A pea seed mutant affected in the differentiation of the embryonic epidermis is impaired in embryo growth and seed maturation

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SUMMARY

During legume seed development the epidermis of the embryos differentiates into a transfer cell layer which mediates nutrient uptake during the storage phase. This specific function of the epidermal cells is acquired at the onset of embryo maturation. We investigated this process in the pea seed mutant E2748. The epidermal cells of the mutant embryo, instead of turning into transfer cells, enlarge considerably and become vacuolated and tightly associated with adjacent seed tissues. Expression of a sucrose transporter gene that is upregulated in wild-type embryos differentiates into a transfer cell layer which represents the interface between the embryo and adjacent tissues. In maturing embryos epidermal cells are characteristically rectangular in shape and densely cytoplasmic. In contrast, underlying parenchyma cells are isodiametric, highly vacuolated and divide in both anti- and periclinal planes (Borisjuk et al., 1995). When morphogenesis and organogenesis of the young legume embryo has been accomplished the abaxial epidermal cells differentiate into transfer cells (Bonnemain et al., 1991). These are characterised by finger-like ingrowths of the cell wall at the boundary with transport-active cell surfaces and, thus, exhibit a polarity in terms of wall modification. The wall ingrowths increase the surface of the plasma membrane so as to mediate efficient short-distance transfer of solutes. Within developing seeds, transfer cells are found strategically located, in the vicinity of 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 layer of barley (Weschke et al., 2000) and in the cotyledon epidermis of V. faba and pea (Weber et al., 1997; Tegeder et al., 1999). Transfer cell formation in legume cotyledons occurs at a certain stage of development and represents a regional specialisation to improve embryo nutrition. In V. faba, transfer cells develop at areas of contact of the embryo and the seed coat. It has been suggested that this modification is associated with stimuli coming from neighbouring compartments (Weber et al., 1997). The factors

INTRODUCTION

During embryogenesis of dicot plants protoderm formation is the first recognisable stage of histogenesis. In Arabidopsis this occurs by the 5th cell division (Mansfield and Briarty, 1991). Within the protoderm the cell division plane is strictly anticlinal leaving all daughter cells in the same surface layer. This sets the protoderm as an independent cell lineage (Steeves and Sussex, 1989). The protoderm is an element of radial patterning and there are mutations known that disrupt this pattern (Mayer et al., 1991). The Arabidopsis knolle mutation causes incomplete cytokinesis and affects a syntaxin-like protein involved in vesicle transport. The mutant lacks a protoderm and is therefore defective in radial pattern formation (Lukowitz et al., 1996). Protodermal cells develop the epidermis, which includes the outer cell layer of the shoot apical meristem, and persist throughout the life cycle of the plant (Laux and Jürgens, 1997). The epidermis functions as a protective layer and is involved in solute exchange. It has a key role in development where it controls shape determination, organogenesis and organ fusion (for reviews, see Becraft, 1999; Lolle and Pruitt, 1999).

In legume seeds such as faba bean (Vicia faba) or pea (Pisum sativum) the protoderm is established during the late globular stage. Its cells divide anticlinally and form an epidermis representing the interface between the embryo and adjacent
controlling transfer cell formation are unclear but metabolic signalling may be involved. Exposure to hexose sugars induces transfer cell formation in V. faba embryos (Offler et al., 1997; Farley et al., 2000) whereas high levels of sucrose are inhibitory (Offler et al., 1997; Weber et al., 1997).

Physiological studies indicate that the control of sucrose uptake resides in these cells and is mediated by an energy-dependent H\(^+\) co-transport (McDonald et al., 1995). Accordingly, transfer cell formation is coupled with up-regulated expression of transport-related genes encoding transporters for sucrose (Harrington et al., 1997; Weber et al., 1997; Tegeder et al., 1999), hexoses (Weber et al., 1997), amino acids (Tegeder et al., 2000) and H\(^+\)-ATPases (Harrington et al., 1997). In V. faba cotyledons, increased expression of the sucrose transporter gene is accompanied by accumulation of large amounts of sucrose in the underlying tissue (Borisjuk et al., 2001). The increase in sucrose is expected to induce expression of storage-associated genes within these cells (Weber et al., 1998b). The sucrose is thought to be transferred via plasmodesmata, indicating that transfer and underlying parenchyma cells represent a symplastic domain (McDonald et al., 1995; Tegeder et al., 1999).

Cotyledonary differentiation occurs gradually starting from the inner adaxial region and progressing outwards. The first cells to differentiate increase in size and accumulate starch and storage proteins whereas the outermost cells underneath the epidermal transfer cells remain smaller and mitotically active generating additional cells to the interior (Hauxwell et al., 1990; Borisjuk et al., 1995).

