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Transfer cell induction in cotyledons of *Vicia faba* L.

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Summary. Immediately prior to seed fill, a dermal transfer cell complex, comprised of epidermal and subepidermal cells, differentiates on the abaxial surface of the cotyledons in seed of *Vicia faba*. Over the period of differentiation of this complex in vivo, the principal sugars of the seed apoplasmic sap change from hexoses, glucose and fructose, to sucrose. Cotyledons were removed from seeds before differentiation of the transfer cell complex and cultured for 6 days on an agar-based medium in the dark with their abaxial surface in contact with a medium containing either 100 mM hexoses (glucose and fructose in equimolar concentrations) or 100 mM sucrose. On both media, cotyledon growth rate was maintained throughout the culture period at, or above, that of in vivo grown cotyledons of equivalent developmental age. When cotyledons were cultured on a medium containing glucose and fructose, epidermal cells of both the ab- and adaxial surfaces developed wall ingrowths on their outer periclinal walls and their cytoplasm became dense, vesicular, and rich in mitochondria. Extensive ingrowth deposition also occurred on walls of the subepidermal cells and several rows of underlying storage cells where they abutted intercellular spaces. This latter ingrowth development was apparent on both cotyledon surfaces, but extended into more of the underlying cell layers on the abaxial surface at the funicular end of the cotyledon. In in vivo grown cotyledons, such ingrowth development is restricted to the subepidermal cells of the abaxial surface. Ingrowth morphology was commensurate with that of transfer cells of in vivo grown cotyledons. In contrast to the observed induction on a medium containing glucose and fructose, cotyledons cultured with sucrose as the sole sugar source exhibited no ingrowth deposition or small wall ingrowths in some abaxial epidermal cells. While the potential sugar signalling mechanism is unknown, this culture system offers an exciting opportunity to explore the molecular biology of transfer cell development.

Keywords: Cotyledon culture; Sugar signals; Transfer cells; Transfer cell induction; *Vicia faba*.

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Abbreviations: DAA days after anthesis; GC-MS gas chromatography and mass spectrometry; PAR photosynthetically active radiation; RGR relative growth rate; SCM standard culture medium.

Introduction

Immediately prior to seed fill, a dermal transfer cell complex comprised of epidermal and subepidermal cells (McDonald et al. 1996a) differentiates on the abaxial surface of the cotyledons in seeds of *Vicia faba* (Bonnemain et al. 1991, Johansson and Walles 1994). The wall ingrowth labyrinth of the epidermal transfer cells is extensive and polarized to their outer surface (Offler et al. 1989). In addition, small ingrowths may occur along the outer periclinal walls of the subepidermal cells where they abut intercellular spaces. This dermal transfer cell complex only differentiates on the abaxial surface of the cotyledon, that is, the surface juxtaposed to the enclosing seed coat. However, it is responsible for 80 percent of the sucrose accumulated by the cotyledons from the seed apoplasm (McDonald et al. 1996a). The significant role of these transfer cells in sucrose accumulation by the cotyledons (McDonald et al. 1996b) has been confirmed recently by the co-localization of genes or their products responsible for membrane transport of sucrose (Harrington et al. 1997). We demonstrate that the culture system described in this paper offers an exciting opportunity to study the induction and subsequent development of these transfer cells.

Developing seeds of *V. faba* have a number of features which are advantageous for studying the biolo-

gy of transfer cells. First, the cellular isolation of the filial cotyledons and maternal seed coat allows removal of cotyledons without mechanical damage. Second, transfer cell differentiation occurs over a short, definable period of seed development (Bonnemain et al. 1991) thus, cotyledons can be selected for experimentation prior to wall ingrowth deposition. Finally, the cells potentially receptive to induction form the cotyledon epidermis, thus are directly accessible to inductive agents provided in a bathing medium.

Successful protocols for growing cotyledons of grain legumes in liquid medium with or without continuous shaking have been reported (e.g., soybean, Raper et al. 1984, Egli 1990; pea, Stafford and Davies 1979, Wang et al. 1987; broad bean, Barratt and Pullen 1984, Dekhuijzen et al. 1988). However, to study induction and suppression of transfer cell development in cotyledons, a protocol using an agar-based growth medium was required to allow a specified cotyledon surface to be exposed to the medium. The work to establish this protocol for *V. faba* cotyledons is presented together with experiments which comment on regulatory agents of transfer cell development.

Material and methods

Plant material

Field bean (*Vicia faba* L. cv. Fiord) plants were grown singly in a steam-pasteurized potting mixture of 3 parts course sand and 1 part vermiculite in 17.5 cm diameter pots under glasshouse conditions (14-h photoperiod; day/night temperature, 25/16 °C). At the onset of flowering, plants were transferred to controlled environment cabinets (14-h photoperiod, 70% relative humidity and day/night temperature, 19/14 °C). Metal halide (1000 W) and incandescent lamps provided a photosynthetically active radiation (PAR) (400–700 nm) incident on the upper leaves. The PAR was stepped as follows: 0–2 h at 60; 2–4 h at 200; 4–10 h at 400 $\mu mol/m^2 \cdot s$ and thereafter the reverse sequence to give the 14-h photoperiod. A mineral supplement of full-strength Hoagland's solution was provided twice weekly at the approximate rate of 50 ml per pot. To ensure optimum pod set and subsequent seed growth, plants were pruned to four flowering axes. Each axis was decapitated above the sixth flowering node and one pod was permitted to develop at each node. Flowers were tagged and hand-pollinated at anthesis.

In vivo cotyledon growth

Fresh and dry weights were obtained for cotyledons of the distal seed of pods harvested between 5 and 37 days after anthesis (DAA). Dry weights were recorded after drying seed at 80 °C for 48 h in a forced-draught oven. The linear regression of the natural log of cotyledon dry weight against DAA was used to compute relative growth rate (RGR, g/g·d). In addition, the relationship between fresh and dry weight for cotyledons of dry weights up to 0.03 g was established (Fig. 1 C) and is fitted by the second-order polynomial equation,

$$y = 0.27x^2 + 9.14 \cdot 10^{-2}x + 1.21 \cdot 10^{-4} \quad (1)$$

where y is cotyledon dry weight (g) and x is cotyledon fresh weight (g). $r^2 = 0.986$ (adjusted for 79 degrees of freedom).

Assuming that cultured and in vivo cotyledons grow similarly, Eq. (1) was used to obtain estimates of dry weight of cotyledons prepared for histology either before (day 0 control) or after culturing (day 6). An equivalent relationship has been established and used to calculate starting dry weight for studies of growth of soybean seed in vitro (Obendorf et al. 1984). The dry weight estimates were then used to compute cotyledon RGR during culture.

Criteria for selection of cotyledons for culture

Pod and seed criteria: Pods were harvested and pod and seed length, breadth, and width (mm) were measured prior to obtaining the fresh and dry weight (g) of cotyledons from each seed. For cotyledons of up to 0.01 g dry weight, the regression coefficient was computed for the relationship between cotyledon dry weight and each pod and seed parameter.

Cotyledon criterion: Based on light and electron microscope examination of developing seeds, cotyledons which exhibited no transfer cell development had a fresh weight of up to 0.003 g (Wardini and Offler unpubl.). The corresponding dry-weight value was estimated by Eq. (1).

