Differentiation of legume cotyledons as related to metabolic gradients and assimilate transport into seeds

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Abstract

Legume seed development is closely related to metabolism and nutrient transport. To analyse this relationship, a combination of biochemical, histological and transgenic approaches was used. Sugars within tissue sections have been quantitatively measured by metabolic imaging. During cotyledon differentiation glucose gradients emerge related to a particular cell type, with higher concentrations in non-differentiated premature regions. Sucrose increases at the beginning of maturation in a layer underneath the outer epidermis expressing a sucrose transporter. Sucrose distribution is initially controlled by uptake activity and the permeability within the parenchyma and, later on, also by differences in growth and starch accumulation. Increased sucrose levels are accompanied by increased levels of sucrose synthase and ADP-Glc pyrophosphorylase mRNAs, but carbon flux into starch is initially still low. Rates increase at a stage when hexose concentrations become low, allowing increased flux through the sucrose synthase pathway. Transfer cell formation represents a regional specification of the cotyledonary epidermis for embryo nutrition characterized by increased transport-active cell surfaces and up-regulated expression of transport-related genes. The E2748 pea seed mutation blocks epidermal differentiation into transfer cells and leads to the loss of epidermal cell identity. Embryos with impaired epidermis cannot tolerate elevated levels of sucrose and respond with disorganized growth. The E2748 gene product is required for transfer cell formation in developing cotyledons with no other function during plant growth. Seed coat permeability provides a hypoxic environment for embryo development.

However, at maturity, seed energy supply is not limited indicating fundamental developmental and metabolic adaptations. Results from transgenic seeds show that altered expression of single genes induces complex and unexpected changes. In AGP-antisense seeds the block in starch synthesis leads to pleiotropic effects of water and nitrogen content and induces temporal changes in seed development.

Key words: Carbon flux, cotyledon differentiation, legumes, metabolism, nutrient transport, seed development, starch, sugars.

Introduction

To analyse how seed metabolism is connected with growth and development and how biosynthetic pathways are controlled by transport processes requires an integrated experimental approach. This includes biochemical and histological methods as well as transgenic approaches and genomic tools. The seeds of Vicia faba or Pisum sativum offer excellent models and due to their special morphology and large size are accessible to a wide range of different methods.

Growing embryos acquire strong sink strength at the onset of maturation, actively importing assimilates from the surrounding maternal tissue via the apoplast. Phloem unloading, post-phloem transfer and transport processes of assimilates within the maternal and embryonic seed tissues thus became a major subject of investigation and, during recent years, considerable progress has been made towards a better understanding of these mechanisms (for recent reviews: Patrick and Ofller, 1995, 2001; Weber et al., 1997a, 1998a).

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A growing seed is a heterogeneous, highly organized system consisting of different organs. Its development is largely controlled at the metabolic level. Sugars and nitrogen, in addition to their nutritive role, act as signals which regulate and influence development (Wobus and Weber, 1999a, b). Therefore, new research tools are needed to investigate seed metabolism on a spatial resolution by imaging metabolites directly in tissue sections (Borisjuk et al., 1998, 2002a) and, in the future, also by non-invasive NMR techniques (Köckenberger, 2001). A long-term goal is to visualize in vivo metabolite fluxes quantitatively.

Transgenic approaches are applied to investigate whether specific pathways or single enzymes, as well as assimilate transporters, exert rate-limiting roles on storage product synthesis and composition as well as on seed maturation. Available results so far show an enormous complexity and flexibility of seed development and metabolism, creating highly pleiotropic phenotypes, even if the expression of single genes has been changed. Analysis of such phenotypes needs sophisticated experimental approaches.

Recent results are described here using a range of different techniques in order to provide a better understanding of how a seed functions. This new information, along with previous knowledge on the cellular pathway and the physiology of assimilate import into seeds, will allow a more integrated understanding of seed development and metabolism. Such knowledge also has a significant potential for applications based on manipulating seed growth and development, and thus for agricultural yield.

