes, except in the *aba,abi3* mutant where a considerable amount of starch was

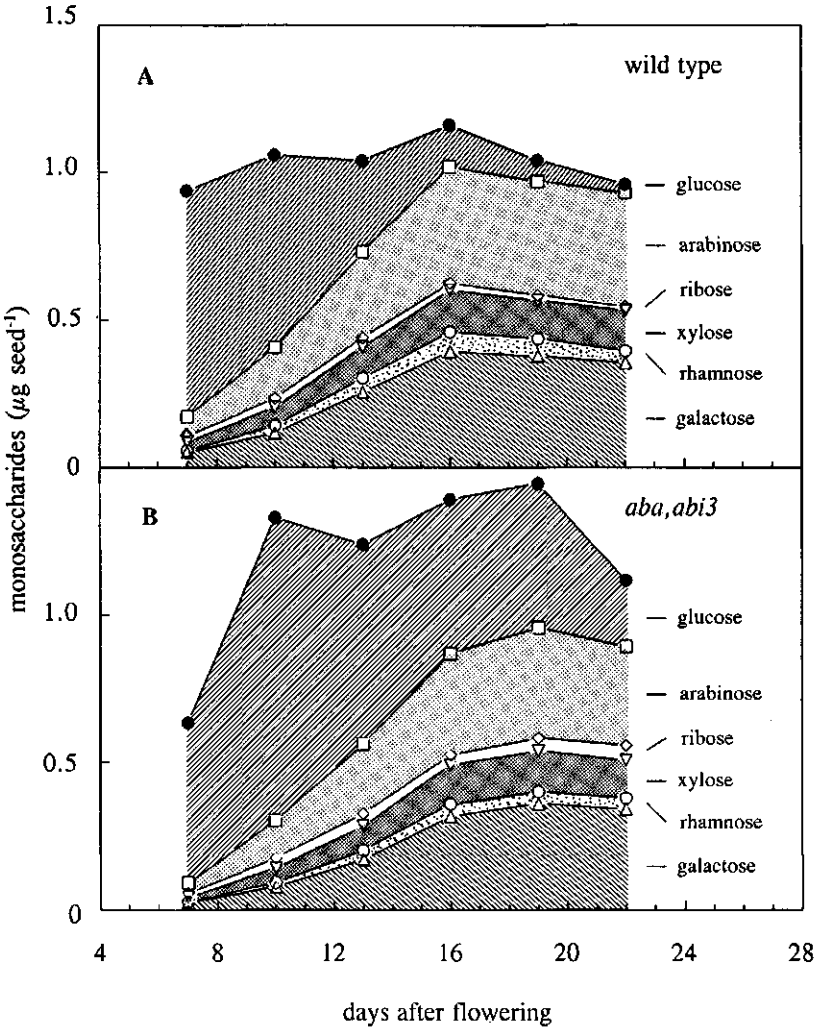

Figure 6.5 Cumulative levels of monosaccharides after total hydrolysis of a methanol-insoluble residue from homogenates of seeds of wild-type (A) and *aba,abi3* (B) *Arabidopsis* plants. Data are mean of triplicate samples.

left. The differences in starch level in Figure 6.6B are even more pronounced when expressed on a dry weight basis: at 10 days after flowering 15.1 % of the dry weight of *aba,abi3* seeds consists of starch *versus* 6.7 % in wild-type seeds, at 19 days after

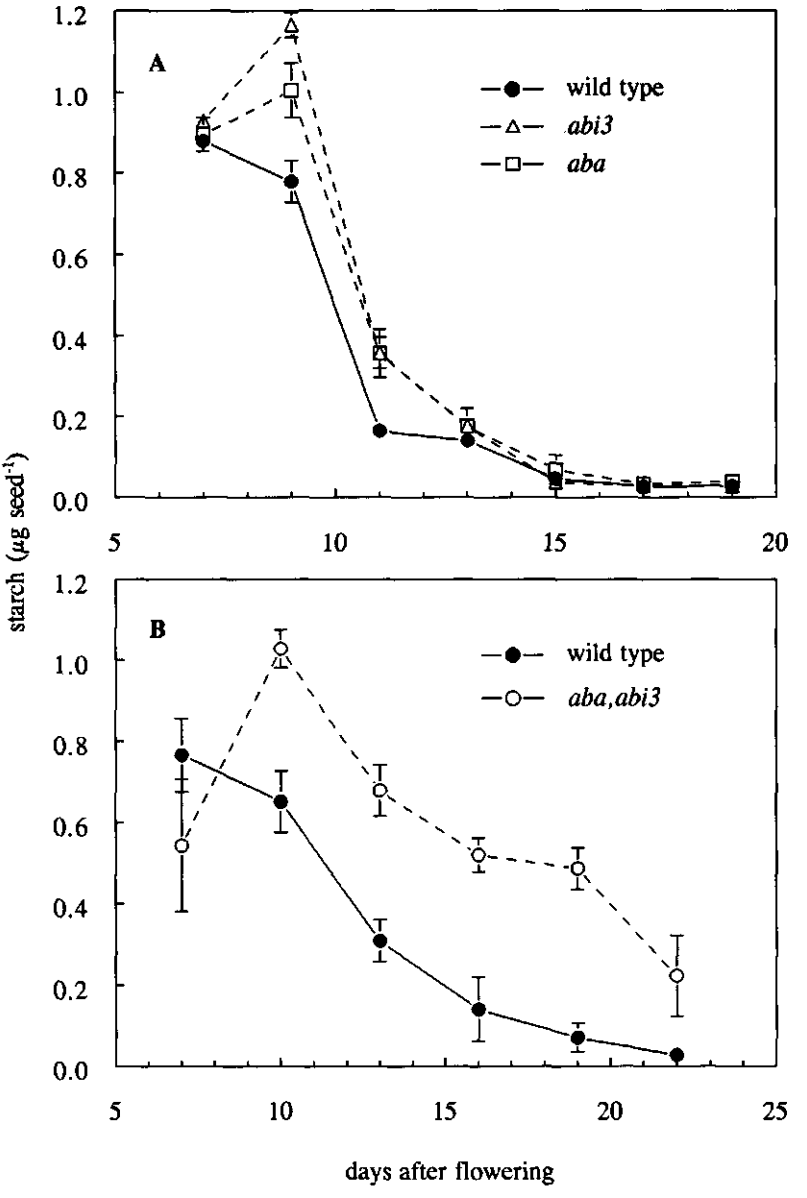

Figure 6.6 Levels of starch during seed development in seeds of wild-type, *abi3* and *aba* (A) or wild-type and *aba,abi3* (B) *Arabidopsis* plants. Data are mean ± SD of triplicate samples.

flowering the ratio was 2.9 % *versus* 0.4 %.

Discussion and conclusion

The use of hormone mutants

This Chapter deals with the effects of ABA on deposition of storage material in seeds of three *Arabidopsis* mutants. The effects observed were not always similar for these mutants, *e.g.* seeds of the single mutants *abi3* and *aba* accumulated triacylglycerols to the same levels as wild-type seeds, whereas *aba,abi3* seeds had reduced levels. Apparently the lower level of ABA or the decreased sensitivity to ABA in the single mutants was not enough to influence storage lipid biosynthesis. However, the combination of an elevated threshold level for this response and a decreased hormone level in the double mutant was effective. This was not surprising, since other phenotypic aberrations, such as vivipary, lack desiccation tolerance, lack of pigmentation in mature seeds, inhibition of accumulation of storage proteins, are also restricted to this recombinant (Koornneef *et al.*, 1989; Meurs *et al.*, 1992).

Fatty acid composition

With respect to triacylglycerol accumulation, at least three effects were discerned: 1. mutants accumulated considerably less long-chain fatty acids in their seeds, 2. seeds of the recombinant *aba,abi3* contained only half the amount of total triacylglycerols of the wild type and the single mutants, 3. seeds of the recombinant *aba,abi3* had less 18:1.

Mutant plants had lower levels of 20:1 in the seeds and a concomitant increase in 16:0, 18:1 and 18:2. These data confirm the observations of Finkelstein and Somerville (1989) and Holbrook *et al.* (1992), who observed an increase in the fraction of long-chain fatty acids (20:1 and 22:1) in *Brassica* embryos cultured *in vitro* after incubation with ABA. Moreover, Holbrook *et al.* (1992) found increased incorporation of precursors in long-chain fatty acids in cell-free homogenates of *Brassica* embryos after pre-incubation with ABA. From their work it is clear that ABA directly interferes with the activity or presence of the elongase enzyme(s), and not with the presence of cofactors or malonyl-CoA or the transport of 18:1 from the plastids to the site of elongation. Since the incubation times with ABA in their studies were rather long (three to seven days), it is not possible to decide whether ABA directly inhibited the enzyme(s) or acted on the level of gene expression.