Culture protocol

Culture procedure

Pods (no larger than 7.0 × 7.5 mm, breadth times width) were excised with a sterile scalpel blade and placed on ice. Harvested pods were surface sterilized for 20 min in a 1% (v/v) NaOCl solution containing 0.01% (w/v) SDS with constant agitation, rinsed in sterile distilled water (three 5-min rinses) and maintained in the sterile distilled water until required. Morphologically normal seed were removed from pods using sterile techniques and the non-green cotyledons were excised from seeds of 4.5–5.5 mm in length. One cotyledon was transferred to a sterile 9-cm diameter plastic petri dish with the abaxial surface (unless otherwise stated) in contact with 25 ml of culture medium. The other cotyledon (day 0 control) was weighed and used to determine initial dry weight or fixed for histological assessment of transfer cell development. Petri dishes containing the cotyledons, were sealed with Parafilm and placed in the dark (unless specified otherwise) at 27–28 °C for 6 d. At the completion of this period, the fresh weight of the day 6 cotyledon was obtained before being used to obtain final dry weight or being fixed. A 6-place balance (Mettler Micro Balance, Zurich, Switzerland) was used for weighing all cotyledons used in the culture procedure.

Standard culture medium

The standard culture medium (SCM) was modified from that of Murashige and Skoog (1962). Changes to the Murashige and Skoog medium were as follows: Additions were $FeSO_4 \cdot 7H_2O$ (9.3 mg/l), casein hydrolysate (250 mg/l), 62.5 mM asparagine, and 0.01% citric and malic acids. Edamin, glycine, IAA, and kinetin were omitted. The osmolality of this medium was 250 mOsmol/kg. The pH of the medium was adjusted to 5.7–5.8 with 1 M KOH or HCl prior to

addition of 0.8% (w/v) agar and autoclaving at 121 °C and 18 lb/in^2 for 18 min.

Assessment of culture conditions and media components

The SCM, with 100 mM sucrose as the sole sugar source, was used in all experiments undertaken to determine the optimal culture conditions. No change in the sugar composition of the medium was detected following autoclaving (data not shown). Cotyledons were cultured in darkness for 6 d with their abaxial surface in contact with the medium. Cotyledon RGR (g/g·d) was computed at the completion of culture and compared with the in vivo RGR to assess the benefit, or otherwise, of the parameter under test.

Osmolality and sugar composition

Osmolality

Coats of seeds between 4.5 and 5.5 mm (pre-transfer cell development, 0.003 g fresh weight) and between 8 and 12 mm [post-transfer cell development, 0.07 g fresh weight (equivalent weight to 6-d cultured cotyledons)] were split in half along the integumentary fusion line in a humid environment to minimize evaporative loss of water. A microcapillary pipette was used to aspirate a 1–2 µl sample of the apoplasmic solution located between the seed coat and cotyledons. Subsamples (1 nl) of the collected apoplasmic solution were placed immediately under a layer of water-saturated paraffin oil. Solution osmolality was measured for 7 replicate seed with a nanolitre cryo-osmometer (Clifton Technical Physics, New York, NY, U.S.A.) calibrated with NaCl standards.

Sugar composition

The apoplasmic solution of seeds prior to pre-transfer cell development (4.5 and 5.5 mm, 0.003 g fresh weight) was collected by the aspiration method described above. For post-transfer cell development seeds (8 and 12 mm, 0.07 g fresh weight) collection was by centrifugation of their seed coats at 1400 **g** for 15 min. Samples were stored at –20 °C in derivatisation tubes and the sugar composition of apoplasmic solution was assayed by GC-MS.

Light and electron microscopy

Whole cotyledons were fixed on ice in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 10 mM sucrose buffered at pH 7.0 with 25 mM cacodylate/acetate. The fixative was changed three times over 4.5 h followed by three 0.5-h rinses in buffer. Tissue was then postfixed in 1% OsO_4 overnight at 4 °C. Following three 0.5-h rinses in ddH_2O, tissue was dehydrated on ice through a 10%-step graded ethanol series changed at 45-min intervals. Infiltration was at room temperature through a 20%-step graded series to 100% LR White (Polysciences, Inc., Qld., Australia) over 9 h. After daily changes of 100% resin for 6 d, the tissue was embedded in gelatin capsules by polymerization at 60 °C for 24 h.

For light microscopy, sections, 0.5 µm thick, were cut onto water with glass knives and an Ultracut Reichert microtome. Sections were stained for 1 min with 0.5% safranin and counterstained for 10 s with 1% Stevenel's blue. The sections were viewed and photographed with a Zeiss Axiophot photomicroscope. For electron microscopy, sections, 80 nm thick, were cut on a diamond knife, stained with uranyl acetate and lead citrate for 10 min each and viewed on a Jeol JEM 1200 Ex II electron microscope.

Results

Characteristics of cotyledon growth

Growth rate

The relationship between cotyledon dry weight and stage of seed development (DAA) is presented in Fig. 1 A. These data display the same growth pattern as that of larger-seeded cultivars of *V. faba* (Briarty et al. 1969, Dekhuijzen and Verkerke 1986) including cv. Coles Dwarf Prolific for which a clear correlation is reported between development of the cotyledon dermal transfer cell complex and commencement of the linear phase of seed fill (Bonnemain et al. 1991) (Fig. 1 A). This correlation suggests that transfer cell initiation occurs over a 6-d period before seed filling commences. Examination of cotyledons of this cultivar (Fiord) for transfer cell development (Wardini and Offler unpubl.) established that a dry weight of 0.0003 g was the upper limit of cotyledon weight appropriate for transfer cell induction studies. The cotyledons exhibited an in vivo RGR of 0.226 g/g·d computed from the linear regression of the natural logarithm of cotyledon dry weight against DAA (r^2 = 0.833). This RGR has been used as a benchmark for comparing growth of cultured cotyledons.

Pattern of cotyledon growth

In seeds of *V. faba*, embryo development commences at the funicular end and the cotyledons gradually expand to fill the embryo sac enclosed by the seed coat (Fig. 1 B) (see also Johansson and Walles 1994, Borisjuk et al. 1995). Differentiation of the abaxial epidermal cells to form transfer cells commences when this surface first comes into physical contact with the seed coat (Wardini and Offler unpubl.). Prior to transfer cell initiation, the cotyledons occupy approximately 70% of the embryo sac and are not in contact with the seed coat (Fig. 1 Bi). Contact between the cotyledons and seed coat commences at the end distal from the embryonic axis (Fig. 1 Bii) and is complete when the cotyledons fill the embryo sac (Fig. 1 Biii). Over the expansion phase, progressively more of the cotyledon abaxial surface comes into contact with the seed coat (compare Fig. 1 Bii to Biii). Further expansion involves both the seed coat and the cotyledons and is accompanied by thinning of the seed coat (compare Fig. 1 Biii to Biv).

Cotyledon pairs

In investigations of seed development in grain legumes by in vitro culture of the cotyledons, fre-