Seed development is a series of differentiation events

In legume embryos such as faba bean or pea the cotyledons differentiate into highly specialized storage organs. The histodifferentiation of V. faba embryos has been described and classified into seven stages. Stages I to III describe organogenesis and morphogenesis. Stages IV to VII cover early to late cotyledon development (Borisjuk et al., 1995). Young cotyledons of stage IV are highly mitotically active. During stage V the inner cells increase in size whereas in the outer abaxial region mitotic activity is still maintained. Thereby, differentiation occurs gradually, starting from the inner adaxial region progressing to the outer layers. Because the differentiation proceeds in a wave-like manner the cotyledons represent a heterogeneous population of cells of different physiological ages over most of their development. The main storage phase is entered at stage VI and the cotyledons approach maturity at stage VII when cell expansion and storage activities stop first in the centre and then gradually in the more outer regions. The pattern of starch accumulation correlates well with cell expansion and endopolyploidization, but is spatially distinct from the pattern of mitotic activity (Hauxwell et al., 1990; Borisjuk et al., 1995, 1998). For a general scheme see Fig. 1.

The developmental gradient within growing cotyledons can be described on different levels: (i) on a structural and morphological level visualized by differences in cell size, degree of vacuolization and endopolyploidization (size of nuclei) as well as the accumulation of starch grains and storage protein bodies, (ii) on the level of gene expression visualized by in situ hybridization of mRNA encoding storage proteins and (iii) by the spatial distribution of metabolites within differentiating tissue. In order to understand how a seed functions it is important to know how these gradients are organized and controlled.

Fig. 1. The spatial analysis of sucrose concentration was performed directly in plant tissue sections by bioluminescence imaging. The figure shows a schematic representation of the tissue structure of a section of a V. faba cotyledon of stages V–VI, (field α). Transfer cells are indicated by a red line, the grey area indicates the adaxial region which mainly contains elongated cells (yellow dots). The white area highlights the abaxial region with mainly mitotically active cells (black dots). Starch accumulation is shown by iodine staining (field β). Field γ shows an in situ hybridization of the sucrose transporter SUT1 and the sucrose imaging is seen in field δ. Sucrose concentrations are higher within the abaxial region. Only in the tips of the cotyledon sucrose levels are clearly lower (yellow and green colour). Sucrose uptake into cotyledons occurs via epidermal transfer cells. In this particular cell layer a sucrose transporter gene is expressed (see in situ hybridization in field β as bright signals). Transfer cells cover the storage tissue containing high concentration of sucrose indicating that SUT1 is responsible for sucrose accumulation. Iodine staining shows starch distribution with higher amounts in regions with high sucrose. Scale bars are: 20, 60, 80, 110, 140, and 160 mM for the dark blue, light blue, green, yellow orange, and red colours, respectively. ab, abaxial region; ad, adaxial region; tc, transfer cells.
and what the implications are for metabolism and development.

**Measuring local distribution of glucose and sucrose within differentiating cotyledons**

During the differentiation of the embryo sugar and metabolite concentrations change on both a quantitative and a spatial level. Existing methods to quantify sucrose, for example, enzymatic determination or chromatography require tissue homogenization and extractions. Thereby, a mixture of cell types is always analysed which differ in their physiological and differentiation state. Sugars can be visualized in situ by Schiff's reagents reactions, however, this method can hardly be used for quantification as it is relatively unspecific and highly destructive. Non-invasive techniques, such as spectrometry and NMR analysis (Köckenberger, 2001), have exciting potential for the study of the distribution of metabolites in plants in vivo. However, the spatial resolutions are still fairly low. Therefore, a bioluminescence-based method has been applied which allows the quantitative measurement of the distribution of metabolites in tissue sections with higher resolution (Borisjuk et al., 1998, 2002a). The method couples the reaction of a metabolite of interest to the oxidation of glucose-6-phosphate and the redox system of a luciferase light reaction (Walenta et al., 1990; Mueller-Klieser and Walenta, 1993). An appropriate enzyme mix is overlaid on tissue sections and the spatial distribution of the bioluminescence intensity is registered by microscopy, which is proportional to the tissue concentration. A photon counting processor then yields images of the local distribution of the given metabolite. The method can be applied to image the concentration of glucose, sucrose or, in principle, any other metabolite where the reaction can be coupled to the enzyme assay. It allows a spatial resolution close to single cell dimension.

