



RESEARCH PAPER

# Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: analysis of tobacco seed development and effects of overexpressing apoplastic invertase

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## Abstract

Wild-type tobacco (*Nicotiana tabacum* L.) seed development was characterized with respect to architecture and carbohydrate metabolism. Tobacco seeds accumulate oil and protein in the embryo, cellular endosperm and inner layer of the seed coat. They have high cell wall invertase (INV) and hexoses in early development which is typical of seeds. INV and the ratio of hexose to sucrose decline during development, switching from high hex to high suc, but not until most oil and all protein accumulation has occurred. The oil synthesis which coincides with the switch is mostly within the embryo. INV activity is greater than sucrose synthase activity throughout development, and both activities exceed the demand for carbohydrate for dry matter accumulation. To investigate the role of INV-mediated suc metabolism in oilseeds, genes for yeast INV and/or hexokinase (HK) were expressed under a seed-specific napin promoter, targeting activity to the apoplast and cytosol, respectively. Manipulating the INV pathway in an oilseed could either increase oil accumulation and sink strength, or disrupt carbohydrate metabolism, possibly through sugar-sensing, and decrease the storage function. Neither effect was found: transgenics with INV and/or HK increased 30-fold and 10-fold above wild-type levels had normal seed size and composition.

**This contrasted with dramatic effects on sugar contents in the INV lines.**

Key words: Fatty acid synthesis, hexokinase, hexose, invertase, seed development, storage product synthesis, sucrose, sugar-sensing, tobacco.

## Introduction

Factors regulating fatty acid synthesis and controlling total oil content in oilseed crops are still poorly understood (Thelen and Ohlrogge, 2002). Control of flux from acetyl CoA to lipid is shared between fatty acid synthesis and lipid assembly, with the former reactions having most control (Ramli *et al.*, 2002). The contribution of upstream reactions, from sugar entry into the seed to its conversion to acetyl CoA, has not been determined. Given that the capacity for sucrose metabolism often correlates with the sink-strength of storage organs, for example, in pea (Dejardin *et al.*, 1997), a significant degree of control over oil accumulation in seeds may lie in the pathway(s) of sucrose catabolism and entry into metabolism.

INV and HK constitute one of two means by which sucrose can enter metabolism. They may influence seed development and storage function through their direct metabolic effects, or through their involvement, direct or

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Abbreviations: CW INV, cell-wall (apoplastic) invertase; dpa, days post-anthesis; DW, dry weight; FA, fatty acid; FW, fresh weight; hex, hexose; HK, hexokinase; HP, hexose phosphate; INV, invertase; SuSy, sucrose synthase; suc, sucrose; SE, standard error; WT, wild-type.

indirect, in sugar-sensing pathways. High INV and a high hex-to-suc ratio is found in young seeds of cereals including maize (Cheng and Chourey, 1999), rice (Hirose *et al.*, 2002), and barley (Weschke *et al.*, 2000), faba beans (Weber *et al.*, 1997), and in the earliest stages of potato tuber development (Viola *et al.*, 2001). It has been proposed that in early seed and tuber development, INV activity and the resultant high hex-to-suc ratio maintain high rates of cell division. By contrast, INV and hexose appear to have little role in the provision of carbon for storage product accumulation, indeed the INV pathway may be incompatible with the establishment and maintenance of storage function in some systems (Weber *et al.*, 1995; Wobus and Weber, 1999; Weschke *et al.*, 2003). Coincident with the shift from cell-division to storage product accumulation, there is a shift from INV to SuSy-mediated sucrose metabolism, and from high hex-to-suc to high suc-to-hex in both seeds and tubers. The evidence of a causal relationship between the changes in sucrose catabolism and storage function is strongest for starch synthesis (Borisjuk *et al.*, 2003), but also exists for protein synthesis (Sanchez-Romero *et al.*, 2002). Less is known of how oil accumulation is established, and whether levels of hex, suc, or their ratio are determinants for oil synthesis in developing seeds. In the oilseeds of *Arabidopsis*, around the time that oil accumulation begins there is a minor drop and then a large increase in the hex-to-suc ratio. A second phase of accumulation is preceded by a rise then a fall in hex-to-suc ratio (Baud *et al.*, 2002). It is not clear which, if any, of these changes are controlling oil synthesis. Lack of such knowledge is hampering attempts towards the genetic engineering of oilseed metabolism (White and Benning, 2001). Although the effects of manipulating invertase expression have been tested on various sink organs (Dickinson *et al.*, 1991; Weber *et al.*, 1998; Tang *et al.*, 1999; Fernie *et al.*, 2000; Neubohn *et al.*, 2000), no such work on an oilseed has yet been reported. To study the role of INV in oilseed development and metabolism, INV in tobacco seed was over-expressed and it was targeted to the apoplast. The INV believed to be important in development of starchy seeds and tubers is apoplastic (acidic, cell-wall invertase). The napin promoter was chosen in order to achieve expression throughout the storage period (Demoor, 1992) in both embryo and endosperm (Scarafoni *et al.*, 2001) and also in the maternal seed coat (Stalberg *et al.*, 1993). The use of INV from yeast avoided the potential for a suppression of activity by the INV inhibitor, which is present in tobacco but does not affect yeast INV (Greiner *et al.*, 1998).