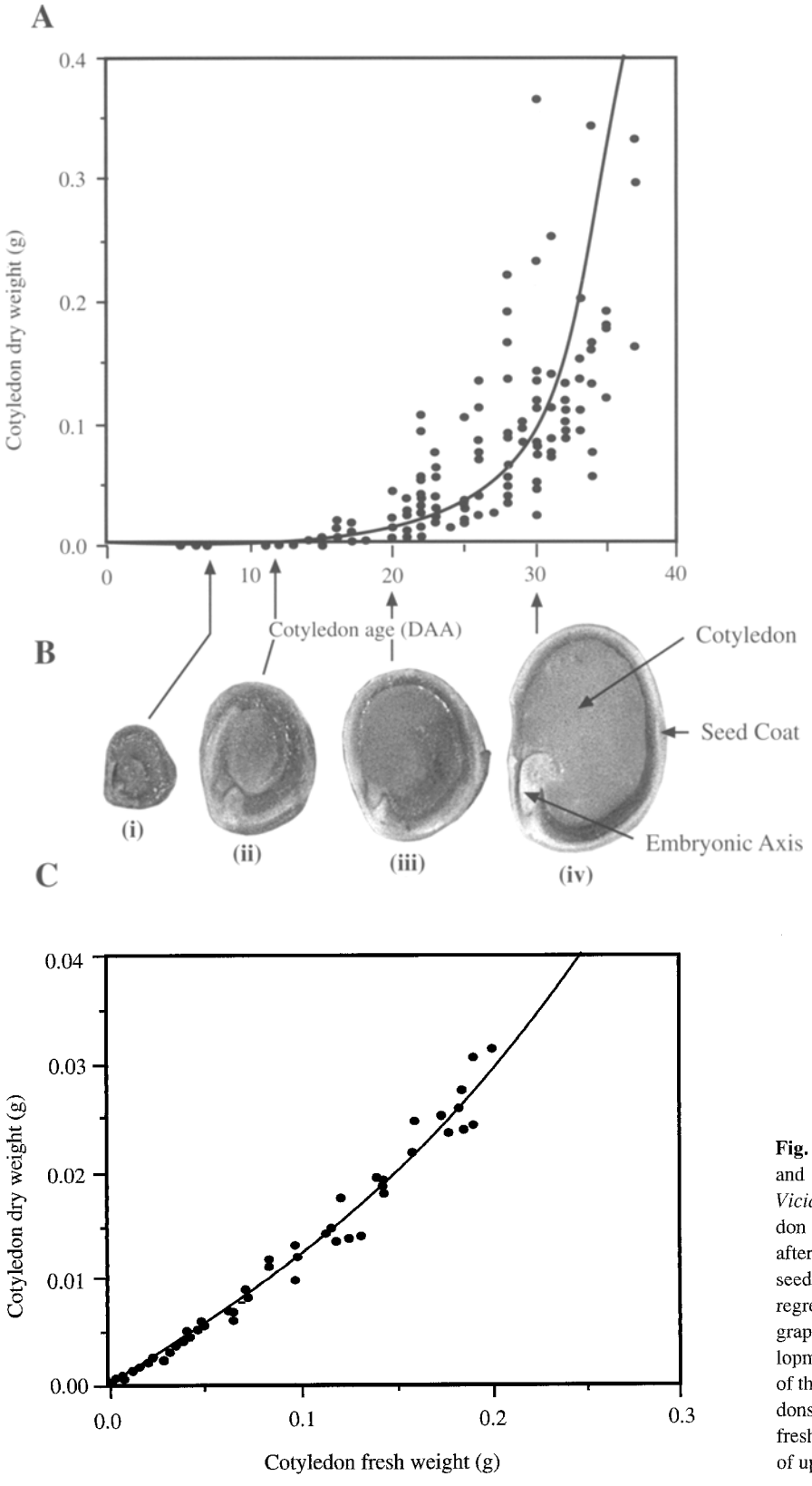


Fig. 1 A–C. Characteristics of cotyledon growth and seed morphology of developing seed of *Vicia faba*. **A** The relationship between cotyledon dry weight (g) and chronological age (days after anthesis) of seed. Data are from the distal seed sampled from 143 pods. The curvilinear regression has an r^2 value of 0.833. **B** Photographs of longitudinally dissected seed of developmental ages commensurate with development of the dermal transfer cell complex of the cotyledons. **C** The relationship between cotyledon fresh and dry weight. Data are for 80 cotyledons of up to 0.03 g dry weight

quently one cotyledon has been used to obtain initial weight data (e.g., Dekhuijzen et al. 1988, Egli 1990). The validity of this approach was verified by comparing dry weights of cotyledon pairs (weight range 0.002–0.203 g). No significant dry-weight difference was found between pairs of cotyledons ($t_{56} = 0.6499$; $p < 0.05$). Assessment of the cotyledon pairs for transfer cell development also revealed no differences (data not shown). Thus, based on this outcome, one cotyledon of each pair has been used as a day 0 control for the other member which has been subjected to the test culture conditions.

Development of a culture protocol

Selection of cotyledons for culture

To select pods containing seeds with cotyledons appropriate for culturing, regression coefficients of cotyledon dry weight (0.0003 g or less) and physical parameters of pods and seeds were determined (Table 1). These coefficients indicate that cotyledon dry weight is correlated more closely with seed rather than pod parameters with the best correlation being seed length (Table 1). Values to use as selection criteria were calculated for each correlated pod and seed parameter using the regression equations (Table 1). Pods and seeds selected by these values contained cotyledons of dry weights of up to 0.0003 g. Based on these values (Table 1), pods of 7.0 mm × 7.5 mm (breadth times width) were harvested and seeds of lengths of 5.5 mm or less were used for the final selection.

Assessment of culture parameters

To establish a culture protocol suitable for transfer cell induction experiments, both media requirements and culture conditions were assessed using in vitro versus in vivo cotyledon RGR (g/g·d). An agar-based, modified Murashige and Skoog (1962) medium (SCM) with 100 mM sucrose as the sole sugar source was used. The choice of 100 mM sucrose as the sugar source reflected the sugar composition and concentration of seed apoplasmic sap during seed fill (McDonald et al. 1996a). A 6-d culture period was selected based on the time frame of development of the abaxial epidermal transfer cells in vivo (Fig. 1 A).

The influence of light on cotyledon growth rate has been assessed previously with conflicting results (positive effect: Thompson et al. 1977, Raper et al. 1984; no effect: Dyer et al. 1987, Dekhuijzen et al. 1988). The issue was therefore revisited by culturing cotyledons with their abaxial surface in contact with the medium either under low light (fluorescent lights, PAR, 16 $\mu mol/m^2 \cdot s$; 16-h photoperiod; cf. Raper et al. 1984) or in the dark. Cotyledon RGR (Fig. 2 A) exceeded the in vivo value (0.226 g/g · d), but there was no significant treatment effect. Thus, for simplicity, to minimize condensation in the petri plates and photosynthetic effects, in all subsequent experiments cotyledons were cultured in the dark.

Cotyledon RGR over the 6-d period was found to be independent of the cotyledon surface (ab- or adaxial) in contact with the agar medium (Fig. 2 B). At least 8 cotyledons could be cultured on 25 ml of medium in a 9-cm diameter petri plate without a significant reduction in their RGR (Fig. 2 C). Further, subculturing at day 3 had no significant effect on cotyledon RGR over 6 d of culture (Fig. 2 B). However, after only 3 d in culture, the RGRs of cotyledons was significantly higher (30–50%) than that recorded after 6 d (Fig. 2 B). This result is consistent with the growth pattern reported by Dekhuijzen et al. (1988) for *V. faba* cotyledons cultured in the absence of plant growth regulators and presumably reflects the depletion of endogenous pools. The observed decline in growth rate was not however considered a detrimental feature of the culture system since 6-d RGRs exceeded in vivo rates.