Accordingly, glucose concentrations were mapped in tissue sections of differentiating faba bean cotyledons. The patterns of the local distributions are compared with tissue cell-type, mitotic index and the distribution pattern of starch. During cotyledon differentiation, gradients in the glucose concentration emerge which are related to a particular cell type. Higher concentrations are found in non-differentiated premature regions of the cotyledon, whereas mature starch-accumulating regions contain particularly low concentrations of glucose. The glucose distribution pattern is, therefore, related to the developmental gradient. These data provide first evidence for steep glucose gradients across developing plant embryos and favour the idea that in developing plant tissues sugar gradients can have morphogenic functions (Borisjuk et al., 1998).

Because sucrose becomes the predominant sugar during cotyledon maturation, the method was extended in order to image the local concentrations of sucrose within V. faba cotyledons covering the main stages of histo-differentiation (Borisjuk et al., 2002a). Young embryos before the storage phase (stage IV) contained moderate levels of sucrose which were evenly distributed. At the onset of maturation (stage V) high concentrations (>140 mM) were present within a tissue layer of 10–15 cell rows covering the outer half of the cotyledons. Toward the inner region the sucrose concentration dropped to only ~30 mM over a distance of ~400 μm. This layer was directly underneath the epidermis, which is expressing a sucrose transporter gene, indicating that epidermal transporters caused the high sucrose accumulation in the underlying tissue. At stage V the sucrose gradient was inversely compared to that of cell size and starch. Cells within the interior were larger, contained starch but lower sucrose. However, during the main storage phase (stage VI) actively elongating and starch-accumulating cells contain the highest sucrose concentrations (Fig. 2), which were correlated with transcript levels of sucrose synthase and ADP-Glc pyrophosphorylase, indicating a signalling function for sucrose to induce starch biosynthesis on the gene expression level.

**The high hexose environment of the early stages is controlled by the seed coat**

During early seed development seed coat-associated invertases (Weber et al., 1995) create an environment of high hexose sugars within the endosperm vacuole and the embryo at a time when mitotic activity proceeds in the cotyledons (90–100 mM and 40 mM for hexoses and sucrose, respectively, Borisjuk et al., 2002a). This special sugar status seems to promote growth by cell division. Large-seeded genotypes of V. faba have a prolonged activity of cell wall-bound invertase in their seed coats, produce more cells in the embryo and reach the mature stage at a later time. The resultant longer period of high hexose conditions is accompanied by a longer cell division phase increasing the final cell number (Weber et al., 1996a). Accordingly, the emerging glucose gradients within V. faba cotyledons are correlated to mitotic activity (Borisjuk et al., 1998). Studies with the growth-deficient pea embryo E2748, show that this characteristic change in the presence of the principal sugar is not dependent on normal embryo growth, but is instead controlled by the maternal seed coat (Borisjuk et al., 2002b).

**Sucrose is accumulated through transporters within the epidermal transfer cells**

Mitotically active embryos contain moderately low levels of sucrose without major spatial differences. From the stages V to VI a rapid increase of sucrose occurs in a tissue-layer directly underneath the outer epidermis.
Within the epidermal cells a sucrose transporter gene is upregulated, presumably causing this high accumulation in the underlying tissue. It has been shown that in faba bean and pea embryos 75–90% of the total sucrose uptake is mediated by active transport via the outer epidermis (McDonald et al., 1996; Tegeder et al., 1999) thus exerting major control on sucrose uptake. The sucrose distribution in stage V is, therefore, mainly determined by uptake activity. However, the permeability within the storage parenchyma cells may also play a role. Symplasmic conductivity within maturing cotyledons of stages VI to VII is high due to numerous plasmodesmatal connections (McDonald et al., 1995). The cell population during stages IV to V embryos is also symplasmically connected, although permeability is somewhat restricted compared to the older stages (L Borisjuk et al., unpublished results).

In stage VI cotyledons, sucrose concentration is high in regions accumulating starch and lower in the tip regions with young and dividing cells where concentrations of starch are also lower. Thus, a sucrose gradient in stage VI cotyledons exists between strongly elongating, starch-storing cells and non-differentiated regions. In stage VI cotyledons, higher sucrose occurred in areas with high starch accumulation, a situation which is clearly different from that in stage V. It has been shown that, in potato tubers, sucrose moves into faster growing regions irrespective of their higher steady-state concentrations of sucrose (Merlo et al., 1993). Thus, sink strength and the ability to attract assimilates is controlled by both growth by cell expansion and starch synthesis (Engels and Marschner, 1986; Merlo et al., 1993). It is concluded that the intra-cotyledonal distribution of sucrose at stage VI, but not V, is partially controlled by differences in growth and starch accumulation. The pattern present in stage VII cotyledons supports this conclusion. Here a distinct gradient of sucrose concentration is evident. The region with highest values (~140 mM) consists of a small region of cells which are still expanding and accumulating starch, whereas the cells located in the more inner region have reached maturity and contain only ~20 mM (Borisjuk et al., 1995).