Although there has been much work on manipulating quality and quantity in oilseeds, with some success (Thelen and Ohlrogge, 2002), there has been no attempt to increase flux into oil synthesis by increasing the capacity for sucrose breakdown and entry into metabolism. If the predominant sacrolytic activity is invertase, hexose phosphorylation is essential for sucrose utilization, and hexokinase may

therefore have high control over oil synthesis. Alternatively, hexokinase may influence oil synthesis via sugar-sensing and the control of seed development. There is no definitive evidence for a direct role in signalling (Halford *et al.*, 1999), but with such a central role in sugar metabolism, manipulation of hexokinase may influence seed storage processes indirectly. Therefore HK was over-expressed, alone and in combination with INV, under the same napin promoter.

To interpret any impacts of manipulating INV and/or HK, it is important to characterize the carbohydrate metabolism of the wild-type developing seed. To determine whether tobacco seed development follows the model of switches in sugar metabolism described above, the developmental changes in sugars and enzymes were determined, and these were compared with the patterns of seed development and storage product accumulation.

## Materials and methods

### Construction of transformation vectors

Standard recombinant techniques were used to replace the duplicated-enhancer CaMV 35S promoter of pBI-525 (Datla *et al.*, 1993) with the promoter from the BngNAP1 napin gene (Basczynski and Fallis, 1989; provided by C Basczynski, Pioneer Hi-Bred). The resulting plasmid, pNAP-525, contained the BngNAP1 napin promoter fused to the alfalfa mosaic virus (AMV) translational enhancer and a multiple cloning site separating the promoter from the nopaline synthase (nos) terminator region. The yeast invertase gene (*suc2*) fused to a 0.23 kb fragment encoding the N-terminal region of potato proteinase inhibitor II (PI) and the octopine synthase (ocs) terminator (von Schaeuwen *et al.*, 1990; kindly provided by L Willmitzer) was cloned into pNAP-525. Cloning of the PI/yeast invertase construct was facilitated by incorporating an *NcoI* site at the start codon of the PI sequence using PCR. The 3.2 kb *HindIII*–*EcoRI* fragment containing the napin-AMV promoter fused to the PI/yeast invertase construct was subsequently subcloned from pNAP-525 into the binary vector pRD400 (Datla *et al.*, 1992) to form the vector pSMPI-INV.

Yeast hexokinase A (Stachelek *et al.*, 1986) was amplified by PCR from pRB-142 (kindly provided by D Botstein, Stanford University). The primers used for amplification incorporated an *NcoI* site at the start codon of the hexokinase coding sequence and a *BamHI* site immediately following the termination codon to facilitate cloning into pNAP-525. Using standard recombinant techniques, a *HindIII* site was introduced 3' to the nos terminator to enable the subsequent cloning of the hexokinase expression cassette. The expression cassette comprising the napin promoter, the AMV translational enhancer, the hexokinase A coding sequence, and the nos terminator was cloned into the *HindIII* site of pSMPI-INV to form the vector pHEX-INV. The hexokinase expression cassette was also cloned into the binary vector pBS:hph (provided by R Datla, Plant Biotechnology Institute; unpublished) to create pHEX:hph.