Analysing seed mutants can contribute to our understanding of transfer cell formation and seed development (Wang and Hedley, 1993; Liu et al., 1996). The pea seed mutant E2748 was originally obtained by chemical mutagenesis (Johnson et al., 1994). The seeds grow slowly and abort before complete maturation. The embryo at later stages adheres to the maternal seed coat and the outermost cell-layer is enlarged and highly vacuolated. The embryo appears to lack a proper epidermis or, alternatively, the epidermal layer is abnormal. Therefore, E2748 has been classified as a cellular mutant (Johnson et al., 1994). It is not known whether the abaxial surface of the embryo becomes ontogenetically fused with the seed coat and dedifferentiates (Siegel and Verbeke, 1989) or whether adhesion occurs in the absence of the epidermis. Because transfer cell formation is also affected, assimilate supply could be impaired. The mutant provides a suitable model to study nutrient uptake into the embryo, filial-maternal interactions, as well as maturation and differentiation events in the embryo. We have found that the epidermal cells of E2748 mutant embryos cannot form transfer cells and subsequently lose epidermal cell identity at the beginning of seed maturation. As a consequence co-ordinated maturation is affected and the cotyledon tissue adopts callus-like features. Thus, the E2748 gene product is required for epidermal differentiation of developing cotyledons and indirectly for the maintenance of epidermal cell identity.

E2748 mutant seeds are lethal the plants were maintained as heterozygotes. In selfed heterozygous plants 25% of the seeds have a homozygous mutant phenotype (Johnson et al., 1994). The use of heterozygous plants allowed both mutant and wild-type seeds to be analysed from the same pod and thus provided suitable controls of the same age. To determine fresh and dry weight accumulation, seeds were weighed before and after drying, then embryos removed and weighed separately.

The volume of vacuolar fluid in the endosperm cells was determined by gradual sampling from embryo sacs of freshly harvested seeds at different developmental stages using a micro-syringe.

**Extraction and determination of sugars and starch**

Procedures were performed as described by Heim et al. (Heim et al., 1993). Briefly, soluble carbohydrates were extracted in 80% ethanol at 80°C and sucrose, glucose and fructose were determined enzymatically. The remaining insoluble material was used for starch determination after solubilisation in 1 M KOH and hydrolysis with amyloglucosidase.

**Fixation, sectioning and histochemical staining**

Seeds or parts of seeds were fixed in 2.5% glutaraldehyde, 50 mM sodium cacodylate (pH 7), or in 4% (w/v) paraformaldehyde, 50 mM potassium phosphate buffer (pH 7.0), under slight vacuum for 4 hours at room temperature, rinsed in cacodylate buffer, dehydrated and embedded in Paraplast Plus (Sherwood Medical, St. Louis, Mo., USA). Sections were cut at 1-10 μm on a Microtome, transferred on poly-L-lysine-treated slides (Sigma Diagnostics, München, Germany), and dried overnight at 45°C. Sections were stained with Toluidine Blue according to the methods of Gerlach (Gerlach, 1977) and Carson (Carson, 1990).

**Electron microscopy**

Segments of cotyledons were sliced into small pieces and immersed in a fixative of 2% paraformaldehyde, 0.5% glutaraldehyde in 50 mM potassium phosphate buffer, 5 mM EGTA pH 7.2, 5 mM CaCl\(_2\), 3% sucrose. After 2 hours at room temperature the fixation was continued overnight on ice with freshly prepared fixative. All the following steps were performed on ice. Specimens were washed in buffer twice (10 minutes each) and treated for 2 hours with 1% osmium tetroxide in buffer. The tissue was washed twice with buffer and double-distilled water, for 10 minutes each, and stained overnight in an aqueous 2% uranyl acetate solution. After washing twice with double-distilled water the specimens were dehydrated in an ethanol series, infiltrated with Spurr’s resin (Plano, Marburg, Germany) and polymerised thermally (24 hours, 70°C). Ultrathin sections were cut perpendicular to the epidermis and stained with uranyl acetate and lead citrate for ultrastructural observations.

**RNA isolation and hybridisation techniques**

RNA isolation, cDNA labelling and northern hybridisation were carried out as described previously (Heim et al., 1993). In situ hybridisation was performed according to Weber et al. (Weber et al., 1995). The following cDNA fragments were used as probes after labelling with \([^{32}P]dCTP\) or \([^{33}P]dCTP\): the complete cDNAs of V. faba SUT1 (Weber et al., 1997), VFK1 (Ache et al., 2001), ATP1 (Weber et al., 1998a), SUS1 (Heim et al., 1993), VfAGPL and pPGM (our unpublished results). Signals on filters were quantified using a phosphi-imager.