The decreased 20:1 levels in *aba* and *abi3* seeds (Figure 6.3) conflicts with the data of Finkelstein and Somerville (1990) who found similar 20:1 levels in wild-type and the *aba* mutant. A possible explanation is that the threshold level of ABA needed for this response is around the endogenous ABA level in *aba* mutants (at least twenty times lower than in wild-type seeds: Karssen *et al.*, 1983). The culture conditions of the plants may slightly have influenced the endogenous ABA level in seeds. We experienced a rather large variation between batches of plants, not only with respect to seed weight or accumulation of lipids, but also to the overall development and appearance of the plants; the plants used for the present experiments were of relatively good quality, had high fruit yield and good seed set, and mostly seeds of the firstly initiated siliques were used. Maybe these conditions contributed to low endogenous seed ABA levels and subsequently to low 20:1 levels.

Another effect of ABA was the lower amount of total triacylglycerols in *aba,abi3* seeds (Figure 6.2). Also in other systems an effect of ABA on total neutral lipid accumulation was observed: Finkelstein and Somerville (1989) reported a 60 % higher fatty acid content in zygotic *Brassica* embryos cultured *in vitro* on ABA, as compared to basal medium; Dutta and Appelqvist (1989) found a three- to fourfold increase of storage lipid levels after ABA treatment of carrot (*Daucus carota*) somatic embryos; Kim and Janick (1991) observed an increase of total fatty acid content by 34 to 53 % in celery (*Apium graveolens*) somatic embryos after ABA addition to the culture medium; and Holbrook *et al.* (1992) reported a 40 to 50 % higher overall fatty acid content in *Brassica* microspore-derived embryos. However, in all those *in vitro* systems ABA is routinely added to the cultures to prevent precocious germination, and the presence or absence of ABA may result in totally different developmental stages at the time of harvest for lipid determination. This is clearly demonstrated by the work of Finkelstein and Somerville (1989), who found that the effect of ABA on lipid accumulation could be fully replaced by treatment with high osmoticum, this effect of osmoticum not being mediated by increased levels of endogenous ABA (Finkelstein and Crouch, 1986).

Our results prove that the effect of ABA on lipid accumulation also occurs during *in planta* development of seeds. One might argue that seeds of the *aba,abi3* mutant also differ from wild-type and single-mutant seeds with respect to lack of desiccation tolerance and that those seeds will not accumulate storage material because they come into a premature vivipary-like germination mode (Meurs *et al.*, 1992). However, the effects on lipid accumulation in the *aba,abi3* mutant were already observed at six days after flowering

(Figure 6.2), and at this stage the developmental program with respect to germination is similar in all genotypes (Koornneef *et al.*, 1989).

Wild-type and mutant seeds also differed in the relative amount of 18:1 (Table I). The elevated levels of 18:1 in the single mutants *abi3* and *aba*, combined with the slightly increased levels of 18:2, 18:3 and 16:0, most likely reflect a compensation for the decreased levels of 20:1. Analogous to the situation with other mutants deficient in the elongation of 18:1 (James *et al.*, 1990; Lemieux *et al.*, 1990; Kunst *et al.*, 1992), the increase in 18:1 is the highest, whereas the desaturation pathway seems less favourable (Appleby *et al.*, 1974). However, seeds of the double mutant *aba,abi3* showed a markedly decreasing level of 18:1 towards the end of development (Table I; De Bruijn *et al.*, 1993), and more elevated levels of 18:2 and 16:0 than in single-mutant seeds. The increase in 18:2 level supposes a higher desaturase activity in this mutant.

Carbohydrate composition

The overall pattern of dry matter distribution in single-mutant seeds was not very different from that in wild-type seeds: levels of total storage lipids (Figure 6.2A), starch (Figure 6.6A) and protein (Meurs *et al.*, 1992) show only minor differences. However, double-mutant seeds contained only half the amount of lipid (Figure 6.2B) and protein (Meurs *et al.*, 1992) as compared to wild-type seeds. The interesting question is whether those seeds have an alternative for storage of their reserves, since it is clear from Figure 6.1 that seeds of both genotypes achieved the same maximum dry weight. To compensate for the lower lipids levels, double-mutant seeds accumulated more soluble carbohydrates (Figure 6.4B) and starch (Figure 6.6B) than wild-type seeds, and the decrease in starch during development was not only retarded but a considerable amount of starch was left in 'mature' seeds. Nevertheless, a rough calculation indicates that the lower lipid and protein content in the double mutant is not fully balanced by the increase in carbohydrate levels.

The transient peak in starch content of wild-type and single-mutant seeds agrees well with previous findings on other cruciferous seeds (Norton and Harris, 1975; Romano *et al.*, 1984; Fischer *et al.*, 1988). In general, starch functions in those seeds as a temporary buffer of carbon, and in mature seeds hardly any starch is present (Siddiqui and Wood, 1977; Thibault *et al.*, 1989).

In the study of Ackerson (1984), *in vitro* cultured soybean embryos accumulated starch and proteins at the expense of lipids, when ABA was added to the culture medium.

Apparently, these data conflict with the data of the present study, but again it has to be stressed that these embryos germinate immediately in the absence of ABA. In developing kernels of maize, no effects of increased ABA levels (caused by exposure to water stress) on starch content were observed (Ober and Setter, 1990). Myers *et al.* (1990) observed a lower number of starch granules in cultured maize kernels when treated with ABA, but this effect was thought to be secondary to earlier effects which decreased endosperm cell number in the kernels.

Comparison of the presented data gives some insight into the timing of the processes that are affected by ABA. Early during development, the accumulation of lipids in seeds of the *aba,abi3* mutant was inhibited (Figure 6.2). The surplus of assimilates or metabolites (either from increased lipase activity or from a block in the fatty acid synthesis) is channeled to starch and this led to a peak in starch content at 10 days after flowering (Figure 6.6); during the following days starch breakdown was retarded and hexose levels increased (Figure 6.4). At the end of development, a fraction of the *aba,abi3* seeds precociously germinated; this caused a further degradation of starch and a sharp increase in sucrose levels (Figures 6.4, 6.6).

In conclusion, the deficiency of ABA and the lack of responsiveness to ABA in seeds of the *aba,abi3* mutant reduces the accumulation of storage lipids in favour of carbohydrates. Although substantial evidence has arisen that ABA does not affect the long-distance transport of assimilates to seeds (Schroeder, 1984; Quarrie *et al.*, 1988; Figure 6.1; Chapters 3, 4 and 5), ABA may play a role in the distribution of these assimilates among the various types of reserve material within the seed.

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7 General discussion and conclusion

The effects of ABA on seed growth and development have been studied in detail in this thesis. It was hypothesized (Chapter 1) that ABA stimulates the transport of assimilates towards developing seeds, and influences the allocation of these assimilates among the various type of reserve materials in the seed. In the next paragraphs, the influence of ABA on the route of assimilates from carbon fixation in source leaves to storage in sink tissues is discussed. Successively, effects of ABA on photosynthesis, phloem loading, phloem transport, phloem unloading and processing of assimilates within the sink are considered.

Effects of ABA on the production of assimilates

Parameters such as biomass production and harvest index are related to the processes of photosynthesis and assimilate partitioning (Chapter 1). The first process that can be influenced by ABA in this chain of processes is the regulation of gas exchange of the leaves. It is well known that increase of ABA in leaves (*e.g.* caused by drought stress) leads to a net loss of potassium from the stomatal guard cells, resulting in reduced turgor and closure of the stomatal pore (MacRobbie, 1991). However, stomatal closure is not only crucial to the decrease of transpiration rates but will also result in lowered intercellular CO_2 levels and subsequently depressed photosynthesis. This link between transpiration and assimilation was shown in grape vines: plants receiving double the normal amount of irrigation water had lower levels of ABA in leaves and xylem sap, and higher transpiration and assimilation rates (Loveys, 1991).

Apart from the indirect influence of ABA on photosynthesis *via* stomatal closure, several non-stomatal effects of ABA have been described. Stomata of *Lemna* cannot be closed by ABA, nevertheless, plants treated with ABA showed a remarkable decrease in the net photosynthetic rate (Bauer *et al.*, 1976; Tillberg *et al.*, 1981). Initially, some evidence arose that this decrease could be explained by an inhibition of rubisco (ribulose-1,5 bisphophate carboxylase, one of the key enzymes in carbon fixation) by ABA (Poskuta *et al.*, 1972; Ryć and Lewak, 1980), but, more recently, this effect has been doubted (Ward and Bunce, 1987). Several other contradictory results of ABA application were also described: inhibition of Hill reaction (Bauer *et al.*, 1976), increased photosynthetic activity (McLaren and Smith, 1977), and no effect on photosynthetic rate (Mawson *et al.*, 1981). Effects of ABA on photosynthesis derived from the changes in partial pressure of CO_2 in the intercellular spaces after ABA application (Raschke and Hedrich, 1985; Fischer *et al.*, 1986) are probably based on erroneous calculations (Mansfield *et al.*, 1990). Apparently, the role of ABA in the control of processes related to carbon fixation is not yet understood.