### Plant growth and transformation

*Nicotiana tabacum* cv. Petit Havana, SR1 plantlets were germinated and grown *in vitro* and leaf discs were transformed with *Agrobacterium tumefaciens* as described previously (Miki *et al.*, 1993). Transgenic lines were transferred to a greenhouse and allowed to grow to maturity in 8" pots filled with a potting mix consisting of soil:Greenworld Original Grower Mix (Mount Elgin, ON, Canada): sand:perlite (1:3:1:1 by vol.) with a small amount of dolomite lime.

The plants were treated weekly with fertilizer (20:20:20 by vol.) and Hoaglands 2 solution. On flowering, bags were placed over the individual plants to prevent cross-pollination. Seeds were collected and stored at room temperature until analysed.

#### Fatty acid analysis

The moisture content of fresh seeds was determined by weighing samples before and after overnight drying at 70 °C in a desiccator. Seed lipids were extracted by grinding in a glass homogenizer using the hexane-isopropanol procedure (Hara and Radin, 1978) adapted for plant material (Pomeroy *et al.*, 1991). Fatty acid methyl esters were prepared in 1.5 M methanolic HCl (Supelco, Bellefonte, PA) with pentadecanoic acid as an internal standard (Pomeroy *et al.*, 1991). GLC analysis was performed on a Hewlett Packard 5880 gas chromatograph with a 15 m×0.25 mm Bonded FSOT capillary column (Alltech Associates, Deerfield, IL) at 180 °C and an He flow rate of 1 ml min<sup>-1</sup> (Pomeroy *et al.*, 1991).

#### Carbohydrate analysis

The residue from lipid analysis was dispersed in 10 ml of water and heated in a boiling water bath for 5 min then filtered through a 0.45 µm filter attached to a syringe. Quantitative carbohydrate analysis was carried out by HPLC with a Carbo-Pak PA1 column (4.6×250 mm) (Dionex, Sunnyvale, CA) fitted with a Carbo-Pak PA1 pre-column (4.6×36 mm). Samples were injected from a Waters model 717 auto injector (Waters Ltd, Mississauga, ON) and eluted sugars were detected with a Waters model 464 pulsed amperometric detector. The carbohydrates were eluted using a ternary mobile phase consisting of 1 M NaOH (A), water (B), and 1 M NaOOCCH<sub>3</sub> (C) flowing at 1 ml min<sup>-1</sup> programmed as follows: A=15% throughout; at initial equilibrium B=83%, C=2% which was maintained for 2 min then B was reduced to 81% and C increased to 4% linearly over 18 min to elute all oligosaccharides. The column was flushed with 35% B and 50% C over 6 min before returning to the starting conditions. A standard mixture of glucose, fructose, sucrose, and raffinose (Sigma) was prepared from stock solutions and used for calibration and as an external standard for determining elution volumes.

#### Protein determination

Total seed protein levels for individual transgenic lines was determined by Kjeldahl analysis. Seeds samples were ground in a Udy Cyclone Sample Mill (Fort Collins, CO) and dried for 1 h at 140 °C. Kjeldahl analysis was performed on a Kjeltec Auto 1030 Analyser (Tecator, Hoganas, Sweden) according to the manufacturers instructions.

The developmental profile of protein accumulation was determined using the BioRad Protein Assay (Hercules, CA).

#### Extraction of seed for enzyme assays

Seed (0.2 g) was ground to a powder under liquid N<sub>2</sub> in a mortar. One millilitre of extraction buffer was added: 100 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES)-KOH pH 7.4, 5 mM MgCl<sub>2</sub>; 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulphonyl-fluoride (PMSF); 5 mM dithiothreitol (DTT), 1 ml l-1 Triton X-100, 200 ml l-1 glycerol, and 5 mM thiourea. The powder was ground further while the extraction buffer thawed, and the resultant extract was transferred to a close-fitting all-glass homogenizer. The mortar was washed with 0.2 ml extraction buffer and this was transferred to the homogenizer. The extract was homogenized for 3 min, then transferred to an Eppendorf tube. The homogenizer was washed with 0.2 ml buffer and this was added to the rest. The extract was centrifuged at 14 000 g at 4 °C for 3 min.