**Imaging of local sucrose concentrations**

Analysis of local sucrose concentrations in cryosections was performed by quantitative bioluminescence imaging and single photon counting. Cryosections, adhered to a cover glass, were laid upside down on a glass slide with a rectangular casting-mold. The mold was filled with a solution containing an enzymatic cocktail to link sucrose cleavage, via the oxidation of glucose-6-phosphate and
NADH+/NADPH + +H+ redox system, to the FMN-oxidoreductase/ luciferase light reaction of the marine bacterium Photobacterium fischeri (Borisjuk et al., 2002). All enzymes and coenzymes were obtained from Boehringer Mannheim (Mannheim, Germany).

Staining with carboxyfluorescein and detection of fluorescence

The fluorescent dye 5-(6) carboxyfluorescein diacetate (CFDA) was prepared according to the method of McDonald et al. (McDonald et al., 1995). Surgically isolated embryos or parts of seeds were incubated in a medium containing 100 mM sucrose, Hoagland nutrients and CFDA at a concentration of 100 µg/ml, pH 6.0. Isolated embryos of normal and mutant seeds were incubated in parallel in media with or without CFDA dye at room temperature. Embryos were then washed in fresh incubation medium without CFDA and observed under epifluorescence using a Zeiss Axiophot Photomicroscope fitted with blue excitation filter E 450-490, a F 510 chromatic beam splitter and LP 420 barrier filter. Alternatively, labelled embryos were analysed by confocal microscopy.

RESULTS

The E2748 pea seed mutation affects embryo growth during the maturation phase

Up to the onset of maturation, mutant and wild-type seeds are hardly distinguishable. Both have similar size and fresh weights. Thereafter wild-type embryos become the largest seed organ whereas mutant embryos never grow big enough to occupy the endosperm cavity (Fig. 1).

Fresh weight increase of mutant seed coats was similar to that of wild type (Fig. 2A). Only from approx. 250 mg seed weight onwards (later cotyledon stage) were values ~10-20% lower. Decreased embryo growth is compensated for by larger endospermal vacuoles leaving seed fresh weight nearly unchanged up to approx. 100 mg seed weight (Fig. 2B). At this stage morphogenesis and cell division have largely terminated and maturation has begun, characterised by rapid fresh weight gain, cell expansion and storage product synthesis. Mutant embryos grew slowly during that phase and reached only ~10% of the wild-type values (Fig. 2B).

The volumes of the endospermal vacuoles increased similarly up to ~150 mg seed weight for both wild type and mutant (Fig. 2C). Thereafter, it decreased continuously to zero in the wild type, because the space was occupied by the fast growing embryo. In the mutant, endospermal vacuoles continued to increase until the seeds reached ~250 mg.

The results indicate that in the mutant, it is predominantly embryo growth that is affected, whereas seed coat growth was nearly unchanged. Growth arrest occurred at the beginning of the seed’s maturation phase.

Retarded embryo growth is not due to limited carbohydrate supply

Reduced growth of mutant embryos suggested that nutrients could be limiting. Sugars are normally unloaded from the seed coat into the endospermal vacuole and are taken up by the embryo. We therefore measured the concentrations of sugars within the endospermal vacuole. Despite the large differences in volumes between mutant and wild type, the sugar concentration as well as the ratio between hexoses and sucrose

![Fig. 1. Hand cut sections of E2748 mutant and wild-type seeds harvested from the same pod at different developmental stages. e; embryo; v; endospermal vacuole; sc; seed coat. Scale bars: 3 mm.](image-url)

![Fig. 2. Developmental parameters of E2748 mutant and wild-type seeds. Fresh weight accumulation of seed coats (A) and embryos (B), and endospermal vacuole volumes (C). Data points represent single measurements. See also legend of Fig. 13, for staging of pea seed development.](image-url)
were not different up to ~250 mg seed weight (Fig. 3). For both mutant and wild type the initial high concentrations of hexoses (~100 mM glucose plus fructose in seeds of 50 mg) decreased to below 5 mM in seeds of 200 mg. Conversely, the relative low levels of sucrose in seeds of 50 mg (~30-40 mM) increased to ~130 mM in seeds of 200 mg. This characteristic switch in the presence of the principal sugars occurs at the onset of maturation and has been suggested to act as a metabolic control element (Weber et al., 1995).

Retarded embryo growth of the mutant was not therefore due to limiting carbohydrates. Moreover, the change in the hexose to sucrose ratio within the endospermal vacuole was also found in the mutant and is apparently independent of normal embryo growth.

**Mutant embryos are not able to accumulate normal levels of sucrose**

Embryo maturation is characterised by high concentrations of sucrose, which were similar in mutant and wild type up to ~100 mg seed weight (Fig. 4A, early cotyledon stage). Subsequently, in the wild type concentrations steadily increased to ~150 μmol/g followed by a slight decrease, but in the mutant sucrose levels began to decrease to ~60 μmol/g in the mutant embryo of 200 mg seed weight. Sucrose levels in both types of seed coats were initially higher than in embryos. Concentrations then slightly decreased in wild-type coats of 150 mg seeds onwards whereas in the mutant coats levels were ~10-20% higher (data not shown).