In summary, the sucrose pattern is initially controlled by uptake activity and permeability within the parenchyma. Later on, differences in growth and starch accumulation also affect intra-cotyledonal distribution.

**Legume embryos develop under hypoxic conditions**

Specific morphological characteristics of the seed cause oxygen deficiency within embryonic tissues. Using optical sensors O2 profiles were measured across developing seeds of *V. faba* and *P. sativum* (Rolletschek et al., 2002a). Within the seed coat, O2 concentration decreased sharply to ~3% towards the inner border. Thus, the low seed coat permeability provides a hypoxic environment for legume embryo development. O2 concentration in embryonic tissue changed during development with the lowest levels at the early stages. However, there was no gradient of O2 concentration across the embryo tissue, probably due to the presence of a net of air-filled intercellular spaces which allow high diffusion. Measured in darkness, O2 levels were below 3%, but increased upon illumination, indicating that photosynthesis significantly contributes to internal O2 levels. ATP levels and energy charges were low in very young embryos only, otherwise maintaining a constant...
higher value. To analyse the regional energy supply within differentiating cotyledons ATP levels were measured using the bioluminescence method (L Borisjuk et al., unpublished data). ATP concentrations were always highest within the axis. In prestorage cotyledons the level was low, but increased strongly in the course of further development, starting from the abaxial region of cotyledons and moving towards the interior. Greening pattern, chlorophyll distribution and photosynthetic activity within embryos temporally and spatially corresponded to the ATP distribution, implying that the overall increase of the energy state is associated with the greening process. ATP pattern was associated with the photosynthetic capacity of the embryo. The general distribution pattern, as well as the steady-state levels of ATP, and did not change upon dark/night conditions. Starch accumulated at the highest rates when the cotyledons became fully green and contained high ATP levels implying that a photoheterotrophic state was required to ensure high fluxes. ADP-glucose and UDP-glucose measured biochemically did not show large fluctuations. Except for the early stages fermentative activity did not play a major role during embryo maturation. Obviously, specific mechanisms prevent seed tissues from becoming anoxic during development. The results show that despite the low oxygen levels the cotyledons apparently did not become energy-limited, indicating fundamental metabolic adaptations as has been analysed in potato tubers (Geigenberger et al., 2000).

**Sugar gradients and its implications for cell differentiation and metabolism**

Cell differentiation starts during early stage V in the centre of the cotyledon whereas peripheral cells are still dividing. The developmental gradient is, therefore, inversely oriented compared to the sucrose distribution pattern and its increasing concentration is unlikely to be the signal for cell elongation. The situation, however, may be different during older stages when high sucrose is clearly increased within actively growing and storing cells. Cell expansion must be accompanied by both an increase of turgor and a loosening of cell walls (Cosgrove, 1997). Thus, osmolytes are needed to provide the driving force for water influx to increase turgor. In stage V cotyledons sucrose is lower within the inner expanding regions and, therefore, may play only a minor role as an osmolyte. Alternatively, the cells could accumulate inorganic ions, especially potassium (Patrick, 1984). In the endospermal vacuole, as well as in the storage parenchyma, the K⁺-concentration is very high at stage V (L Borisjuk, H Rolletschek, U Wobus, H Weber, unpublished results) and could play a role in initiating cotyledonary cell expansion.

Other factors can play a role as well. It has been shown that ectopic expression of a single expansin gene is sufficient to induce cell elongation (Pien et al., 2001). In tomato, specific members of the expansin gene-family are expressed in maturing seeds where they may induce cell expansion. Some are up-regulated by gibberellins (Chen et al., 2001). In this context it is interesting to note that a gibberellin-deficient pea seed mutant (lh2, Swain et al., 1995) is strongly affected in cotyledonary cell elongation whereas morphogenesis is normal (L Borisjuk, H Rolletschek, U Wobus, H Weber, unpublished results).