Knowledge about the hormonal regulation of sucrose and starch synthesis and degradation in the source leaves is scarce. Ackerson (1983) found higher sucrose and starch levels in leaves of a maize line that accumulated more ABA, but it was not clear whether and how the higher carbohydrate levels were related to ABA. The induction of α-amylase by GA in endosperm of cereal grains, and the inhibition of the production of α-amylase by ABA are well-documented phenomena (Garcia-Maya *et al.*, 1990; Black, 1991), but the hormonal regulation of amylases in vegetative tissues is different from that in cereal kernels (Smith *et al.*, 1987).

Effects of ABA on phloem loading

The hormonal regulation of phloem loading is not yet elucidated (Delrot and Bonnemain, 1985; Delrot, 1989). The reports concerning the effect of ABA on phloem loading are contradictory, up to now. Uptake of sucrose by leaf discs of castor bean cotyledons floating on a medium is a model system for the study of phloem loading (Komor, 1977; Hutchings, 1978), and ABA appeared to inhibit this process in castor bean and some other species (Vreugdenhil, 1983). In a study with isolated veins of pea, Estruch *et al.* (1989) reported a similar inhibition. On the other hand, in tobacco leaf discs, a considerable stimulation of sucrose uptake by ABA was observed (Vreugdenhil and Kerckhoffs, 1992).

A possible explanation was that in the tobacco system assimilates were loaded into the mesophyll rather than directly into the phloem; these tissues may react in a different way to ABA (Vreugdenhil and Kerckhoffs, 1992). This may also explain why Wyse (cited in Brenner, 1982), Dörffling *et al.* (1984) and Delrot (1989) found no effect of ABA on phloem loading in sugar beet leaves, maize leaf strips and broad bean leaf discs, respectively.

It has been demonstrated that apoplastic phloem loading of sugars is driven by a sucrose/proton cotransport, and thus by a proton gradient over the sieve element plasma membrane (Komor, 1977; Komor and Orlich, 1986; Delrot, 1989). Regulation of phloem loading by ABA could possibly be explained by an effect on this proton gradient, since such effects have also been described in other systems (Malek and Baker, 1978; Van Bel and Patrick, 1984). However, in the leaf disc system, pH changes of the medium caused by proton-coupled sucrose uptake were not influenced by ABA (Vreugdenhil, 1983). For the same reason, it is unlikely that the effects of ABA are mediated by inhibition of an ATPase necessary for the proton gradient, as hypothesized by Tanner (1980).

Effects of ABA on phloem transport

Although in several reports the observed effects of hormones on assimilate translocation are attributed to an influence on phloem transport (Skene, 1971; Karmoker and Van Steveninck, 1979; Tietz *et al.*, 1981; Penot *et al.*, 1981; Poder *et al.*, 1988), in most cases it is not clear whether the loading or unloading of assimilates is influenced, or the transport process itself. Taking into account the Münch pressure flow hypothesis (Münch, 1930; Chapter 1) for phloem transport, there is no basis for a direct way in which hormones can regulate the flow of assimilates, although hormones may have a function in the reloading of assimilates or in the establishment or modulation of the potassium gradient along the pathway.

The most convincing evidence for a role of hormones on phloem transport came from experiments with decapitated bean plants. Gibberellic acid (GA_3) and cytokinin (kinetin) applied to the stem stump stimulated transport to the site of application (Mulligan and Patrick, 1979; Turvey and Patrick, 1979), but only in the case of auxin (IAA) the hormone needed to be present along the entire length of the internode (Patrick, 1979). Gibberellin and cytokinin also promoted sugar unloading to the apoplast of the stem (Hayes and Patrick, 1985). Effects of ABA on these processes have not been described.

ABA and unloading of assimilates in sink organs

Correlations between ABA level and assimilate transport to reproductive sinks

Most studies on correlations between ABA contents of sinks and the rate of assimilate translocation to these organs were performed on reproductive sinks, since these organs contain high concentrations of ABA during growth (reviews: King, 1982; Black, 1991). The time-course of ABA concentrations in seeds during development is highly dependent on the species. In general, ABA concentrations increase during development, and decrease rapidly during seed ripening (King, 1982). In most cases, some more or less distinct peaks are described (*e.g.* pea: Eeuwens and Schwabe, 1975; Wang *et al.*, 1987; tomato: Groot *et al.*, 1991; bean: Hsu, 1979; Perata *et al.*, 1990), but the results for one species are not always similar, and dependent on the culture conditions (*e.g.* wheat: King, 1976; Lee *et al.*, 1988; Koshkin and Tararina, 1990). In Chapter 2, the ABA concentrations in developing pea seeds were compared with the increase in seed weight: the first peak coincided with the phase of rapid accumulation of dry matter whereas the second peak occurred just prior to maturation of the seeds (Figure 2.5). Some examples of the relations between ABA and growth rate described in literature, are mentioned below (see also Chapter 1, Table 1.I).

Schussler *et al.* (1984) measured ABA concentrations in seeds of three soybean lines having large, medium or small seeds. In all cases, the ABA concentration in seed-coats and cotyledons, was closely correlated with the growth rates of the seeds, large seeds having the highest ABA concentrations. Moreover, cotyledons isolated from seeds containing the highest ABA levels exhibited the largest capacity to accumulate sucrose from a medium, whereas lower sucrose uptake rates were coupled to lower ABA concentrations (Schussler *et al.*, 1984). In another series of experiments, retarded seed growth rates of soybean caused by changes in photoperiod resulted in delayed peaks of ABA concentrations (Morandi *et al.*, 1990). Similar correlations were found for temperature-induced changes in growth rate and ABA levels in pea seeds (Browning, 1980).

The above-mentioned relationships provide evidence for a role of ABA in determining the growth rate of seeds. However, in a more detailed study, Schussler *et al.* (1991) described the correlation between ABA concentrations and growth rate in nine soybean lines. Their conclusion was that the level of ABA in seed-coats and embryos might be

correlated to water uptake and the rapid expansion of the cotyledons, but not to levels of sucrose in these tissues or to the rate of seed dry matter accumulation. Although a positive correlation was found between sucrose uptake rates of excised embryos and ABA levels (similar to that described by Schussler *et al.*, 1984), the authors stressed that this correlation was inherent to the gradually decreasing ABA levels in all lines, and that no cause and effect relationship could be inferred from these data. In comparable studies on cultivars of barley and wheat, no correlation between ABA levels and grain weight was found (Quarrie *et al.*, 1988; Koshkin and Tararina, 1990).

From these studies it becomes clear that one has to be cautious with correlative relationships between hormone levels and phenomena such as seed growth rate. At least part of the ABA in the developing seeds originates from the mother plant and is translocated to the seeds *via* the phloem (Hein *et al.*, 1984), so, it is not surprising that levels of ABA imported into the seeds correlate with levels of imported sucrose and to the growth rate of the seeds. In this case, it is self-evident that changes in growth rate caused by changes in temperature or photoperiod (Browning, 1980; Morandi *et al.*, 1990) will also result in changed ABA levels. Similar trends were found for cytokinins in pea seeds: in a biphasic growth curve, the maxima in the cytokinin content coincided exactly with the maxima in growth rate of the seeds (Burrows and Carr, 1970); these peaks in cytokinin content, however, are considered to be a consequence of accumulation at the end point of the transport pathway (Monselise *et al.*, 1978).

Another complicating factor is that it may be important to distinguish between hormone levels in the various seed tissues. In wheat, ABA concentrations in the embryo are four to ten times higher than in the endosperm, although the endosperm ABA makes up 80 % of the total grain ABA (King, 1982), and soybean seeds have up to twenty times higher concentrations of ABA in the embryonic axis than in the seed-coat and the cotyledons (Hein *et al.*, 1984, but see Schussler *et al.*, 1984). To explain these differences in hormone levels it has to be taken into account that the embryos themselves also produce ABA.

Effects of ABA on the unloading of assimilates in reproductive organs

Many studies have been performed to elucidate a putative role for ABA in the final steps of the assimilate transport pathway: the unloading of photosynthates from the phloem and the subsequent transport to the sink organs, mostly fruits or seeds (Chapter 1, Table 1.I).