Starch levels in embryos were not different up to ~150 mg seed weight. Thereafter, wild-type embryos rapidly accumulated starch whereas the mutant did not and remained constantly low (Fig. 4B). Seed coats of both wild type and mutant contained similar levels of starch between 100 and 200 mg seed weight (~45 mg/g). Thereafter, levels continuously decreased in wild-type coats but were slightly higher (by ~20%) in the mutant coats of 300 mg seeds (data not shown).

Mutant embryos were therefore not able to accumulate normal levels of sucrose and starch. Slightly higher levels in mutant seed coats are probably due to the failure of the growth-arrested embryo to remobilize these products.

**Cotyledonary epidermal cells of the mutant dedifferentiate at the beginning of seed maturation**

In embryos of ~50 mg seeds epidermal cells could already be distinguished from the parenchyma cells by their anticlinal divisions and smaller size (Fig. 5A). Their subcellular structure, however, was similar to that of the underlying parenchyma cells. Nuclei were localised in the middle of rectangular cells occupying the majority of the volume, while chloroplasts, mitochondria and small vacuoles were more or less regularly distributed within the cytoplasm (Fig. 5B). Cell walls were generally thin with almost no difference between outer (facing the endospermal vacuole) and inner (facing the parenchyma) cell walls (Fig. 5C,D).

During further development epidermal cells become more vacuolated and expand mainly periclinally. Differences in size between epidermal and parenchyma cells which could barely be recognised earlier, become apparent (Fig. 5E-H). The epidermis of wild-type cotyledons remained mitotically active during mid-cotyledon stage. Cell divisions occurred equally and exclusively anticlinally (Fig. 5F). As revealed by electron microscopy (Fig. 5G) the outer cell wall was 2- to 3-fold thicker as compared to the inner one and contained some invaginations (Fig. 5H), the first morphological indication of transfer cells.

The smooth surface of wild-type embryos (260 mg seed weight) was formed by a continuous thin epidermal cell layer which covered the storage parenchyma (Fig. 5I). This layer represented a clear boundary between embryo and...
neighbouring tissue. Epidermal cells contained many small vacuoles as in earlier stages. The outer cell wall was approx. 5-fold thicker than the inner one indicating asymmetrical cell wall thickening (Fig. 5K). The cell wall had ingrowths into the cytoplasm (Fig. 5K). The walls of the inner cells had many symplasmic connections to the parenchyma via branched-type plasmodesmata (Fig. 5L). The results showed that epidermal cells of 260 mg seeds had acquired clear morphological features of transfer cells.

To determine the time point when the mutant phenotype becomes first recognizable a total of 15 embryos from heterozygous plants were examined at the heart to early cotyledon stage (up to 50 mg seeds). The probability of a seed being homozygous is 0.25, and at the level of 0.99 a sample of 15 seeds should contain at least one mutant. All embryos underwent complete morphogenesis and the parenchyma was covered by a single-cell layered epidermis. Early development and morphogenesis were therefore not affected.

A mutant phenotype could be identified from early cotyledon stage. At this stage the cotyledon epidermis in the mutant changed in certain areas, most often abaxially or at the tip (Fig. 6C,D). Some cells became more vacuolated and enlarged, forming a highly irregular morphology with a rough surface (Fig. 6C). Periclinally as well as anticlinally oriented cell walls were visible (Fig. 6D). Structural changes often, but not always, occurred in regions where the embryo was in close proximity to adjacent tissues. Mutant embryos at that stage were still freely movable within the endosperm vacuole.
Later on the modified epidermis frequently adhered to neighbouring tissues, mostly to coenocytic endosperm, and its boundary appeared indistinct (Fig. 6E). The outer cell walls (Fig. 6F) were very thin and similar to those of the inner region (Fig. 6G) indicating no morphological features of transfer cells. Because at this stage the cotyledons had both normal and abnormal epidermal morphology we termed this the transition stage. During further development (160 mg seeds) mutant embryos became tightly stuck to the maternal tissue and could not be removed without damage (Fig. 7A, this seed came from the same pod as the wild type in Fig. 5E-H). The surface layer of the cotyledons unusually became enlarged and irregularly shaped (Fig. 7A,B) and epidermal cells could not be clearly distinguished from parenchyma cells (Fig. 7A,B). Most of the cellular volume consisted of numerous vacuoles of different sizes and chloroplasts containing large starch grains, similar to storage parenchyma cells (Fig. 7B). Modified cells often had thicker walls between each other and towards the inner parenchyma (Fig. 7B,C). In 260 mg seeds, cells of the boundary further enlarged and became enormously vacuolated (Fig. 7D, this...
Pea seed mutant affecting transfer cell formation

The cell population as a whole became very heterogeneous in size, degree of vacuolisation and storage product accumulation (Fig. 7E). The histological analysis showed that mutant epidermal cells do not form transfer cells and lost their typical morphology at the beginning of maturation, instead becoming very enlarged and vacuolated. Moreover, the surface responded to contact and formed tight interactions with adjacent tissue. This indicates that mutant cells lost their epidermal character.