Vicia faba seed tissues have been reported to release substantial amounts of phenolic substances (Fakhrai et al. 1989). In initial experiments, a brown stain, probably due to the oxidation of phenolic compounds (Chitty et al. 1994), accumulated on the surface of cotyledons and in the adjacent medium. Evaluation of the effect of organic acids (citric, malic, and ascorbic) and agar concentration (0.6 and 0.8%) on this staining and on cotyledon RGRs (data not shown) led to exclu-

Table 1. Regression coefficients of cotyledon dry weight to pod and seed parameters of *Vicia faba* and selection criterion values for each parameter

Pod or seed parameter	Regression coefficient (r^2)	Selection criterion value (mm)
Pod length	0.205	40.19
Pod breadth	0.525	6.85
Pod width	0.511	7.40
Seed length	0.823	5.50
Seed breadth	0.741	3.40
Seed width	0.796	4.49

Criterion values are based on a cotyledon dry weight of up to 0.0003 g

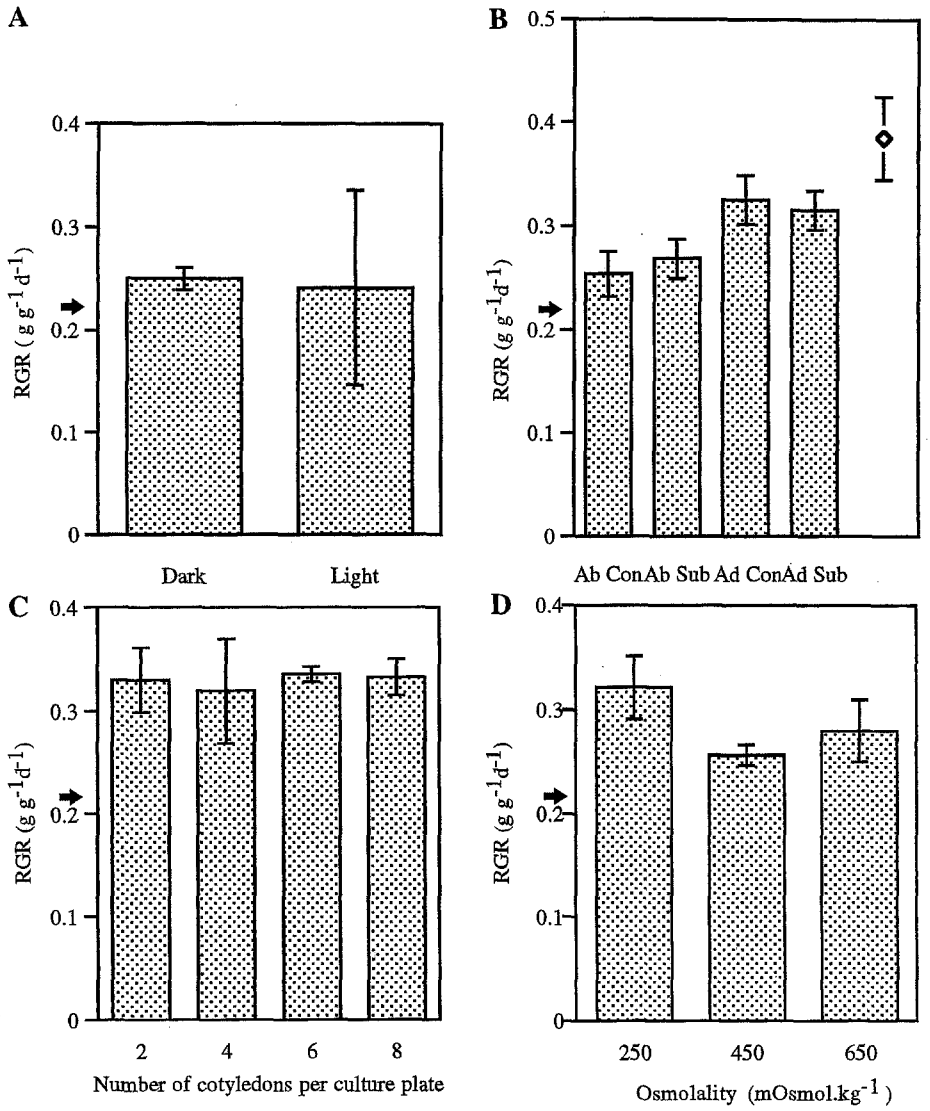


Fig. 2 A–D. The relative growth rates (RGR, g/g·d) of *Vicia faba* cotyledons selected prior to development of abaxial epidermal transfer cells (up to 0.0003 g dry weight) and cultured in the dark for 6 d with their abaxial surface in contact with an agar-based medium. One of the two cotyledons of each seed was used as a day 0 control for the other cotyledon which was cultured. **A** RGRs of cotyledons cultured in the dark or under low light (PAR 16 μmol/m^2·s; 16-h photoperiod). **B** RGRs of cotyledons with their abaxial (*Ab Con*) or adaxial (*Ad Con*) surface in contact with the medium and retained on the same plate over the 6 d of culture or subcultured at 3 d of the culture period (*Ab Sub*) and (*Ad Sub*), respectively. ◇ RGR after 3 d in culture. **C** RGRs of cotyledons cultured on plates containing 2, 4, 6, or 8 cotyledons. **D** RGRs of cotyledons cultured on media adjusted to 250, 450, or 650 mOsmol/kg with betaine. Data are means and standard errors of 8 replicate cotyledon pairs for A, 7–8 for B, and 18 for D; for C, the experiment was replicated 3 times. The RGR (0.226 g/g·d) of in vivo grown cotyledons is indicated by an arrow on the left of each panel

sion of organic acids from the SCM and retention of the use of 0.8% agar. The problem was ultimately rectified by avoiding any damage to the cotyledons during plating out.

The osmolality of the liquid endosperm of pre- and the embryo sac fluid of post-transfer cell seed (0.07 g fresh weight; equivalent size to 6-d cultured cotyledons) was analysed. The osmolality of the liquid endosperm was 595 ± 26 mOsmol/kg and for the embryo sac fluid 445 ± 28 mOsmol/kg. These values were significantly higher than that of the SCM. Thus, the effect of osmolality on cotyledon RGR was tested between osmolalities of 250 and 650 mOsmol/kg with the osmolality of the medium adjusted with betaine. The data (Fig. 2 D) indicate no significant effect of media osmolality on cotyledon RGR. Thus, for subsequent experiments, media osmolality was adjusted to 360 mOsmol/kg with betaine.

In summary, the following culture protocol was adopted for transfer cell induction experiments. One of a pair of cotyledons was cultured on an agar-based (0.8%) MS medium modified as detailed for the SCM, but with the deletion of organic acids. The sole sugar source was 100 mM sucrose with medium osmolality adjusted to 360 mOsmol/kg using betaine. Six cotyledons, with their abaxial surface in contact with the medium, were cultured per plate in darkness at 27–28 °C for 6 d. At the completion of the culture period, cotyledons were processed for histological examination and comparison with their day 0 control cotyledon. Cotyledon RGR was computed with converted fresh weights (Eq. (1)) of the day 0 control and day 6 cultured cotyledons.