In most cases, ABA levels in the sink organs were increased by applying (spraying, dipping, *etc.*) the hormone, or decreased by treatments with fluridone, a carotenoid-biosynthesis inhibitor. The experiments on wheat mentioned in Chapter 1 clearly illustrate the difficulties of this approach, which probably explain the contradictory results.

Release of assimilates from the seed-coat was studied with the empty-seed-coat technique (review: Wolswinkel, 1992). These studies are relatively non-invasive, since damage to the tissues is limited to the holes in the pod wall and seed-coat, and the growth regulators are added to a medium that replaces the apoplastic fluid. ABA slightly stimulated the release of sugars from the seed-coat, but the effects were variable (Gifford and Thorne, 1986; Ross *et al.*, 1987; Clifford *et al.*, 1986, 1987, 1990). Our hypothesis was that effects of ABA might be more pronounced in ABA-deficient mutants and/or at source-limited conditions. However, in studies with an ABA-deficient pea line, phloem import appeared not to be dependent on endogenous ABA levels (Chapter 3).

In some species, ABA was applied to the fruits or seeds after detaching these sink organs from the plant and culturing them on a sucrose medium (wheat: King and Patrick, 1982; Borkovec and Procházka, 1992; pea: Barratt, 1986a; broad bean: Barratt, 1986b; soybean: Schussler *et al.*, 1984). However, in the case of fruit culture, normal pod or seed development is more or less disturbed (pea: Srivastava *et al.*, 1980; Barratt, 1986a). Several authors report that the uptake of sucrose from culture media by isolated embryos is stimulated by ABA (soybean: Brenner *et al.*, 1982; Schussler *et al.*, 1984), although others found negative effects of applied ABA on the growth of embryos or kernels during *in vitro* culture for some days (pea: Davies and Bedford, 1982; broad bean: Barratt, 1986b; wheat: Borkovec and Procházka, 1992).

Another less-invasive and more subtle system to influence the ABA content of sink organs was described by Dembinska *et al.* (1992): in a split-root system with wheat plants, the leaf water potential was maintained at a normal level by water uptake in one half, whereas ABA levels in the spikes were increased considerably by water stress in the other root-half. However, the higher ABA levels did not influence the grain set. Unfortunately, no data were given on final grain size.

In this thesis, the benefits of the use of hormone mutants to study the role of ABA on seed development have been mentioned already (Chapters 1 and 4). Seeds of the tomato *sitiens* mutant, the pea *wil* mutant and the *Arabidopsis aba,abi3* recombinant have growth rates that are similar to or just slightly lower than growth rates of wild-type seeds (Groot *et al.*, 1991; Chapters 2 and 6). A problem with these comparisons is the variation

between individual plants, and the differences in water status between wild-type and mutant plants. Chapters 4 and 5 describe experiments conducted to study differences in sink strength of seeds that differed genetically with respect to their capacity to make ABA and/or their sensitivity to ABA, using seeds growing on the same mother plant. In this experimental setup, the seeds competed for the same source, and differences in growth rate between the seeds were necessarily due to differences is ABA level or sensitivity. Both in the case of pea (ABA-deficient) and *Arabidopsis* (ABA-deficient and ABA-insensitive), even under source-limited conditions, no effect of the genotype of the seeds was observed (Chapters 4 and 5, respectively). This indicates that either ABA is not absolutely required for seed growth, or that the level of ABA is still not below the threshold for this response in these mutants. However, the latter explanation is unlikely, at least in the case of the *Arabidopsis* recombinant.

One might argue that in these experiments (Chapters 4 and 5) the ABA concentration in the genetically different seeds is not different at all, since all ABA that is present during the stage of rapid growth originates from the (ABA-deficient) mother plant and not from the embryos themselves. However, it has been demonstrated for the tomato *sit*^{w} mutant, that heterozygous seeds developing on ABA-deficient mother plants have considerably higher ABA levels than self-pollinated seeds on an ABA-deficient plant, even early during seed development (Groot *et al.*, 1991). Moreover, in the case of *Arabidopsis*, *Aba/aba* seeds developing on *aba/aba* mothers contained four times as much ABA as self-pollinated seeds, at ten days after flowering, and these heterozygous seeds also responded differently with respect to germination as compared to homozygous recessive seeds, even early during development (Karssen *et al.*, 1983). With respect to ABA-insensitivity in *Arabidopsis*, other effects of this mutation were already observed at six or seven days after flowering (Finkelstein and Somerville, 1990; Chapter 6). It seems plausible that, at least in *Arabidopsis*, but most likely also in pea, considerable differences in ABA content and/or sensitivy have existed between heterozygous and homozygous recessive seeds growing on the same mother plant, during the phase of rapid dry matter accumulation.

ABA and non-reproductive sinks

One of the most established effects of ABA with respect to assimilate partitioning to non-reproductive sinks is its influence on the shoot/root ratio. In several studies, application of ABA has led to inhibition of shoot growth and no effect or stimulation of

root growth (*e.g.* Watts *et al.*, 1981; Biddington and Dearman, 1982; Creelman *et al.*, 1990). Corresponding results were found after application of fluridone and with the use of an ABA-deficient maize mutant: lowered endogenous ABA content was associated with inhibition of root elongation and promotion of shoot growth (Saab *et al.*, 1990). These results correspond also with data of ABA mutants in other species (potato: Waggoner and Simmonds, 1966; tomato (*flacca*): Bradford, 1983; pea: Chapter 2). Probably, ABA regulates the adaptation to changing water potentials, favouring enhanced root growth at conditions of low-water potential, at the expense of shoot growth. Such changes will improve the ability to attract water from the soil, and reduce water losses by the leaves (Setter, 1990). Recent evidence has shown that roots react on soil drying by production of ABA that is moved to the shoot *via* the xylem (Zhang and Davies, 1989, 1990). This xylem ABA may not only increase the hydraulic conductivity of the root (Ludewig *et al.*, 1988) and the stomatal resistance of the leaves (Hartung and Davies, 1991; Tardieu *et al.*, 1992) but also indirectly inhibit shoot growth, because of depressed photosynthesis. The results of Nagel *et al.* (1991) do not fit in this hypothesis: they found lower shoot/root ratios in the tomato *sitiens* mutant. The lower relative growth rate of this mutant seemed to be caused by an altered water status, and they concluded that their data do not lend support to the idea that ABA affects biomass allocation in intact plants by locally promoting the sink strength.

Other literature data concerning effects of applied ABA on shoot or root growth are mostly compatible with the hypothesis that ABA favours root growth at the expense of shoot growth. ABA inhibited the transport of assimilates in decapitated bean internodes (Mullins, 1970); Gaither *et al.* (1975) found stimulation of root elongation in pea by ABA; sugar levels in roots of bean seedlings were increased by ABA, whereas sugar levels in stems were unaffected (Karmoker and Van Steveninck, 1979); ABA treatment of wheat plants resulted in reduced plant height (Quarrie, 1982) and reduced shoot growth rate (Hall and McWha, 1981); water deficit-induced increases in ABA, or application of ABA considerably decreased the growth rate of soybean hypocotyls within one or two hours (Bensen *et al.*, 1988). Moreover, ABA was found to stimulate uptake of sugars by sugar beet root tissue (Saftner and Wyse, 1984) and phloem transport of phosphate towards potato tubers (Poder *et al.*, 1988). However, in a few other studies, opposite results were found (McWha and Jackson, 1976; Barlow and Pilet, 1984). It has also to be realized that in many of these studies the effect of ABA was coupled to changes in water status, so it is difficult to say whether ABA was directly responsible for these

effects. This was illustrated by the work of Bensen *et al.* (1988): when seedlings grown at low water potential were rewatered, the recovery in growth rate preceded the decrease of ABA levels.

Effects of ABA on processing of assimilates within reproductive sink organs

Assimilates that arrive in a sink organ are utilized either for respiration or for the synthesis of structural compounds or storage reserves. Little is known about the influence of ABA on respiration, although there is some evidence that ABA plays a role in the suppression of respiration at the end of seed development (F. Tetteroo, unpublished observations). In potato tuber explants, ABA inhibited respiration (Gude *et al.*, 1988). If such an inhibition also occurs during seed development, this might cause lower levels of storage reserves in seeds of ABA-deficient plants.

Photoassimilates transported to seeds are stored as proteins, carbohydrates or lipids.