The gradient of cell differentiation is affected in mutant cotyledons

In the wild type the storage parenchyma is composed of large, expanded cells of increasing size towards the centre of the cotyledons (Fig. 8A). In the mutant, the outer layers of the cotyledon parenchyma at mid-maturation were composed of enlarged irregularly shaped cells whereas towards the inner region the cells were much smaller (Fig. 8B). In the wild type the starch accumulation pattern followed the cell size gradient thereby forming a gradient of maturity. Epidermal cells never contained starch grains (Fig. 8C,E). In the mutant, starch grains were frequently located within the outer layers of enlarged cells, whereas the smaller cells in the inner region contained only a few grains (Fig. 8D,F).

In summary, the loss of the epidermal cell identity was accompanied by unusual enlargement of the outermost cell layers whereas the inner cells remained small indicating that the differentiation gradient within the parenchyma was altered.

![Fig. 8. Cotyledon cell morphology and starch deposition in wild-type (A,C,E) and mutant embryos (B,D,F) at mid-cotyledon stage. The section through the abaxial region of a mutant embryo shows highly vacuolated cells (arrowhead) on the surface and in the outer tissue layers of the cotyledon, whereas cell sizes increase toward the interior (B). After iodine staining in wild-type cotyledons, starch grains are barely recognisable because of the low resolution (C). Starch deposition in the outer tissue layers of the mutant cotyledon (D). In the seed coats of both wild-type and mutant seeds starch accumulation is similar (C,D). Accumulation of starch grains during maturation coincides with the different cell size gradients in both wild-type (E) and mutant cotyledons (F). Scale bars: 125 μm (A,B); 450 μm (C,D); 200 μm (E,F).](image_url)

![Fig. 9. Transcript levels of transport- and storage-associated genes in embryos (A,C,E,G,I,K) and seed coats (B,D,F,H,J,L) of E2748 and wild-type cotyledons during development. The experiment had been repeated twice with similar results. The figure shows representative results from a single experiment. Identical blots were hybridised by sequential rounds of northern hybridisations with probes detecting the following mRNAs: sucrose transporter (PsSUT1; A,B); potassium channel (PsKT1; C,D); H+ -ATPase (PsATP1; E,F); sucrose synthase (PsSUS1; G,H); ADP glucose pyrophosphorylase large subunit (PsAGPL; I,J); plastidial phosphoglucomutase (PspPGM; K,L). The values on the x axis refer to seed fresh weight of wild-type seeds. Black symbols: mutant; white symbols: wild type.](image_url)
Transcript levels of transport-associated genes and those of the sucrose to starch pathway are lower in mutant embryos

RNA isolated from mutant and wild-type embryos and seed coats at four different stages was analysed by northern hybridisation and signals were quantified using a phospho-imager (Fig. 9). Two classes of cDNA probes were used with genes related to transport processes: sucrose transporter (VfSUT1), K+ channel (VFK1) and H+-ATPase (VfATP1) genes, as well as those of the sucrose to starch pathway: sucrose synthase (VfSUS1), large subunit of ADP glucose pyrophosphorylase (VfAGPL) and plastidic phosphoglucomutase (VfpPGM). At 40 mg seed fresh weight, when mutants could not be distinguished from the wild type, the expression levels are identical and represented by a single data point. In wild-type embryos of 150 mg seeds the mRNA levels of transport-associated proteins SUT1, VFK1 and ATP1 are much higher than in the corresponding mutant embryos (Fig. 9A,C,E). At later stages expression levels were lower in both mutant and wild type. A similar pattern was found for SUS1 and pPGM mRNAs (Fig. 9G,K). Transcript levels of AGPL in the wild type were highest later in development (200-300 mg seeds) and ~3- to 5-fold lower in the mutant cotyledons (Fig. 9K). In the seed coat the transcript levels were similar in mutant and wild type (Fig. 9B,D,H,J,L); expression of VFK1 and ATP1 were very low (Fig. 9F,H).

Taken together, the northern analysis indicates that in wild-type embryos expression of transport-associated genes and those of the sucrose to starch pathway are induced and peak at the beginning of the storage phase. Such induction was not observed in the mutant where mRNA levels either decreased or remained constantly low.