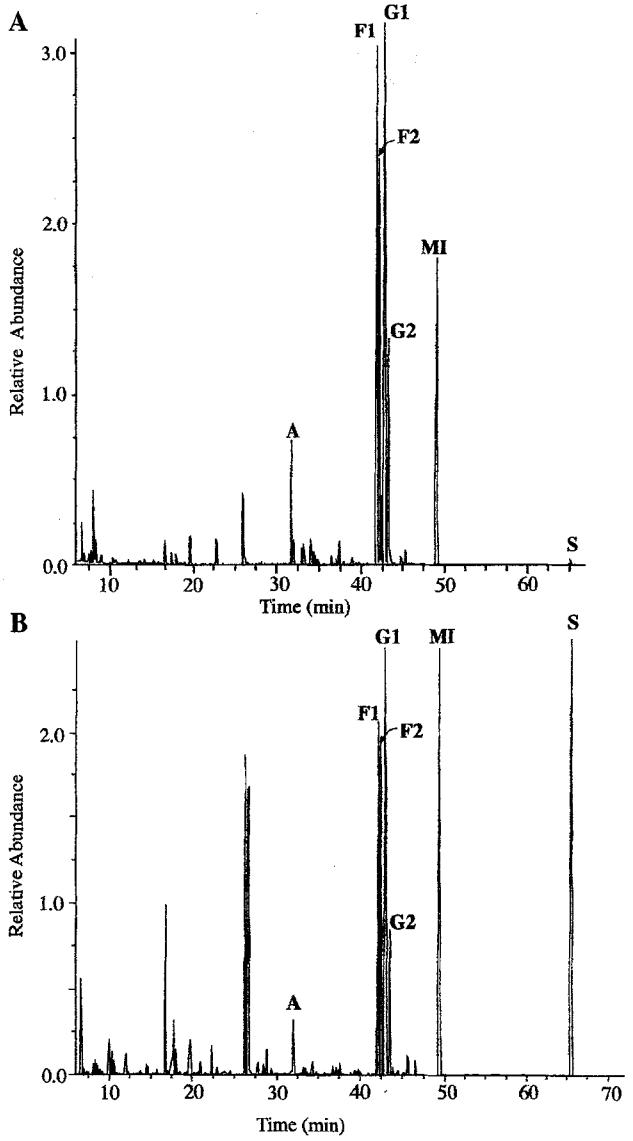


Fig. 3 A, B. Gas chromatography and mass spectrometry (GC-MS) traces of the relative abundance of glucose, fructose, and sucrose in the apoplasmic sap bathing the cotyledons of seed of *Vicia faba*. Traces are for **A** liquid endosperm of seeds prior to transfer cell development (0.003 g cotyledon fresh weight) and **B** the apoplasmic fluid post transfer cell initiation (0.07 g cotyledon fresh weight). *A* Arabinose; *F1* fructose (fraction 1); *F2* fructose (fraction 2); *G1* glucose (fraction 1), *G2* glucose (fraction 2); *MI* myo inositol; *S* sucrose

Induction/suppression of wall ingrowths in cotyledons

For early seed development in some grain legumes (e.g., *Phaseolus*; Smith 1973) and cereals (e.g., *Sorghum*; Maness and McBee 1986), the dominant sugar species of the apoplasmic sap are glucose and fructose whereas sucrose is the principal sugar during the linear phase of seed fill (Smith 1973, McDonald et al. 1995). These reports are consistent with more recent observations of the pattern of developmental expression of seed coat acid invertase and sugar concentrations reported for *Vicia* (Weber et al. 1995a).

The concurrence of changes in the sugar composition of the seed apoplasm and the initiation of transfer cell development was explored. The relative abundance of glucose, fructose, and sucrose in the pre-(liquid endosperm) and post-(apoplasmic sap) transfer cell phases of seed development is presented in Fig. 3. The contribution of sucrose to the overall composition of the apoplasmic sap in contact with the cotyle-

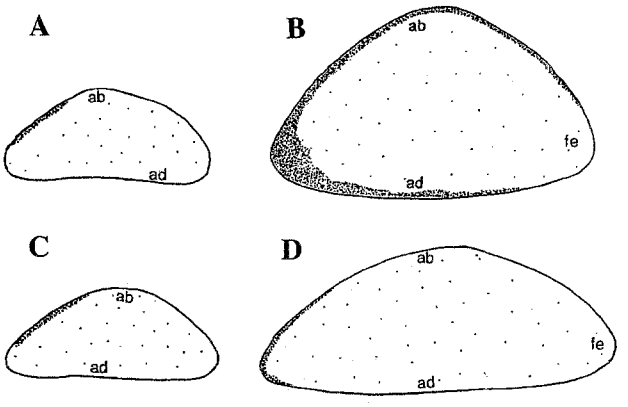
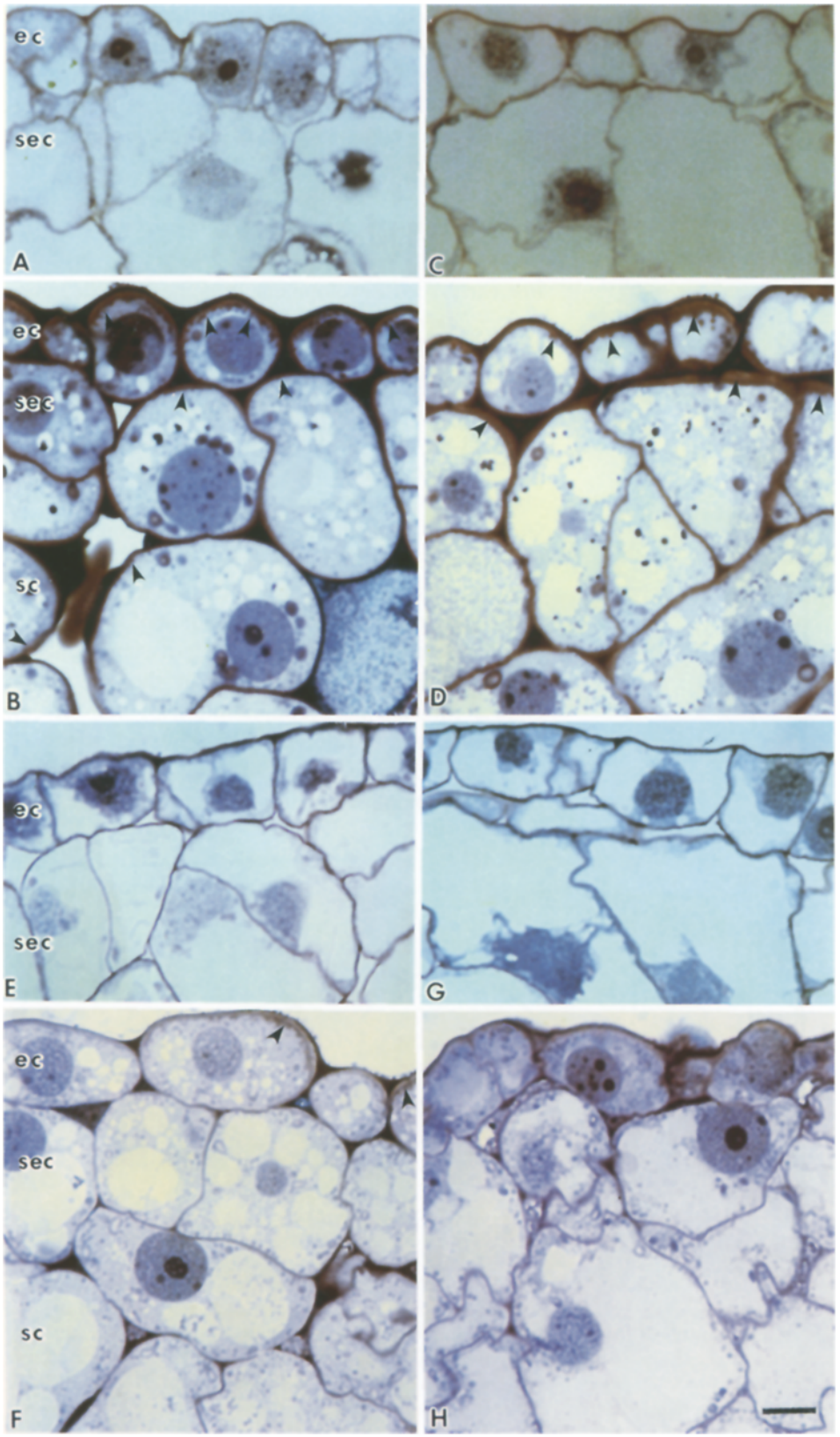