**Carbon flux into storage products**

Sucrose induction of storage-associated gene expression is a well-described phenomenon (Koch, 1996). Accordingly, the increase of sucrose in stage V embryos is accompanied by higher transcript levels of sucrose synthase and ADP-Glc pyrophosphorylase. Sucrose concentration increases during stage V, but starch accumulation rate is low, reflecting a small carbon flux into starch. One possible reason for this low rate may be that hexose concentrations are still high. Hexose levels decrease only during stage VI along with increasing starch accumulation rates. Sucrose cleavage towards starch synthesis is mediated by sucrose synthase (Heim et al., 1993), which catalyses a readily reversible reaction (Geigenberger and Stitt, 1993) and is inhibited by free hexoses (Weber et al., 1996b; Ross and Davies, 1992). The $K_m$ of sucrose synthase in *V. faba* seeds is exceptionally high (169 mM; Ross and Davies, 1992). Flux through sucrose synthase in the breakdown direction depends upon high sucrose levels and upon removal of the cleavage products. Thus, at stage V, sucrose breakdown by sucrose synthase is down-regulated or even reversed because hexoses are high. Although high sucrose is a signal for induction of the starch biosynthetic apparatus at the gene expression level (Weber et al., 1998b) concentrations of hexoses must decrease to increase carbon flux through the sucrose synthase pathway. This could explain the large increase in the starch accumulation rate at stage VI when hexoses become low. The decrease may be catalysed partly by sucrose phosphate synthase, which is up-regulated in maturing parenchyma cells (Weber et al., 1996b). This enzyme could synthesize sucrose at the end of the cell division phase, thereby decreasing the hexoses to sucrose ratio especially in maturing regions.

**Assimilate transport into seeds as related to transfer cell formation**

In legume seeds the sieve elements end in the seed coat. Phloem unloading and subsequent transfer through the seed coat occur passively. Control is probably exerted by the permeability of the plasmodesmata (Patrick and Offler, 2001). Assimilates are unloaded from the inner layer of the seed coat into the endospermal vacuole, most likely via non-selective pores (DeJong et al., 1996). Sucrose and
amino acids are taken up into the embryo by active \( \text{H}^+ \)-co-transport.

When morphogenesis and organogenesis of the young legume embryo has been accomplished, immediately before the start of the storage phase, the abaxial epidermal cells differentiate into transfer cells (Bonnemain et al., 1991). These are characterized by finger-like ingrowths of the cell wall at the boundary that are transport-active cell surfaces and, thus, exhibit a polarity in terms of wall modification. The wall ingrowths increase the transport-active surface. Within developing seeds transfer cells are strategically located in close proximity to the maternal unloading tissue in both donor and recipient cells (Bonnemain et al., 1991). They are found in the basal endosperm of maize (Felker and Shannon, 1980), in the modified aleurone cells of barley (Weschke et al., 2000), as well as in the cotyledonal epidermis of \( \text{V. faba} \) and pea (Weber et al., 1997b; Tegeder et al., 1999). Transfer cell formation in developing legume cotyledons establishes a new function that these epidermal cells acquire at a certain stage of development, and represents a regional specification to ensure embryo nutrition. In \( \text{V. faba} \), transfer cells develop at the contact area of the embryo to the seed coat. Obviously this modification is associated with stimuli coming from neighbouring compartments (Weber et al., 1997b). The factors controlling transfer cell formation are unclear, but metabolic signalling may be involved. Exposure to hexose sugars induces transfer cell formation in \( \text{V. faba} \) embryos (Of\l er et al., 1997; Farley et al., 2000), whereas high sucrose is inhibitory (Of\l er et al., 1997; Weber et al., 1997b).

Transfer cell formation is coupled with up-regulated expression of transport-related genes encoding transporters for sucrose (Harrington et al., 1997; Weber et al., 1997b; Tegeder et al., 1999), hexoses (Weber et al., 1997b), amino acids (Tegeder et al., 2000) as well as \( \text{H}^+ \)-ATPases (Harrington et al., 1997). In \( \text{V. faba} \) cotyledons increased gene expression of the sucrose transporter is accompanied by accumulation of large amounts of sucrose in the underlying tissue (Borisjuk et al., 2002a).