Proteins

The role of ABA in the accumulation of storage proteins in developing seeds has been well studied (*e.g.* Sussex and Dale, 1979; Davies and Bedford, 1982; Finkelstein *et al.*, 1985; DeLisle and Crouch, 1989; Koornneef *et al.*, 1989; Groot *et al.*, 1991; Nambara *et al.*, 1992), although in this case again it is still not clear whether the observed effects are directly or indirectly caused by ABA. Most work on this subject was done either with *in vitro* cultured embryos/embryoids, or *in vivo* with mutants. In *in vitro* cultured embryos or embryoids, addition of ABA to the culture medium results in synthesis of storage proteins (Sussex and Dale, 1979; Crouch and Sussex, 1981), but culture on low osmotic potential has a similar effect (Crouch and Sussex, 1981; Finkelstein and Crouch, 1986; Xu *et al.*, 1990). The relationship between effects of ABA and osmotica is not completely clear, since effects of osmotica are not necessarily mediated by higher ABA levels (Finkelstein and Crouch, 1986; Morris *et al.*, 1988; Barratt *et al.*, 1989) and in some cases storage protein synthesis is initiated or stimulated by osmotica but not by ABA (Barratt *et al.*, 1989), or by ABA but not by osmotica (Rivin and Grudt, 1991). Another indication that ABA is somehow involved, is the decreased level of storage proteins in the ABA-insensitive *abi3* mutant of *Arabidopsis* (Finkelstein and Somerville, 1990) and in the viviparous *vp1* mutant of maize (Rivin and Grudt, 1991), although the ABA-deficient *sit*[w]

mutant of tomato has wild-type storage protein levels (Groot *et al.*, 1991). ABA is also involved in the synthesis of proteins that are not necessarily seed storage reserves: *e.g.* the 'late embryogenesis abundant' proteins (LEA), the 'dehydration-induced proteins' (dehydrins), the 'responsive to aba' proteins (RAB) and the 'early methionine' proteins (Em) (Bray, 1991). The question whether ABA acts by inhibiting water uptake, which affects gene expression (Finkelstein and Crouch, 1986), or that ABA directly increases the transcription rate of the genes responsible for storage proteins (DeLisle and Crouch, 1989) is still unresolved (Bray, 1991).

Lipids

Lipids in seeds are mostly stored as triacylglycerols. In general, oil-containing seeds initially import carbohydrates and convert them to starch, but later during development, starch is degraded and lipids accumulate (Appelqvist, 1975). In the chloroplasts of leaf cells or in the proplastids of oil-seed cells, pyruvate, either derived from degraded starch or from imported sucrose is decarboxylated to acetyl-CoA (Figure 7.1; reviews: Stumpf, 1989; Browse and Somerville, 1991; Huang, 1992; Slabas and Fawcett, 1992). Acetyl-CoA, together with malonyl-CoA, serves as a substrate for the fatty acid synthetase system, which consists of a series of non-associated enzymes that couple acyl chains to acyl carrier proteins (ACPs), and elongate them to stearoyl-ACP (18:0-ACP). The latter

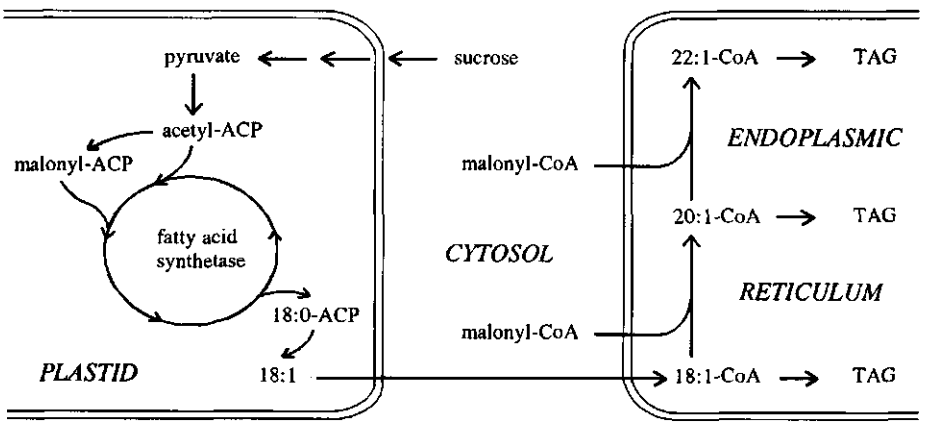

Figure 7.1 Overview of the biosynthesis of triacylglycerols, and the role of the different compartments of a seed cell.

compound is desaturated to oleoyl-ACP (18-1-ACP), and subsequently oleic acid (18:1) is formed as the end-product. Oleic acid can readily be transported from the chloroplast or proplastids to the endoplasmic reticulum, where the fatty acids are eventually further elongated, desaturated and attached to glycerol backbones to form triacylglycerols. The triacylglycerols are stored in small oil bodies, formed by the endoplasmic reticulum. The fatty acid composition of these triglycerides in oil-seeds is very variable, not only among species, but there are also differences among the various seed tissues, and during seed development. Cruciferous seeds contain not only the common 18:1 and 18:2 but also 20:1 and in some cases 22:1 (Appelqvist, 1975; Chapter 6).

Several groups have reported that ABA stimulates the accumulation of lipids during *in vitro* development of embryoids (Finkelstein and Somerville, 1989; Dutta and Appelqvist, 1989; Kim and Janick, 1991; Holbrook *et al.*, 1992). Most likely, ABA prevents in the embryoids primarily germination, which permits further maturation and deposition of reserve food. In the view of these findings, *Arabidopsis aba,abi3* seeds might be compared with embryoids cultured without ABA, and a likely explanation of the cessation of lipid accumulation in this recombinant (Chapter 6) would be that those seeds lack desiccation tolerance and therefore enter into a pre-mature germination mode (Koornneef *et al.*, 1989) that does not need further accumulation of storage material. In *in vitro* systems, it is not possible to distinguish between an effect of ABA on reserve accumulation and its effect of suppressed germination of those embryoids, but hormone mutants allow this distinction. *Arabidopsis aba,abi3* seeds accumulate considerably less storage lipids (Chapter 6), even at stages where they do not differ from wild-type seeds in germination ability (Koornneef *et al.*, 1989). Apparently, the ABA effect on deposition of storage lipids in the double mutant is not coupled to an ABA-induced switch in the developmental program of the embryo. Another important observation in this connection is that the elevated sucrose levels that are typically found in 'mature' *aba,abi3* seeds were not found in germinating wild-type seeds (J.J.J. Ooms, personal communication), indicating that the observed differences in storage reserve level are not the consequence of precocious germination of the mutant seeds.

The results presented in Chapter 6 indicate that ABA either stimulates the synthesis or inhibits the breakdown of triacylglycerols. The possibility that ABA suppresses the activity of lipase, agrees with the findings of Hole *et al.* (1988) and Bai *et al.* (1989), who reported that in developing kernels of the viviparous *vp5* (ABA-deficient) and *vp1* (ABA-insensitive) lines of maize and in a fluridone-treated line, lipid bodies were degraded due

to an increased activity of triacylglycerol lipase. With less specific techniques, similar evidence was derived from the inhibiting effect of ABA on lipase or esterase activity in germinating wheat seeds (Noda and Kanzaki, 1988). Probably the mode of action of ABA in the regulation of storage lipid accumulation is to some extent comparable with the suppression of the gibberellin-inducible α-amylase in developing wheat seeds: although the seeds are capable of α-amylase production, ABA is one of the factors that can block this production (Garcia-Maya *et al.*, 1990). The same mechanism is imaginable for lipid accumulation: lipids accumulate during seed development because their breakdown by lipase is inhibited in the presence of ABA, whereas breakdown during germination is permitted in the absence of ABA.

Apart from this, ABA is also involved in the elongation of acyl chains from 18:1 to 20:1 and 22:1 (Finkelstein and Somerville, 1989; Holbrook *et al.*, 1992; Chapter 6). At present, the nature of the elongase system is unclear (Agrawal and Stumpf, 1985a,b; Taylor *et al.*, 1992; Kunst *et al.*, 1992), which makes it difficult to relate the effects of ABA to this system. Effects of ABA on the level of gene expression is not excluded, since the incubation times with ABA in the study of Holbrook *et al.* (1992) were rather long.

Carbohydrates

In general, carbohydrates are stored as polysaccharides, although several fruits accumulate considerable levels of mono- or disaccharides (Biale, 1960). One of the most common polysaccharides is starch. In cereal seeds, starch is the final storage reserve, but in oil-seeds, starch is just a transient buffer of photosynthates that becomes depleted at the end of development; the breakdown products are subsequently converted to lipids. Little is known about the involvement of ABA in the process of starch accumulation. Radley (1976) observed a decrease of the α-amylase activity when the ABA content of developing wheat grains increased, and suggested that there might be a causal connection between these phenomena. Setter (1980) found both higher ABA levels and considerably elevated sucrose and glucose levels in soybean leaves after depodding, but the starch content of the leaves was unchanged. In *in vitro* cultured soybean embryos and broad bean cotyledons, addition of ABA to the culture medium stimulated the accumulation of starch and proteins, whereas the lipid content decreased (Ackerson, 1984; Barratt, 1986b). However, in maize kernels, no such effects were observed (Myers *et al.*, 1990; Ober and Setter, 1990).