A sucrose transporter gene (Sut1) has been shown to be specifically expressed in the epidermis of *V. faba* and pea...
embryos, with upregulation in transfer cells. We used a Sut1-specific probe for in situ hybridisation experiments with both wild-type (Fig. 10A) and mutant cotyledons of ~60 mg seeds, representing the transition stage (Fig. 10D). In wild-type cotyledons Sut1-specific signals were present within the outermost layer (Fig. 10B, arrowhead, the same section after Toluidine Blue staining is shown in Fig. 10C). This indicated expression within the epidermal cell layer. In mutant cotyledons of the transition stage a similar pattern occurred. However, the signal was somewhat weaker and less regular (Fig. 10E, Toluidine stain in Fig. 10F). In a 160 mg mutant seed (Fig. 10G, Toluidine stain in Fig. 10H) stronger label intensity was present in areas with unusually enlarged cells within the outer layer (Fig. 10I, Toluidine stain in Fig. 10J) whereas it was almost absent from other regions (Fig. 10G, arrowhead). In a wild-type cotyledon of a 200 mg seed (Fig. 10K) the Sut1-specific signal was present within the outer layer with higher intensity in the abaxial region where transfer cells had been formed (Fig. 10L, arrowhead). A steep gradient of label intensity was present decreasing from the abaxial towards the interior indicating lower expression in the parenchyma cells. In a corresponding mutant seed (Fig. 10M) the Sut1-specific labelling pattern was completely irregular. Stronger label occurred within the parenchyma than in the outermost cell layer (Fig. 10N, arrowhead).

Taken together, in wild-type cotyledons Sut1 gene expression was predominantly confined to the epidermis and epidermal transfer cells. In the mutant this was only true for the early transition stage. Thereafter this regularity changed to a more patchwork-like pattern which is typical for callus tissue.

Symplasmic continuity is disturbed within mutant embryos

Parenchyma cells within the mid-region of mutant cotyledons remained smaller and cell expansion and starch accumulation occurred predominantly within the outer regions. This suggested restriction of assimilate transport. To examine symplasmic conductivity we used carboxyfluorescein diacetate (CFDA) and light and laser scanning microscopy. After entering the cell via the apoplast, CFDA is converted into the hydrophilic fluoresophore carboxyfluorescein (CF), which is membrane-impermeable and behaves as a symplasmic tracer. Wild-type and mutant embryos of ~60 mg fresh weight from the same pod were incubated in a CFDA-containing solution. After 10 minutes the fluorescent dye was visible in a continuous layer around the outer boundary of the wild type (Fig. 11A). After 1 hour the tracer was homogeneously distributed across the whole parenchyma tissue indicating complete symplasmic coupling (Fig. 11B,C). In the mutant after 10 minutes the dye had been taken up by only a fraction of parenchyma cells whereas others remained unlabelled (Fig. 11D). After 1 hour irregular patchy labelling occurred (Fig. 11E,F). Only some regions within the parenchyma showed high fluorescence (Fig. 11F) indicating that smaller symplasmic fields were present within the parenchyma.

CFDA was applied for 15 minutes followed by a one hour incubation period without tracer. Immediately after the pulse CF could be detected in both wild-type and mutant embryos (data not shown). After the one hour chase period fluorescence could not be detected in the wild type indicating that equilibration within the parenchyma occurred which diluted fluorescent signals below the level of detection. Only a faint signal was left inside the outer cells (Fig. 11G). In contrast, high labelling was observed within distinct domains of the mutant (Fig. 11H) indicating that the label remained within distinct cell groups and did not equilibrate.

To analyse possible symplasmic connections between mutant embryo and seed coat or endosperm we used seeds weighing ~200 mg in which embryos were tightly adhered to the inner layers of the seed coats. The chalazal part without injuring the embryo and the wounded seed coat surface was brought in contact to CFDA. Tracer is present within the seed coat parenchyma (sc p) and the endospermal tissue (en) but not within embryonic tissue (e) which appears as a black layer. Scale bars: 200 μm (A,B,D,E); 1 mm (C,F).

The results show that in the wild-type cotyledon the symplasmic tracer labelled all parenchyma cells and equilibrated rapidly within the parenchyma indicating high continuity. In the mutant, however, tracer was restricted to the outermost layer of the seed coat (sc p) whereas it remained within the inner layers of the seed coat. A steep gradient of label intensity was present decreasing from the abaxial towards the interior indicating lower expression in the parenchyma cells. In a corresponding mutant seed (Fig. 10M) the Sut1-specific labelling pattern was completely irregular. Stronger label occurred within the parenchyma than in the outermost cell layer (Fig. 10N, arrowhead).