However, for the uptake of amino acids the situation may be different. At least seven amino acid permeases are present in the \( \text{V. faba} \) genome. One of these, \( \text{Vfaat1} \), recognizes a broad range of amino acids with a preference for cysteine, and is strongly expressed in developing seeds. In the cotyledons, \( \text{Vfaat1} \) mRNA was found across the parenchyma tissue, but not in the epidermal layer. Expression of \( \text{Vfaat1} \) reaches a maximum shortly before the beginning of accumulation of transcripts from storage protein genes, consistent with a role in providing cotyledons with amino acids that will be used for the synthesis of storage proteins (Miranda et al., 2001). Similar to the sucrose transporter (Weber et al., 1997b) \( \text{Vfaat1} \) is feed-back-regulated at the gene expression level (Miranda et al., 2001). Therefore, beneath the epidermal transfer cell layer (Tegeder et al., 2000) there is a transport system for amino acids also present on the level of the parenchyma cells.

**A pea seed mutant affected in the differentiation of the embryonic epidermis**

The pea seed mutant \( E2748 \) provides a suitable model to study nutrient uptake into the embryo, and filial±maternal interactions, as well as maturation and differentiation events in the embryo. Homozygous seeds are strongly reduced in growth and abort before complete maturation (Johnson et al., 1994). The epidermal cells of the mutant embryo, instead of turning into transfer cells, become strongly enlarged, vacuolated and tightly associated with adjacent seed tissues. Expression of a sucrose transporter gene which is up-regulated in wild-type transfer cells decreases in the mutant and changes its spatial pattern. This indicates that the outermost cell layer of mutant cotyledons cannot acquire transfer cell morphology, loses epidermal cell identity, and does not function as a sucrose uptake system.

Seed coat growth as well as composition, concentration and dynamics of sugars within the endospermal vacuole are unchanged. Thus, seed coat growth is independent of that of the embryo and seems to be genetically fixed and dependent on the maternal genotype. Furthermore, the seed coat modulates both concentration and composition of sugars within the endospermal vacuole, irrespective of proper embryo growth. This has significant implications for seed development, confirming the importance of the maternal seed tissue in regulating seed size (Davies, 1977; Hedley and Ambrose, 1980; Weber et al., 1996b).

The loss of epidermal identity in the \( E2748 \) mutant has severe consequences for further cotyledon development and is followed by restricted movement of a symplasmic tracer within the parenchyma, the breach of the developmental gradient, lower sucrose and starch levels and initiation of callus-like growth (Borisjuk et al., 2002b). The results show that the \( E2748 \) mutation blocks epidermal differentiation into transfer cells and leads to the loss of epidermal cell identity. The \( E2748 \) gene product most probably controls an important step in this process. As a consequence of the lack of the transfer cell layer, the cells adopt callus-like growth at a time when sucrose increases. This dedifferentiation disrupts further co-ordinated development which ultimately causes seed abortion. On sucrose-containing medium both the \( E2748 \) cotyledons and wild-type cotyledons where the epidermis has been removed artificially, show callus-like growth. Obviously, the embryos with impaired epidermis cannot tolerate elevated levels of sucrose in the culture medium and react with a kind of wound response. This sucrose response does not occur in intact cotyledons because transfer cells provides a barrier. \( E2748 \) embryos can be rescued...
in vitro, thereby the embryo axis develops into a normal and fully differentiated pea plant. In the rescued plant a mutant phenotype only becomes evident in all developing embryos after seed set (L Borisjuk et al., unpublished results). The E2748 gene product, therefore, is required only for transfer cell formation in developing cotyledons and has apparently no other function during normal plant growth. E2748 thus represents an embryo-specific gene controlling the regional specification of the epidermis into a nutrient uptake system.