In the present study, seeds of the ABA-deficient and ABA-insensitive *Arabidopsis*

recombinant *aba,abi3* retained high starch levels and accumulated sucrose during development, in contrast to wild-type and single-mutant seeds (Chapter 6). Whether these high amounts of carbohydrates originated from increased synthesis or decreased degradation and utilization, was not resolved.

At present, no literature data are available on starch or carbohydrate levels in mutants that are disturbed in their fatty acid composition. However, studies on *Papaver orientale* somatic embryos have revealed that exposure to abundant sucrose leads to starch accumulation, whereas *Papaver* seeds are oil-seeds and normally do not contain starch (Hara *et al.*, 1985). This indicates that those seeds have at least the potential to store starch as an overflow mechanism.

Such a mechanism seems to occur in *Arabidopsis aba,abi3* seeds. The reduced accumulation of lipids or increased degradation of lipids in those seeds results in either reduced breakdown of starch, the transient carbon-buffer, or even enhanced starch synthesis, since the breakdown product of lipids (acetyl-CoA) can easily be converted to hexoses or sucrose, *via* the glyoxylate cycle in the glyoxysomes of oil-seeds.

Conclusion

Does ABA influence assimilate partitioning to developing seeds? Above it was shown that ABA may inhibit processes associated with the supply of assimilates to the seeds, such as photosynthesis (either indirectly by decreasing the stomatal conductance, or directly *via* an effect on carbon fixation) and phloem loading. It could be argued from these effects that ABA-deficient plants invest more dry matter in their seeds, which is not true, however (Groot *et al.*, 1991; Chapters 2 and 6). Apparently, ABA does not influence photosynthesis or phloem loading at physiological conditions, or these influences are counteracted by opposite effects at the unloading site of the pathway. Another explanation is that these processes do not limit dry matter transport to seeds, although this seems unlikely.

Application of ABA to plants, fruits or seeds have yielded contradictory results. In this thesis, ABA mutants were used to study effects of ABA on photosynthate transport towards seeds, but no influence of the genotype or ABA was observed (Chapters 3, 4 and 5). At least in those systems, ABA does not play a major role in the determination of the sink strength of reproductive organs. In view of the contradictory evidence in literature, this conclusion may have more universal validity.

With respect to storage reserves in seeds, ABA is not only involved in the regulation of the synthesis of storage proteins, but also in the accumulation of storage lipids (Chapter 6) and the elongation of acyl chains in oil-seeds (Finkelstein and Somerville, 1989, 1990; Holbrook *et al.*, 1992; Chapter 6). As a result of the lower lipid levels in those seeds, carbohydrates accumulate.

In conclusion, ABA does not influence the partitioning of assimilates towards developing seeds, but plays a role in the regulation of their fate after arrival in the seeds.

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This thesis describes the influence of abscisic acid (ABA) on the transport of assimilates to seeds and the deposition of reserves in seeds. It is well-known from literature that ABA accumulates in seeds during development, and that ABA concentrations in seeds correlate rather well with seed size and seed growth rates. However, since ABA is at least partly synthesized in the leaves and transported to the seeds *via* the phloem, a correlation between ABA levels and growth rate can easily be explained as the result of the combined transport of ABA and assimilates. Reports about the effect of applied ABA on transport of assimilates to seeds are contradictory (Table 1.I). Moreover, application of ABA has several disadvantages: the application technique itself may cause artefacts, and the results are difficult to interpret since the endogenous ABA level after application depends on penetration, transport and metabolism in the tissue. For these reasons, we have chosen for a different approach, *viz.* the use of hormone mutants. Two species were used: *Pisum sativum* and *Arabidopsis thaliana*.

Growth and development of the ABA-deficient '*wilty*' mutant of pea is described in detail (Chapter 2). A non-wilty isogenic line was obtained after six successive backcrosses of the mutant with a closely approximating line. The plants were grown at conditions of high relative humidity and cultured on hydroponics, since leaves of ABA-deficient plants fail to accumulate ABA at drought stress and consequently do not close their stomata. For the same reason, mutant leaves have a higher dry matter content than wild-type leaves. The mutant grew slower and especially root growth was reduced; this resulted in a considerably larger shoot/root ratio. Similar effects have been found in ABA-deficient mutants of several other species. This root-growth promotive effect of ABA can be

explained as a measure to prevent an undesirable water status of the leaves by increasing the volume of soil explored under dry conditions.

ABA-deficient plants had fewer and smaller seeds than wild-type plants, but since the mutants plants themselves were also smaller, the weight ratio of reproductive to vegetative parts was similar in both lines. The seeds of mutant plants contained about five times less ABA than wild-type seeds. It was concluded that the lower growth rate of both vegetative and reproductive parts was not directly caused by the lower ABA content of these organs, but by disturbed water relations.

One of the reasons to choose the pea mutant was that transport of assimilates to legume seeds can be studied by the empty-seed-coat technique. After removal of a part of the pod wall and the seed coat, the embryo is replaced by a buffer, while leaving most of the maternal tissue intact. This buffer receives assimilates from the seed-coat and is regularly analysed for the presence of sucrose. The rate of sucrose efflux calculated from the seed-coat into the medium is assumed to be a measure for phloem import, especially during the period of near-constant sucrose release (4-10 hours after the start of the experiment). The effect of ABA on sucrose release was studied by applying various ABA concentrations to the buffer (Figure 3.1) and expressing the amount of sucrose released into these buffers relative to the amount present in a control seed-coat (a surgically modified seed-coat containing buffer without ABA). It was shown that hardly any ABA leaked from one seed-coat to another. The experiments were performed with both wild-type and ABA-deficient plants, either or not at source-limited conditions, since it was assumed that a possible effect of ABA might be more pronounced in ABA-deficient plants and at source-limited conditions. Source-limiting indeed caused a reduction of the sucrose release-rate. However, no effect of ABA on sucrose release could be discerned, irrespective of the experimental conditions.

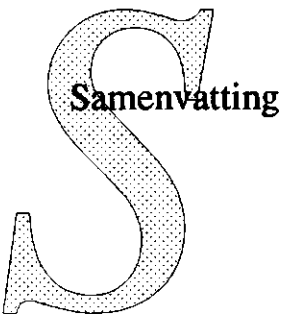
Another advantage of the use of mutants is the possibility to study competition between genetically different seeds, for the same source of assimilates (Figure 1.3). In pea, this was achieved by crossing an ABA-deficient mother plant with pollen from plants that were heterozygous for this trait. Chapter 4 describes experiments on ABA-deficient pea plants bearing pods with both ABA-deficient and ABA-containing seeds in the same pod. Seeds in the same pod usually have the same growth rate. In these pods, the growth rate of the seeds was determined by measuring the diameter of the seeds with a pair of callipers. In a control experiment it was shown that these manipulations (opening of the pod and measuring the seeds) did not disturb the normal growth pattern of the seeds. No

effect of the genotype on the growth rate of the seeds was detected.

Similar studies were performed with *Arabidopsis* mutants (Chapter 5). In one series of experiments, successive flowers of a recombinant of an ABA-deficient and an ABA-insensitive mutant (*aba,abi3*) were alternatingly pollinated with pollen from either wild-type or double-mutant plants. In another series of experiments, a double-mutant that was both ABA-deficient and starchless was used as a mother plant; the amount of available assimilates in these plants was reduced by decreasing the light intensity. The growth rate of the seeds was determined by exposing the mother plants to radiolabelled CO_2 and detecting the amount of radioactivity in the seeds. The weight of the seeds of these crosses was determined on a high-precision balance. In these experiments, again no significant influence of the genotype on either the import of radioactivity or the weight of the seeds could be detected.