Fig. 4. Diagrams of transverse sections of *Vicia faba* cotyledons showing the distribution of cells exhibiting induction of wall ingrowth deposition following culturing for 6 d on media containing either 50 mM glucose and 50 mM fructose (**B**) or 100 mM sucrose (**D**). For each cotyledon pair, the extent of transfer cell development in the cotyledons at the commencement of culturing was obtained from the other cotyledon (**A** and **C** respectively) which was fixed immediately on removal from the seed coat. Stipling indicates regions of wall ingrowth deposition. Bar: 0.5 mm. *ab* Abaxial surface; *ad* adaxial surface; *fe* funicular end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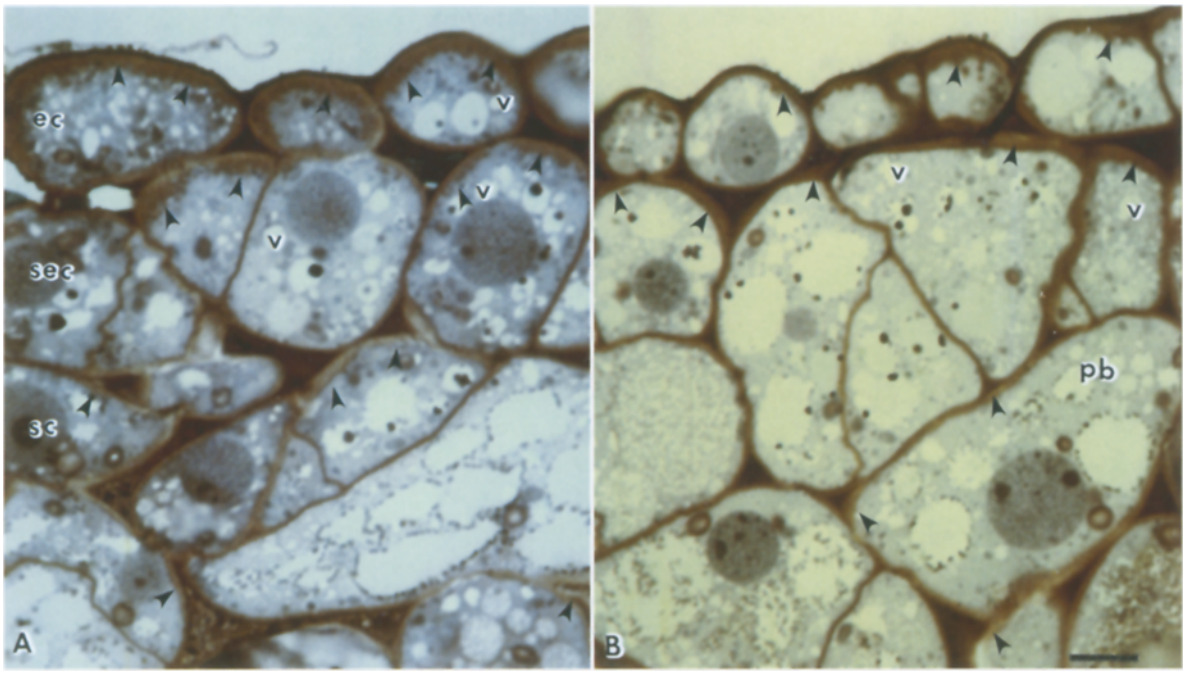


Fig. 6 A, B. Light micrographs of wall ingrowth development in cotyledons of *Vicia faba* cultured on a medium containing 50 mM glucose and 50 mM fructose for 6 d. **A** Portion of the abaxial epidermis and underlying cells distal from the funicular end of the cotyledon juxtaposed to the chalazal vein of the seed coat. Wall ingrowths (arrowheads) occur in the epidermal, subepidermal and storage cells. Note the width of the ingrowth wall of the epidermal cells in **A** compared to that in **B**. **B** Portion of the adaxial surface. Papillate wall ingrowths are indicated by arrowheads. Bar: 10 μm. *ec* Epidermal cell; *pb* protein body; *sc* storage parenchyma cell; *sec* subepidermal cell; *v* vesicle

dons increased dramatically (hexose : sucrose 100 : 1 vs. 3 : 1) during their development (compare Fig. 3 A to B). This significant shift in the relative abundance of hexoses and sucrose (see Weber et al. 1996) coincides with the initiation of transfer cell development in the cotyledons. Thus, the role of these sugars as transfer cell-inducing agents was tested.

Cotyledons were selected using the criteria detailed in Table 1. One of the cotyledons of each pair was weighed and fixed immediately to determine the extent of transfer cell development in the cotyledons at the commencement of culturing (day 0 control). The other cotyledon was cultured in medium containing either 100 mM glucose and fructose (each at 50 mM concentrations) or 100 mM sucrose. After 6 d in culture, cotyledons, together with their day 0 controls, were weighed and prepared for histological examination. The extent of wall ingrowth deposition in the cells of each cotyledon was assessed by light microscope examination of transverse sections of whole cotyledons.

Cultured cotyledons maintained RGRs commensurate

Fig. 5 A–H. Light micrographs of transverse sections of cells of the ab- and adaxial surfaces of cotyledon pairs of *Vicia faba*. One cotyledon of each pair was fixed for histological examination on removal from the seed coat (day 0) and the other following 6 d in culture (day 6). Cotyledons were cultured on a medium containing either 50 mM glucose and 50 mM fructose (B, D) or 100 mM sucrose (F, H). A, C and E, G are respective day 0 controls. **A** and **B** Portions of the abaxial surface of day 0 (**A**) and day 6 (**B**) cotyledons cultured on a medium containing glucose and fructose. Note the development during 6 d of culture of extensive wall ingrowths on the outer periclinal walls of the epidermal cells and the smaller ingrowths on sections of wall adjacent to intercellular spaces of the subepidermal and underlying storage parenchyma cells (**B**, arrowheads). Note the development of a dermal transfer cell complex which is equivalent to that formed in vivo. **C** and **D** Portions of the adaxial surface of day 0 (**C**) and day 6 (**D**) cotyledons cultured on a medium containing glucose and fructose. Wall ingrowth deposition is evident only in the epidermal cells and the ingrowths are small and papillate (arrowheads). **E** and **F** Portions of the abaxial surface of day 0 (**E**) and day 6 (**F**) cotyledons cultured on a medium containing sucrose. Note some thickening of the outer periclinal wall of the epidermal cells and the development of a few small papillate ingrowths in some epidermal cells during the 6 d of culture (**F**, arrowheads). **G** and **H** Portions of the adaxial surface of day 0 (**G**) and day 6 (**H**) cotyledons cultured on a medium containing sucrose. No wall thickening or ingrowth deposition is evident in the epidermal cells in **H** (cf. D). Bar: 10 μm. *ec* Epidermal cell; *sc* storage parenchyma cell; *sec* subepidermal cell