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DISCUSSION

Legume embryos develop inside the seed coat without symplasmic connections to maternal tissues. Nutrients are taken up by transfer cells, which differentiate from cotyledonary epidermal cells at the onset of seed maturation. Thus, transfer cell formation represents a regional specification for assimilate uptake. The E2748 gene product is required for this process and when mutated, cotyledonary epidermis cannot form transfer cells, but instead loses epidermal cell identity which ultimately causes deregulated seed maturation and impaired embryo growth.

The E2748 mutation affects embryo but not seed coat development

Legume seed development can be divided into two phases. The first includes morphogenesis and organogenesis and is determined by mitotic activity. During the second phase embryos rapidly accumulate weight and synthesise storage products; growth occurs by cell expansion (Borisjuk et al., 1995). Embryos of the pea mutant E2748 are not altered in weight gain up to the time when morphogenesis and cell division is terminated (Fig. 2). However, growth is arrested thereafter indicating that the mutation is only associated with seed maturation.

Our results show that seed coat growth is unaffected. Owing to higher volume of the endospermal vacuole, the amounts of sugars are also higher. However, both the concentrations and the developmentally regulated change in the hexoses to sucrose ratio is unaffected (Fig. 3) allowing interesting and important conclusions to be made concerning seed coat-embryo interactions: (i) seed coat growth rate is independent of normal embryo growth and therefore seems to be genetically fixed and dependent on the maternal genotype, (ii) 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., 1996).

E2748 is required for the differentiation of epidermal into transfer cells

A preliminary study reported that E2748 embryos either lack an epidermis or, alternatively, this cell layer adopts an altered morphology (Johnson et al., 1994). We show that young embryos cannot be phenotypically classified as mutant or wild type because complete morphogenesis and organogenesis occur, an epidermis is present (Fig. 6) and no morphological changes are evident. Therefore, mutant embryos at the early stage have a functional epidermis and the E2748 mutation affects a later step in development. This type of mutation is therefore clearly different from the Arabidopsis knolle or keule which affects protoderm formation from the beginning and prevents the organisation of a radial pattern (Lukowitz et al., 1996). First changes in epidermal morphology of E2748 cotyledons occur at the stage when in the wild type the outermost cell layer differentiates into transfer cells. Instead, the mutated surface cells become rough, irregularly shaped, very expanded and vacuolated showing periclinal as well as anticlinal cell divisions and are responsive to contact. In contrast to the wild-type, the ultra-structural features of transfer cells are not present. Thus, accordingly to morphological evidence the outermost cell layer loses its epidermal cell identity.

Transport-associated genes are upregulated during transfer cell formation in faba bean and pea cotyledons (Weber et al., 1997; Harrington et al., 1997; Tegeder et al., 1999). However, in the mutant the expression of these genes is largely reduced (Fig. 9) indicating that transfer cell formation is impaired. In addition, the cell-type specific expression of the sucrose transporter changes. In wild-type cotyledons SUT1 gene expression remains confined to the epidermis and epidermal transfer cells. In the mutant such a pattern is only observed...
until the transition stage (Fig. 10). Thereafter, along with epidermal dedifferentiation, the Sut1 expression pattern changes dramatically. Even stronger labelling occurred within the parenchyma than in the outermost cell layer (Fig. 10N). The loss of the cell-type-specific expression of Sut1 in the outer cell layer of the mutant is another indication that these cells lose their epidermal character.

The sudden accumulation of sucrose immediately preceding the phase of fresh weight increase is caused by Sut1 which is upregulated in the transfer cells (Borisjuk et al., 2002). High sucrose concentration induces storage activity on both the metabolic and gene expression level (Heim et al., 1993; Weber et al., 1998b). In early E2748 mutant embryos sucrose is not increased but remains constantly low at a time when levels in wild-type embryos increase rapidly. The lower sucrose content is accompanied by lower levels of transcripts of storage-associated genes of the sucrose to starch pathway. Thus, reduced growth of mutant embryos is due to the inability to take up sugars rather than to limited carbohydrate availability. This indicates that the outermost cell layer of mutant cotyledons does not function as a sucrose uptake system.

We conclude therefore that the earliest and primary defect in the mutant is the block of differentiation of epidermal into transfer cells that occurs at the beginning of seed maturation. Because this step of differentiation cannot occur, the cells obviously cannot maintain their epidermal identity and respond by dedifferentiating into a callus-like tissue.

**Deregulated cotyledonary development is a consequence of the loss of epidermis**

Sugars are taken up by the transfer cells. Subsequent transfer to the storage parenchyma is symplasmic, mediated by pit-fields of plasmodesmata between cotyledonary cells (Tegeder et al., 1999). Developing embryos represent a single symplasmic domain (Mansfield and Briarty, 1991). The symplasmic path controls metabolite supply and distribution and represents a way to co-ordinate developmental events (Rinne and van der Schoot 1998). Only during further seedling growth are groups of cells isolated, and mosaics of symplasmic domains formed as the plant body is established (McLean et al., 1997).