The supernatant was removed and retained. The pellet was resuspended in 0.5 ml buffer, then centrifuged as above. This second

supernatant was added to the first. The pellet was resuspended in 1.8 ml buffer. For assays on total extract, the supernatant and pellet fractions were combined in equal volumes.

#### Enzyme assays

All metabolites and enzymes were supplied by Roche. All assays were performed at optimum pH, and checked for linearity with time and extract volume.

#### Invertase

Extract (20 µl of supernatant or pellet fractions, for soluble and insoluble activity, respectively) was added on ice to 180 µl assay mix containing 0.1 M sucrose; and either 50 mM Bicine-KOH (*N,N*-bis[2-hydroxyethyl]glycine) pH 7.6 (alkaline invertase) or 50 mM sodium acetate at pH 4.3 or pH 4.7 for soluble and insoluble acid invertase, respectively. At time zero, assays were transferred to 30 °C for 1 h, then to 85 °C for 3 min. Time zero controls were included, in which the incubation at 30 °C was omitted. Prior to heating at 85 °C, assays and controls for acid invertase were alkalized by the addition of 30 µl 1 M TRIS-HCl pH 8. Glucose assays were then performed. Seventy microlitres of assay was added to 190 µl fructose assay mix (100 mM HEPES-KOH pH 7.4, 2.25 mM MgCl<sub>2</sub>, 1.1 mM ATP, 0.2 U hexokinase, and 1.1 mM NADP). Production of G6P from glucose was determined from the increase in absorbance at 340 nm and upon the addition of 0.2 U NADP-dependent G6P dehydrogenase.

#### Sucrose synthase

Extract (20 µl of total extract) was added on ice to 180 µl of assay mix containing 4 mM uridine diphosphate (UDP), 0.2 M sucrose, and either 100 mM HEPES-KOH pH 7 or 30 mM MES (2-[*N*-morpholino]ethanesulphonic acid) plus 30 mM BTP (1,3-bis[tris(hydroxymethyl)methylamino]-propane) pH 5.7. Controls lacked UDP. At time zero, all assays were transferred to 30 °C for 30 min. Reactions were stopped by transfer to 85 °C for 3 min. Fructose assays were then performed, which were identical to the glucose assays for invertase (above), except for the addition of 0.2 U phosphoglucose isomerase (PGI) to the assay mix.

#### Hexokinase

Extract (15 µl of total extract) was incubated at 30 °C for 20 min, in a final volume of 300 µl containing 50 mM HEPES-KOH pH 8, 2.5 mM MgCl<sub>2</sub>; 1.5 mM ATP, 5 mM glucose (glucokinase, GK), or fructose (fructokinase, FK). Controls lacked ATP or hexose. The reaction was stopped by heating at 85 °C for 3 min. Assays were then performed for G6P or F6P, which were identical to the above assays of glucose or fructose, respectively, except that they lacked ATP and hexokinase. There was no significant loss of hexose phosphate during the initial incubation: in control experiments, the G6P added to the assays prior to incubation was fully recovered.

#### Phosphofructokinase, ATP-dependent (PFK)

Extract (5–15 µl of total extract) was incubated at 30 °C for 30 min, in a final volume of 500 µl containing 70 mM MOPS-KOH (3-[*N*-morpholino]propanesulphonic acid) pH 7.5, 2 mM MgCl<sub>2</sub>, 10 mM F6P, and 1 mM ATP. Control reactions lacked ATP. The reactions were stopped by adding 30 µl of 100 ml l-1 perchloric acid, then brought to approximately neutral pH by adding 1 M KH<sub>2</sub>CO<sub>3</sub> (approximately 30 µl, checked by pH paper), and left on ice for at least 5 min. The precipitate was removed by centrifugation at 14 000 g at 4 °C for 5 min, and fructose-1,6-bisphosphate (FBP) assays were performed on the supernatant. FBP assays contained 400 µl of supernatant and 500 µl of assay mix: 70 mM MOPS-KOH pH 7.5, 2 mM MgCl<sub>2</sub>, and 0.4 mM NADH. Production of glycerol from FBP was determined from the increase in absorbance at 340 nm upon the addition of 0.18 U aldolase, 0.5 U glycerol 3-phosphate



dehydrogenase, and 5.5 U triosephosphate isomerase. There was no significant loss of FBP during the initial incubation: in control experiments, the FBP added to the assays prior to incubation was fully recovered.