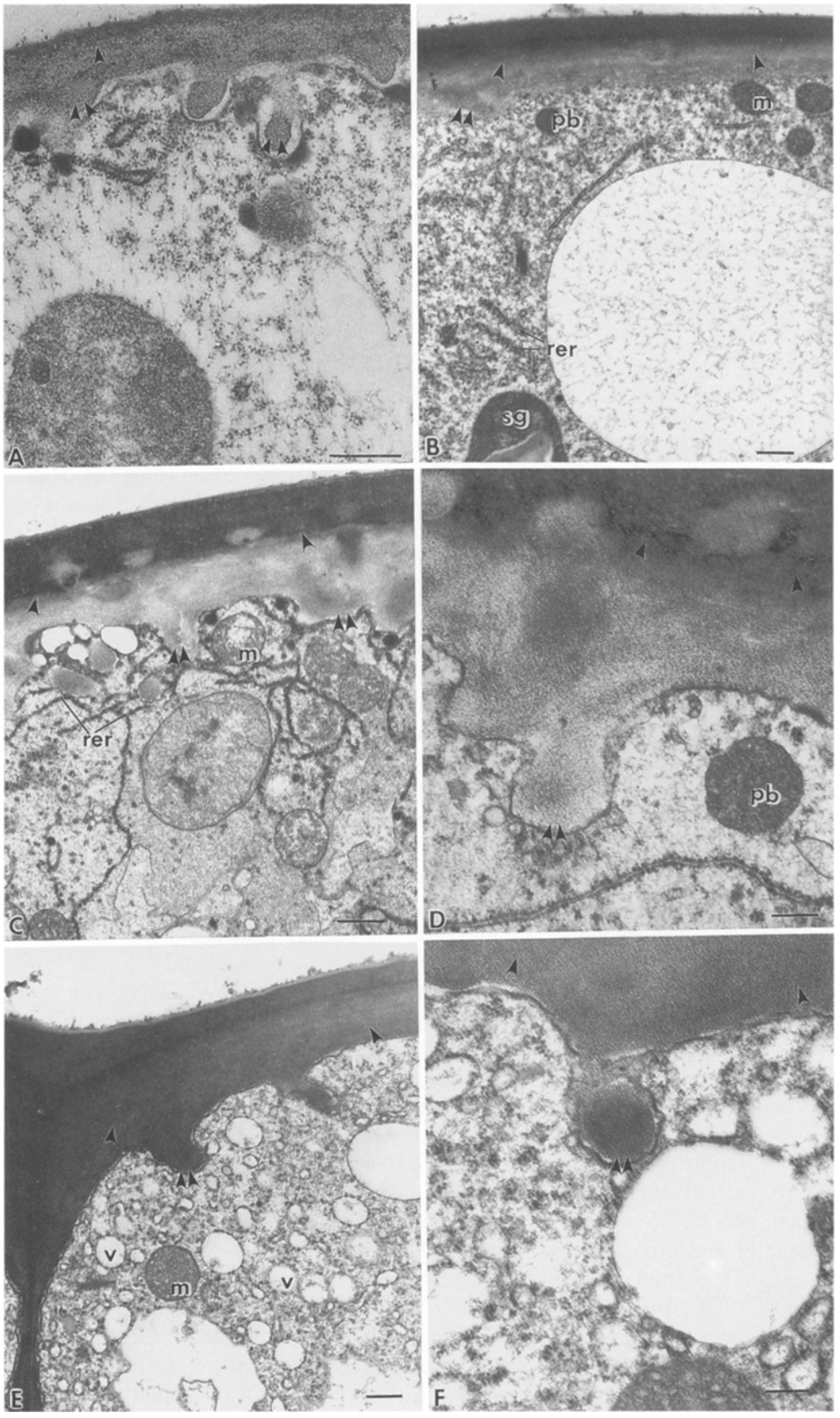
with in vivo rates, but developed an ovoid shape resulting from bowing of their adaxial surface (see Fig. 4). The relative size of day 0 and day 6 cotyledons and the extent of transfer cell development is illustrated diagrammatically in Fig. 4. The day 0 cotyledons (Fig. 4 A, C), exhibited small wall ingrowths in a few abaxial epidermal cells located distal from the funicular end of the cotyledon. By comparing day 0 and day 6 cotyledons (compare Fig. 4 B to A, and Fig. 5 B, D to A, C), it is apparent that wall ingrowth deposition was induced in the epidermal cells of both the ab- and adaxial surfaces of cotyledons cultured on medium containing glucose and fructose. The subepidermal and up to 3 rows of the underlying storage parenchyma cells of the abaxial surface also exhibited wall ingrowths (Fig. 6 A) and small ingrowths were evident in storage parenchyma cells underlying the adaxial epidermis (Fig. 6 B). Induction of wall ingrowth deposition in storage parenchyma cells was concentrated towards the cotyledon margins which in vivo underly the chalazal vein of the seed coat (Fig. 4 B).

The pattern of wall ingrowth development differed for the ab- and adaxial surfaces of the cotyledons. On the abaxial surface, wall ingrowth deposition in the epidermal and subepidermal cells closely resembled that of the epidermal transfer cell complex developed in in vivo grown cotyledons (Fig. 5 B). Ingrowths were polarized to the outer periclinal walls of cells (Figs. 5 B, D and 6 A, B), with the greatest deposition evident in the epidermal cells where an ingrowth wall labyrinth was developing (Figs. 5 B and 6 A). Where wall ingrowths occurred in subepidermal and storage parenchyma cells, they were restricted to those portions of the walls adjacent to intercellular spaces (Figs. 5 B and 6 A). In comparison, the ingrowths of the outer periclinal wall of epidermal cells of the adaxial surface were smaller and papillate (compare Fig. 6 B to A). Irrespective of their location, ingrowth walls exhibited a morphology equivalent to that observed in vivo (compare Fig. 7 C, E to A). A significant thickening of the wall had occurred from which discrete ingrowths protruded. The existence of ingrowths in storage parenchyma and adaxial epidermal cells was surprising, as no such wall development has been observed to occur in these cells in vivo (cf. Offler et al. 1989).

Cytosolic reorganization accompanied wall ingrowth deposition. In comparison with the vacuolated cells of day 0 cotyledons (Fig. 5 A), the cytosol of the abaxial epidermal and subepidermal cells of day 6 cotyledons (Fig. 5 B) was dense with extensive rough endoplasmic reticulum and numerous vesicles and mitochondria (Fig. 7 C, D). In addition, the initial stages of formation of protein bodies were evident in the subepidermal and storage parenchyma cells (Fig. 6 A). All cells had a prominent nucleus. Cytosolic reorganization had also occurred in the adaxial epidermal cells of day 6 cotyledons (compare Fig. 5 C to D), but with less development of mitochondria and endoplasmic reticulum (compare Fig.7 C to E). A difference in the level of vacuolation of the subepidermal cells of the abaxial and adaxial surfaces was also apparent (compare Fig. 6 B to A).

When cotyledons were exposed to sucrose rather than the hexoses (compare Fig. 4 D to B) minimal wall ingrowth deposition was initiated. The induction of wall ingrowths was limited to the abaxial epidermal cells and generally occurred only in cells distal to the funicular end of the cotyledon. Furthermore, in those cells exhibiting wall ingrowth induction, the extent of the ingrowth wall was substantially reduced relative to ingrowth walls of cells of cotyledons exposed to glucose and fructose (compare Fig. 5 F to B). The original outer periclinal wall of the abaxial epidermal cells had become thickened (Fig. 7 B), but only a few small discrete ingrowths protruded from this thickened wall. These small ingrowths, however, did exhibit the same morphology as those induced by exposure to glucose and fructose (Fig. 7 C–F) and

Fig. 7. Electron micrographs of wall ingrowth development on the outer periclinal walls of epidermal transfer cells of in vivo and cultured cotyledons of *Vicia faba*. Cotyledons were cultured for 6 d on media containing 50 mM glucose and 50 mM fructose (**C–F**) or 100 mM sucrose (**B**). **A** Portion of the ingrowth wall of an epidermal transfer cell from an in vivo grown cotyledon of an equivalent fresh weight to 6 d cultured cotyledons. Note the thickened wall (arrowheads) from which discrete ingrowths protrude (double arrowheads). **B** Portion of an epidermal cell from the abaxial surface of a cotyledon cultured on a medium with sucrose as the sole sugar source. Note the thickened wall (arrowheads) and small wall ingrowth (double arrowheads). The cytoplasm is dense with small lengths of rough endoplasmic reticulum, some mitochondria, and starch grains. **C** and **D** Portion of the ingrowth wall of an epidermal cell from the abaxial surface of a cotyledon cultured on a medium containing glucose and fructose (**C**) and an enlargement illustrating one ingrowth (**D**). **E** and **F** Portion of the ingrowth wall of an epidermal cell from the adaxial surface of a cotyledon cultured on a medium containing glucose and fructose (**E**) and an enlargement of one ingrowth (**F**). Bar: *in* A, B, C, and E, 0.5 μm; in D and F, 0.2 μm. *m* Mitochondria; *pb* protein body; *rer* rough endoplasmic reticulum; *sg* starch grain; *v* vesicle


A
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those of in vivo grown cotyledons (Fig. 7 A). When compared with cells of day 0 controls, the cytoplasm of these "transfer cells" was dense (compare Fig. 5 E, G to F, H) and characterized by numerous mitochondria and short lengths of RER (Fig. 7 B). Significantly, both starch and protein storage bodies had developed (Fig. 7 B). The adaxial epidermal cells also exhibited a dense cytoplasm after 6 d in culture (Fig. 5 H) but, in general, this did not appear to be accompanied by wall deposition.