The possible effect of ABA on the deposition of reserve material in seeds was studied with some *Arabidopsis* mutants. *Arabidopsis* is a crucifer and its seeds initially accumulate starch which is degraded and converted to lipids during seed maturation. Seeds of the ABA-deficient (*aba*) and the ABA-insensitive (*abi3*) mutant and their recombinant (*aba,abi3*) were collected during development and their lipid and carbohydrate composition was analysed and compared with wild-type seeds. The maximum dry and fresh weight of the seeds was not influenced by the genotype. All mutants had considerably reduced levels of eicosenoic acid (20:1) in the triacylglycerol fraction as compared to wild-type seeds; it is concluded that ABA is involved in the regulation of elongation of fatty acids. The total amount of neutral lipids in seeds of the single mutants was similar to that in wild-type seeds (about 30-35 % on a dry weight basis), but double-mutant seeds contained only half this amount. On the other hand, double-mutant seeds had elevated levels of starch and soluble sugars. Apparently, the blockade in lipid synthesis in these mutants is so strong that it results in starch accumulation and finally in accumulation of soluble sugars. It is concluded that both the presence of ABA and the sensitivity to ABA are required for normal acyl-chain elongation and lipid accumulation; the absence of both factors results in a higher proportion of the imported assimilates being stored as carbohydrates.

From the above-mentioned experiments, it was concluded that ABA has no major influence on the long-distance transport of assimilates, at least not in the species *Pisum sativum* and *Arabidopsis thaliana*. However, ABA appears to be involved in the distribution of assimilates over the various types of storage material during seed development.



Samenvatting

Inleiding

Aan hogere planten kunnen ruwweg twee delen onderscheiden worden: de vegetatieve (wortels, stengels en bladeren) en de generatieve organen (vruchten en zaden). Tijdens de ontwikkeling van de meeste vruchten en zaden worden aanzienlijke hoeveelheden assimilaten (bouwstoffen, vnl. suikers en aminozuren) aangevoerd vanuit de andere delen van de plant, de vegetatieve organen. Assimilaten worden namelijk gemaakt in de groene delen van de plant (vnl. de bladeren) en door het floëem in de vaatbundels via de stengels getransporteerd naar de generatieve organen. Bij het beschrijven van dit assimilaten-transport worden vaak de begrippen 'source' en 'sink' gebruikt: de 'source' is de bron, de plaats waar assimilaten gemaakt worden, en de 'sink' is de bestemming, de plaats waar assimilaten opgeslagen of verbruikt worden. Meestal zijn er meerdere 'sources' aanwezig (de bladeren, de stengels) en zijn er ook meerdere 'sinks' die concurreren om de beschikbare hoeveelheid assimilaten. De verdeling van assimilaten over de verschillende 'sinks' aan de plant is een belangrijk proces, en is van invloed op de verhouding tussen de oogstbare en de niet-oogstbare delen (de zgn. 'harvest index').

Vraagstelling en omschrijving van de onderzoeksmethode

Op welke manier wordt de verdeling van assimilaten gereguleerd? Het is duidelijk dat de 'source' van belang is voor de hoeveelheid assimilaten en het tijdstip waarop die beschikbaar zijn, maar er zijn geen aanwijzingen dat de 'source' en het transportsysteem

invloed hebben op de bestemming van de assimilaten. Blijkbaar speelt de 'sink' een belangrijke rol bij deze verdeling; de vraag is echter welke factoren van invloed zijn op zijn concurrentie-capaciteit, zijn aantrekkingskracht voor assimilaten (de zgn. 'sink strength'). Zijn plantehormonen hier mogelijk bij betrokken? Het onderzoek dat in dit proefschrift beschreven is, betreft de vraag of abscisinezuur (ABA, een plantehormoon) een rol speelt bij het transport van assimilaten naar zaden.

In de literatuur worden verschillende redenen genoemd die het aannemelijk maken dat de aanwezigheid van ABA in zaden bepalend is voor de 'sink strength'. Het is bekend dat ABA in vele soorten tijdens de ontwikkeling ophoopt in de zaden, vooral tijdens de fase waarin de zaden een hoge groeisnelheid hebben. Verder is gebleken dat cultivars (van sojaboon) met kleinere zaden een lagere ABA-concentratie hebben dan cultivars met grotere zaden. Bovendien is in verschillende gevallen gevonden dat toedienen van ABA aan zaden het transport van assimilaten naar die zaden stimuleerde.

Het is echter niet zo eenvoudig om definitief uitsluitsel te krijgen over de invloed van ABA op de assimilatenverdeling. Er zijn immers diverse vragen te stellen bij de hierboven genoemde onderzoeken. ABA wordt namelijk voor een deel in de bladeren gemaakt en vervolgens naar de zaden getransporteerd via dezelfde route als de assimilaten. Dat betekent dat het niet zo verwonderlijk is dat ABA juist tijdens de fase van snelle groei ophoopt in de zaden. Ook een verband tussen zaadgrootte en ABA-concentratie is dan eenvoudig te verklaren: grotere zaden hebben meer assimilaten en dus ook meer ABA geïmporteerd.

Wat betreft de experimenten waarin ABA is toegediend aan de zaden: op dit gebied worden er diverse tegenstrijdige resultaten genoemd in de literatuur (zie Tabel 1.I). Een belangrijk nadeel van deze experimenten is dat ABA moet worden toegediend: het wordt gespoten, geïnjecteerd, of de organen worden gedompeld in een oplossing. Het is dan ook altijd nodig om te bepalen of het ABA wel in het plantmateriaal binnendringt, wat de concentratie is in het weefsel en of het daar niet wordt afgebroken of omgezet. Bovendien is bekend dat ABA een belangrijke rol speelt in de waterhuishouding van de plant (toedienen van ABA veroorzaakt sluiting van de huidmondjes), en die waterhuishouding is mede van belang voor het in stand houden van het floëemtransport. Als door toedienen van ABA het transport van assimilaten beïnvloedt wordt, moet dus ook gecontroleerd worden of dat niet het indirecte gevolg is van een invloed op de waterhuishouding.

In het onderzoek dat in dit proefschrift beschreven wordt, is gekozen voor een andere benadering. Er is gebruik gemaakt van zgn. hormoonmutanten: planten die een veranderde

hoeveelheid van een hormoon of een veranderde gevoeligheid voor een hormoon hebben. Er is gekozen voor twee soorten: de erwt (*Pisum sativum*) en de zandraket (*Arabidopsis thaliana*).

Karakterisering van de erwtemutant

Van de erwt werd de '*wilty*'-mutant gebruikt. De naam '*wilty*' geeft al aan dat deze plant een verlaagd ABA-gehalte heeft, zgn. ABA-deficiënt is; doordat de bladeren minder ABA bevatten kan de plant zijn huidmondjes niet zo goed sluiten en verwelken (= to wilt) de bladeren snel. ABA-deficiënte planten worden dan ook gekweekt op hydrocyltuur en bij een hoge luchtvochtigheid. In Hoofdstuk 2 is deze mutant uitvoerig vergeleken met het wildtype. Het bleek dat de mutant trager groeide en veel minder wortels had. Bovendien had de mutant kleinere zaden en minder zaden dan het wildtype. De gewichtsverhouding tussen de reproductieve en de vegetatieve organen was echter vrijwel gelijk. Hoewel de zaden van de mutant ongeveer vijf keer minder ABA bevatten dan de zaden van het wildtype, leek het aannemelijk dat de lagere groeisnelheid van de verschillende organen van de mutant veroorzaakt werd door de verstoorde waterhuishouding, en niet zozeer door het lagere ABA-gehalte.

'Empty seed-coat'-techniek

Eén van de redenen om de erwt te kiezen was dat deze zich goed leent voor een van de technieken die gebruikt wordt om het transport van assimilaten naar zaden te bestuderen, de 'empty seed-coat'-techniek. Bij deze techniek wordt een luikje gemaakt in de peulwand en vervolgens wordt een opening gemaakt in de zaadhuid van verschillende naast elkaar liggende zaden (zie Figuur 3.1). Via deze opening wordt het embryo verwijderd en de lege zaadhuid wordt gevuld met een vloeistof. De zaadhuid blijft overigens verbonden met de peulwand en de rest van de plant; bovendien gaat de plant gewoon verder met het transport van assimilaten naar de zaadhuid. De snelheid waarmee de plant assimilaten exporteert naar de vloeistof in de zaadhuid is een maat voor het floëemtransport. Door nu aan deze vloeistof verschillende concentraties ABA toe te voegen, kan de invloed van ABA op het transport van assimilaten worden bestudeerd. Dit experiment is onder verschillende omstandigheden uitgevoerd: zowel met wildtype als met ABA-deficiënte erwteplanten (in het laatste geval was een groter effect van toegediend ABA te

verwachten) en na ontbladeren van de planten (de 'sinks' moeten dan sterker met elkaar concurreren omdat er minder assimilaten beschikbaar zijn). In geen enkel geval kon een duidelijk effect van ABA worden aangetoond (Hoofdstuk 3).