**Changing metabolic pathways in seeds**

In order to change seed metabolism seed mutants can be used or transgenic means can be applied to change the expression of genes encoding enzymes for potential rate-limiting functions, for example, in assimilate uptake and of the sucrose to starch pathway. The scheme in Fig. 3 outlines a possible strategy of how to change metabolic pathways in seeds. Major approaches could be: (i) manipulating assimilate supply to the seed by changing the expression of transporters for sucrose and amino acids as well as of enzymes which energize membrane transport; (ii) increasing assimilate flux into amino acid biosynthesis by over-expressing, for example, PEP carboxylase or aspartate kinase of bacterial origin which is not feed-back-inhibited by the plant; or (iii) decreasing flux into starch biosynthesis by inhibiting ADP glucose pyrophosphorylase (AGP), plastidic glucose-6-P translocator (GPT) or plastidic phosphoglucomutase (pPGM) with possible compensatory increases of assimilate flux into protein biosynthesis. Results from these approaches will increase current knowledge on how specific pathways or single enzymatic steps exert a rate-limiting role on storage product synthesis and composition as well as on seed maturation. At the same time desirable changes that improve seed composition and yield may be achieved.

**Antisense-inhibition of ADP-glucose pyrophosphorylase in seeds decreased starch and increased protein content but creates a pleiotropic phenotype**

To analyse whether a specific down-regulation of one storage product causes a compensatory change on others, transgenic *V. narbonensis* seeds expressing ADP-glucose pyrophosphorylase (AGP) in antisense-orientation and therefore causing a block in starch biosynthesis were investigated. AGP antisense-inhibition changes the storage product composition in mature cotyledons. In addition, cotyledonary development is substantially altered. Transgenic seeds contain more sugars and water and have a longer seed-filling phase (Weber et al., 2000). Both AGP-activity and ADP-Glc levels were strongly decreased, but starch was only moderately reduced and contained less amylose. The flux control coefficient of AGP to starch accumulation was as low as 0.08, i.e. AGP exerts low control on starch biosynthesis in *Vicia* seeds. Mature cotyledons have increased contents of lipids, nitrogen and sulphur. Protein content was higher, in particular due to increased sulphur-rich albumins. The globulin fractions of storage proteins had a lower legumin to vicilin ratio. Isolated cotyledons partitioned less 14C-sucrose into starch and more into soluble sugars without measurable change for the protein fraction. Respiration of isolated cotyledons and activities of the major glycolytic and carbohydrate-metabolizing enzymes were not affected. Sucrose and the hexose-P pool were increased, but UDP-glucose, 3-PGA, PEP, Pyr, ATP, and ADP were unchanged or even lower, indicating that carbon partitioning changed from starch to sucrose without affecting the glycolytic and respiratory pathway to any great extent (Fig. 4). Soluble compounds were increased, but osmolality remained unchanged, indicating compensatory water influx resulting in higher water contents. Developmental patterns of water and nitrogen accumulation suggest a
coupled uptake of amino acids and water into cotyledons. It is concluded that, due to higher water uptake, transgenic cotyledons take up more amino acids which become available for protein biosynthesis, leading to a higher protein content. Obviously, a substantial part of amino acid uptake into *Vicia* seeds occurs passively and is osmotically controlled and driven by water influx.

Understanding the relationship between the metabolic and structural changes during seed development can provide insights into yield-related processes. Relationships similar to those described here for the AGP-antisense seeds have been observed previously by authors working on soybean yield physiology. In general, seed fill duration often correlates with yield (Egli, 1994). For high yield it is important to maintain a steady sink activity throughout the seed-filling period (Hanson, 1991; Jenner *et al.*, 1991). Selecting soybean genotypes for increased seed fill duration often correlates with yield (Egli, 1994). For high yield it is important to maintain a steady sink activity throughout the seed-filling period (Hanson, 1991; Jenner *et al.*, 1991). Selecting soybean genotypes for increased seed fill duration often correlates with yield (Egli, 1994). For high yield it is important to maintain a steady sink activity throughout the seed-filling period (Hanson, 1991; Jenner *et al.*, 1991). Selecting soybean genotypes for increased seed fill duration often correlates with yield (Egli, 1994).

Future prospects

In future studies it will be important to address which mechanisms control the differentiation events within developing cotyledons, the partitioning and the flux of assimilate into the different storage product classes. With this aim in mind it is necessary to analyse the mode of expression of transporters, regulatory genes and enzymes and to test their rate-limiting role either in transgenic approaches or mutants.

The availability of cDNA array filters for pea or the model legume *M. truncatula* will provide tools to analyse gene expression profiles in wild-type seed development. The pattern can then be compared with those derived from the authors’ collection of transgenic models and mutants.

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