Discussion

Vicia cotyledons provide a unique experimental system to study the control of transfer cell development. Differentiation of an epidermal transfer cell complex on their abaxial surface occurs within a short (6 d), defined period of seed development (Bonnemain et al. 1991). Cotyledons can be removed from the seed coat without cellular damage and their growth rates are maintained in culture (Fig. 2) (Dekhuijzen et al. 1988). We have capitalized on these features and established a protocol which enables manipulation of initiation of wall ingrowth deposition.

The culture protocol is adapted from those used previously for *Vicia* cotyledons (Barratt and Pullen 1984, Dekhuijzen et al. 1988) and accommodates culturing smaller cotyledons with a specified surface in direct contact with the medium. The agar-based medium allowed cotyledons to be specifically oriented and a matched control to be identified for each treatment cotyledon. Coincidentally, the epidermal cells of the small cotyledons were not damaged as was the case when a liquid medium with shaking was used (data not shown). The media composition and culture conditions of our protocol differ only slightly from those of Barratt and Pullen (1984) and Dekhuijzen et al. (1988). The temperature regime adopted (27–28 °C) supports optimal growth (Dekhuijzen et al. 1988); the use of sucrose as the sole sugar source is consistent with previous protocols for *Vicia* (Barratt 1984, 1986; Dekhuijzen et al. 1988), pea (Wang et al. 1987), and soybean (Raper et al. 1984), and the sucrose concentration used is in the mid-range of those reportedly used for *Vicia* (100 mM; 1–5 mM, Barratt 1984, 1986; 200 mM, Dekhuijzen et al. 1988). This protocol does differ from others in the use of betaine as an osmoticum.

The initial fresh weight of the cotyledons used in our investigations was a maximum of 0.003 g. Cotyledons of this weight were between 10 (Dekhuijzen et al. 1988) and 300 (Barratt and Pullen 1984; Barratt 1984, 1986) fold smaller than those used by other authors to study development of *V. faba* seed. When cultured, the growth of the small cotyledons followed the same pattern as that reported for larger cultured cotyledons (cf. Dekhuijzen et al. 1988) and attained a RGR above in vivo growth rates (Fig. 2). Since this RGR was unaffected by culturing up to 8 cotyledons per petri plate (Fig. 2), the formation of nutrient depletion zones around the cotyledons was clearly not an issue over the required culture period.

Transfer cell induction was effected by culturing cotyledons on a medium containing the hexoses glucose and fructose. Deposition of wall ingrowths was initiated in the cells of the epidermal cell complex on the abaxial surface of cotyledons (Fig. 5 B), the epidermal cells of the adaxial surface (Fig. 5 D) and up to 3 rows of storage parenchyma cells. Transfer cell differentiation in *Vicia* cotyledons in vivo is restricted to the abaxial epidermal transfer cell complex (Offler et al. 1989, Bonnemain et al. 1991). Deposition of wall ingrowths in other cells therefore suggests that, under the culture conditions, induction of gene regulation of processes leading to localized deposition of wall materials has occurred as opposed to triggering ingrowth deposition in pre-programmed cells. Furthermore, our observations suggest that, the restricted development of transfer cells in vivo has a physiological and not a pre-programmed genetic base. The morphology of the wall ingrowths deposited in the cells of the cultured cotyledons mirrored that of wall ingrowths of in vivo grown cotyledons (compare Fig. 7 A to C–F) substantiating the claim of induction of a "normal" sequence of gene regulation.

In contrast, when cotyledons were cultured on medium containing 100 mM sucrose, wall ingrowth deposition was substantially reduced and restricted to the epidermal cells of the abaxial surface of the cotyledon. Concurrent with this restricted wall ingrowth formation, sucrose appeared to induce starch and protein storage (Fig. 7 B). An equivalent outcome of culturing on high concentrations of sucrose has been reported previously for *Vicia* (Dekhuijzen et al. 1988, Weber et al. 1996), pea (Corke et al. 1990), and soybean (Obendorf et al. 1984) cotyledons. Those authors observed stimulated starch and protein storage, premature cessation of mitosis, and larger cells, all of which are characteristics consistent with establishing the storage phase of cotyledon growth (see also Yang et al. 1990).

The induction/suppression of wall ingrowth develop-

ment in the cotyledons in response to the sugar species presented to their abaxial surface points to sugar-responsive gene regulation of transfer cell development. There is a rapidly growing body of evidence highlighting the importance of carbohydrate-modulated gene expression in regulating sugar metabolism (Koch 1996). For example, in maize, sucrose synthase and invertase genes have been shown to be differentially expressed during development in relation to shifts in carbon allocation (Xu et al. 1996). For developing seed of *V. faba*, increasing sucrose levels are reported to stimulate sucrose synthase gene expression (Heim et al. 1993). Indeed, high levels of sucrose are consistently reported to enhance expression of genes involved in carbohydrate utilization and storage (Koch 1996). The responsiveness of cell differentiation to sugar signals has not been as well documented. Sugar/auxin balance controls differentiation of tracheids versus phloem cells (Fukuda and Komamine 1985), but to our knowledge, initiation of differentiation within a developing organ in response solely to a sugar signal has not previously been reported (see also Koch 1996). Thus, it is conceivable, and indeed likely, that during seed development, both the differentiation of the key sugar-transporting cells and cotyledon metabolism are sugar-modulated and interdependent. In vivo, across the period of differentiation of the cotyledon epidermal transfer cell complex, the principal sugar species of the seed apoplasmic sap changes from glucose and fructose to sucrose (Fig. 3) (Smith 1973, Maness and McBee 1986). Thus, the induction of transfer cell differentiation in vivo could be by developmentally-controlled upregulation of genes controlling wall ingrowth deposition or in response to changing sugar species in the apoplasmic sap. On the other hand, a high concentration of sucrose appears to concurrently suppress wall ingrowth deposition (Fig. 5) and enhance sucrose-metabolizing gene expression (Weber et al. 1995b). Conceivably, sucrose could act to down-regulate gene(s) controlling wall ingrowth deposition with the degree of down-regulation specifying the extent of wall ingrowth deposition (compare Fig. 5 F to B). Alternatively, the responsiveness to hexoses may be developmentally-programmed since pre-transfer cell cotyledons are bathed in hexoses in vivo. However, the initiation of wall ingrowth deposition in the adaxial epidermal and storage parenchyma cells of cotyledons presented with glucose and fructose (Fig. 6) suggests a sugar species-dependent response. The initiation of differentiation in cells other than those known to differentiate in vivo could also be in response to exposure to higher sugar levels as for the "feast" response of Koch (1996). These issues will be addressed in subsequent papers.

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