Groeimetingen aan erwtezaden

Een ander voordeel van het gebruik van mutanten is de mogelijkheid om door middel van kruisingen twee type zaden aan één (mutante) moederplant te laten ontstaan: zaden die als gevolg van zelfbestuiving het genotype van de moederplant hebben en dus ABA-deficiënt zijn, en zaden die door kruisbestuiving met een wildtype plant wel in staat zijn om ABA te maken (zie Figuur 1.3). Deze zaden concurreren met elkaar om dezelfde assimilaten en verschillen alleen van elkaar wat betreft hun ABA-gehalte. Door nu de groeisnelheid van de zaden te meten kan het effect van ABA hierop worden vastgesteld. Dit type experiment is eerst uitgevoerd met de erwt (Hoofdstuk 4); bij de erwt werd een type kruising uitgevoerd waardoor zelfs genetisch verschillende zaden binnen dezelfde peul verkregen werden. Zaden binnen dezelfde peul hebben vrijwel altijd dezelfde groeisnelheid. De groeisnelheid van de zaden werd gemeten in peulen die voorzichtig langs de rugnaad waren geopend (zie Figuur 4.1); de diameter van de zaden werd gemeten met een speciaal aangepaste schuifmaat. Na het meten werd de peul gesloten met een stukje parafilm, zodat de zaden gewoon verder konden groeien. In controle-experimenten werd vastgesteld dat zowel het openen van de peul als het meten, geen effect op de groeisnelheid hadden. In peulen waarin zich zowel ABA-deficiënte als ABA-houdende zaden bevonden, kon echter geen verschil in groeisnelheid van de zaden worden geconstateerd. Blijkbaar had de aanwezigheid van ABA in de zaden geen effect op de groeisnelheid.

Radioactieve labeling van *Arabidopsis*-zaden

Een dergelijk experiment is ook uitgevoerd met *Arabidopsis* (Hoofdstuk 5). In dit geval werd eerst een dubbelmutant gebruikt, die zowel ABA-deficiënt als ABA-ongevoelig was (*aba,abi3*); bloemen van deze plant werden afwisselend bestoven met stuifmeel van dezelfde planten en stuifmeel van wildtype planten. In een tweede type experiment werd een dubbelmutant gebruikt die niet alleen ABA-deficiënt was maar ook geen zetmeel kon maken, de hoeveelheid beschikbare assimilaten kon in deze planten worden verminderd

door ze bij een lagere lichtintensiteit te plaatsen. Omdat de zaden van deze planten zo klein zijn is het erg moeilijk om de groeisnelheid van de zaden vast te stellen door de afmetingen van elk zaad te bepalen. Met een zeer gevoelige balans was het wel mogelijk om de zaden te wegen; bovendien kon de import van assimilaten in de zaden gemeten worden door de bladeren bloot te stellen aan radioactief gelabeld CO_2, de hoeveelheid radioactiviteit die zich na een bepaalde periode in de zaden bevindt is dan een maat voor de 'sink strength' van de zaden. Ook in deze experimenten kon geen enkel effect van de aanwezigheid van ABA of de gevoeligheid voor ABA op de groei van de zaden worden vastgesteld.

De samenstelling van reservestoffen in *Arabidopsis*-zaden

Tenslotte werden enkele *Arabidopsis*-mutanten gebruikt om de invloed van ABA op de bestemming van de assimilaten in het zaad te bestuderen (Hoofdstuk 6). *Arabidopsis*-zaden zijn wat hun samenstelling betreft vergelijkbaar met die van koolzaad: de zaden slaan aanvankelijk zetmeel op maar maken hier tijdens het afrijpen lipiden (vet, olie) van. Zaden van zowel de ABA-deficiënte (*aba*), de ABA-ongevoelige (*abi3*) mutant en de dubbelmutant (*aba,abi3*) werden verzameld gedurende hun ontwikkeling en geanalyseerd wat betreft het gehalte aan lipiden en suikers. Het bleek dat wildtype en enkelmutant *Arabidopsis*-zaden voornamelijk lipiden opslaan (tot 30-35 % van hun drooggewicht in rijpe zaden), maar dat dubbelmutantzaden slechts de helft hiervan bevatten. Het bleek dat juist de aanmaak van langketen-vetzuren in de dubbelmutant sterk was geremd. Overigens bevatten de enkelmutanten ook minder van deze langketen-vetzuren, maar ze waren wel in staat om dezelfde lipidgehaltes te maken als de wildtypezaden. Uit recente literatuur is gebleken dat ABA mogelijk het enzym remt dat nodig is voor de verlenging van vetzuren. De zaden van de *aba,abi3*-mutant bevatten een verhoogde hoeveelheid suikers en zetmeel ten opzichte van de andere lijnen. Blijkbaar is de verstoring in de vetzuursynthese in deze mutant zodanig dat niet alle zetmeel kan worden omgezet in lipiden, zodat uiteindelijk zetmeel en oplosbare suikers ophopen in de zaden.

Conclusies

Uit dit proefschrift blijkt dat ABA geen of geen belangrijke invloed heeft op het transport van assimilaten naar zaden, tenminste niet in de soorten *Pisum sativum* en *Arabidopsis*

thaliana. ABA blijkt echter wel van invloed te zijn op de verdeling van assimilaten over de verschillende vormen van reservestoffen in het zaad: de aanwezigheid van ABA en de gevoeligheid voor ABA in *Arabidopsis* zijn noodzakelijk voor één of meerdere van de stappen in de synthese van lipiden.

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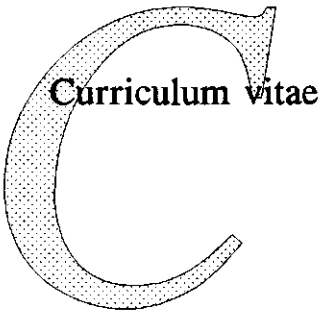

De schrijver van dit proefschrift, Stephanus Marinus de Bruijn, werd geboren op 28 mei 1962 in Sint-Maartensdijk. Van augustus 1974 tot mei 1980 bezocht hij het Mollerlyceum te Bergen op Zoom. Na het behalen van het diploma (Atheneum B) werd in september 1980 gestart met een studie planteziektenkunde aan de toenmalige Landbouwhogeschool in Wageningen. De doctoraalfase van de studie bestond uit een verzwaard hoofdvak entomologie en een hoofdvak plantenfysiologie. Beide vakken resulteerden in een publikatie. Na een tien maanden durende stageperiode bij de afdeling Gewasbescherming van Duphar ('s-Graveland) werd in november 1987 het doctoraalexamen (oude stijl) behaald. Van december 1987 tot en met november 1991 heeft hij een promotie-onderzoek verricht bij de vakgroep Plantenfysiologie van de Landbouwuniversiteit te Wageningen; het daar verrichte onderzoek heeft geresulteerd in dit proefschrift. Sinds april 1993 is de auteur in tijdelijke dienst van de Landbouwuniversiteit, waar hij aan de vakgroep Plantenfysiologie onderzoek verricht aan het koolhydraatmetabolisme gedurende de knolvorming bij aardappel.

Epiloog

'... *Gy siet bysonderlyk in dese observati, de blintheyd van het menschelyk verstant, het welk door soo veele eeuwen geleert heeft, dat dit Kruyt geen Bloemen nog Saden besit, soo dat men dit selfs van jongs op, en in de Woordenboeken aan de kinderen leert. Daarom soo is het niet als de Goddelyke genade, van de welke alle goede wetenschappen en kennisse afkomen, dat in dese laatste tyden het contrarie ontdekt word; en dat de waarheyd uyt de duysternissen voorbreekt. Hierom was het te wenschen myn Heer, dat men syn oordeel en verstant ook in veele andere saken voor verdagt hield, alsoo ons dese observatie middagklaar vertoont, hoe seer ook de aldergauste verstanden tot nog toe hebben kunnen dwalen. Het welk alsoo het omtrent een sigtbaare saak geweest is; wat is het dan niet te vreesen myn Heer, dat men omtrent de onsigtbaare dingen komt mis te tasten, en in alderhande verkeerde oordeelen geinvolveert te worden, tot nadeel en veragting van onse naasten, die juyst met ons van geen een opinie syn? Waarom daar niet beter is, als syn selven altyt te misvertrouwen, en in voorsigtigheyd te wandelen, alsoo den ellendigen Mensch in alles onwetend is, en dat syne waare kenisse niet en bestaat, als in syn swakheyd en ydelheyd wel te kennen: die niets in sig selven besit, en die alles van GOD moet ontvangen: in wiens Gratie ik U myn Heer beveele.*'

Jan Swammerdam (1738) Briefwijse verhandeling van het Varen-Kruyd Manneken van Dodoneus. In: Bybel der Natuure, Leiden, fol. 906-910.