A symplasmic tracer distributes rapidly within wild-type cotyledons and moves freely within the parenchyma (Fig. 11A-C) indicating high symplasmic continuity. In the mutant, the tracer is taken up only by a fraction of the cells, and hardly equilibrates (Fig. 11D-F). The loss of epidermal cell identity therefore causes restricted symplasmic conductivity in the underlying parenchyma. Evidently this affects assimilate transfer into and within cotyledons as shown by a considerably lower sucrose concentration internally (Fig. 12). In addition, cell morphology is characteristically changed. Whereas the cells within the outer layers accumulate high amounts of sucrose and starch and enlarge and vacuolise, the inner cells accumulate less sucrose and starch and remain small. Thus, the co-ordinated pattern of maturation and cell expansion (Borisjuk et al., 1995) is disturbed and the gradient of developmental maturity is altered. The cotyledonary tissue adopts properties of unorganised callus growth. These alterations seem not to be a direct effect of the mutation because enzymatic removal of the outermost cell layer alone reduced growth and was sufficient to cause disturbed differentiation of parenchyma cells similar to that seen in the mutant (unpublished results). We conclude that the E2748 cotyledons lose epidermal character and as a consequence, co-ordinated maturation of storage parenchyma cells does not occur.

Several other mutations are known that affect epidermal morphology and function (Becraft, 1999). In Arabidopsis at least nine mutated loci cause epidermal fusion. Some affect only floral fusions (Verbeke, 1992), others leaf fusions as well (Sinha, 2000; Lolle et al., 1998). Epidermal fusion is frequently due to impaired epicuticular wax synthesis. Most of the Arabidopsis fusion mutants however do not show epidermal dedifferentiation (Yephremov, 1999). The E2748 mutation must therefore affect a more profound process because it causes loss of epidermal cell identity and seed abortion. This indicates that the fusion phenotype in the E2748 cotyledons is not the primary defect but is only the consequence of the loss of the epidermis. There are some similarities between E2748 and the crinkly4 (cr4) mutant of maize which strikingly affects epidermal morphology and also causes adherence. cr4 epidermal cells are abnormally large and irregularly shaped. Cell junctions within the epidermal layer are less tight than in wild type (Becraft et al., 1996). It has been concluded that cr4 is necessary for proper epidermal differentiation. cr4 encodes a receptor kinase, implying that external signalling could induce epidermal differentiation although the signal is not known.

**Epidermal differentiation regulates response to external sugars**

It is not known what triggers the differentiation of epidermal

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**Fig. 13.** Schematic view of major histological changes in E2748 (1m–5m) and wild-type (1–5) embryos. The underlying colour indicates the sugar status from a high hexose to sucrose ratio (H>S) to a low hexose to sucrose ratio (H<S) changing during development. The E2748 mutation blocks epidermal differentiation. The mutant phenotype is not recognisable in cotyledons of the early stage (1, <50 mg seed weight). Primary differences in epidermal morphology occur during early cotyledon stage (transition from 2m to 3m, ~60 mg seed weight) when cells of the outer cell layer of the mutant become vacuolated (3m) instead forming transfer cells (3). Stages 4 (~160 mg seed weight) to 5 (~260 mg seed weight) represent the main storage phase. Development is arrested in the mutant (4m – 5m). For further details see text.
into transfer cells. There is some evidence that metabolic signalling is involved. Transfer cell formation in vitro can be promoted by hexoses but not sucrose (Offler et al., 1997; Farley et al., 2000; Weber et al., 1997). Accordingly, under in vivo conditions transfer cell formation occurs at the time when the environment in which the embryo develops is dominated by a high ratio of hexoses to sucrose (Fig. 3).

Morphological changes occur in mutant cotyledon cells at the time when sucrose becomes high in wild type. Possibly the mutant embryos, with impaired epidermis, cannot tolerate elevated levels of sucrose in their environment and its outermost cells react to sucrose with a kind of wound-response which induces callus-like growth thereby preventing coordinated cotyledon development. This hypothesis is underlined by earlier results, which showed that in vitro sucrose feeding to young embryos also induces callus-like growth. In contrast, older embryos at the mid-cotyledon stage do not respond (Weber et al., 1996) (unpublished results). Obviously the sucrose response occurs only before transfer cells have been formed whereas an intact transfer cell layer provides a barrier preventing underlying parenchyma cells from responding with callus-like growth.

In summary, we showed 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 a transfer cell layer, the cells adopt callus-like growth at a time when sucrose increases. This dedifferentiation disrupts a further coordinated development which ultimately causes seed abortion (for a summarising scheme see Fig. 13).

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