#### *Phosphofructokinase, PPI-dependent (PFP)*

Assays (on 2–6  $\mu$ l of total extract) were identical to those for PFK, except that 15  $\mu$ M fructose-2,6-bisphosphate was added, and 1.5 mM inorganic pyrophosphate (PPI) replaced ATP. Controls lacked PPI.

## Results

### *Morphology of tobacco seed during development*

Tobacco seed consist of a seed coat, endosperm, and embryo. The seed coat consists of three different cell layer types. The outermost layer of large epidermal cells is one cell thick. The inner cell walls are quickly lignified between 8–10 dpa (Fig. 1a, b) and forms a strong shell around the developing seed by 14–16 dpa (Figs 1c, 3). This establishes the seed size and characteristic surface texture. The cells do not contain protein and oil bodies. The middle layer of thin-walled parenchyma cells connects to the vasculature supply to the seed at the funiculus, at which the vasculature bundles end (Fig. 2). This layer is about three cells thick and does not accumulate protein or oil bodies. The cells collapse as the embryo develops and expands within the seed, between 14 dpa and 20 dpa (Figs 1c, 3). The innermost layer of the seed coat is 1–2 cells in thickness and is rich in protein and oil bodies by 12 dpa (Figs 1c, 4a). The inner cell wall is thick and separates the seed coat from the endosperm. There is also a thin secondary cell wall separating these organs (Fig. 4a).

The endosperm surrounds the developing embryo, but is separated from it by a large apoplastic space (Figs 3, 4b). It consists of 3–4 homogeneous layers of cells. These accumulate large amounts of protein and oil bodies throughout

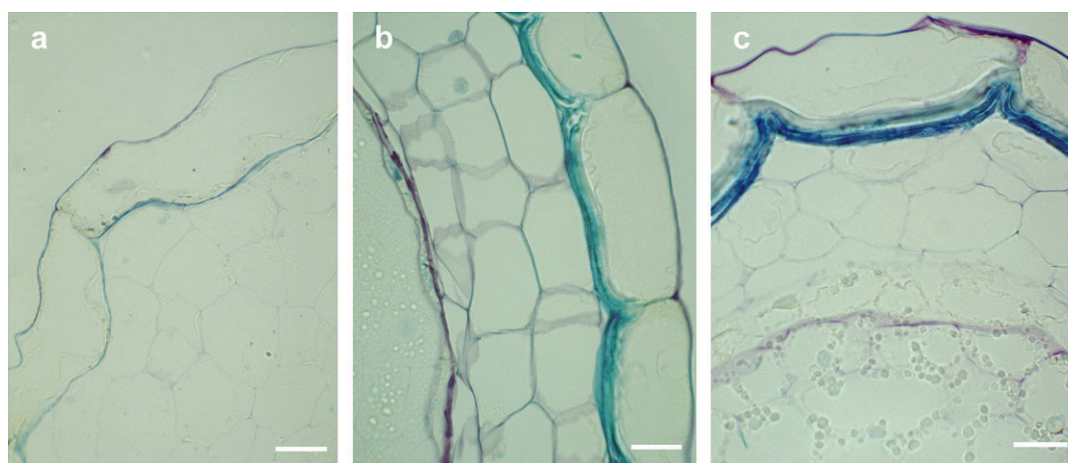
development, beginning at about 10 dpa. In tobacco, the cellular endosperm persists throughout development.

The developing embryo reaches the cotyledon stage by about 14–16 dpa and thereafter accumulates large amounts of protein and oil bodies. These protein and oil bodies are much smaller in size than those in the endosperm and accumulate at a slower rate than in the endosperm (Fig. 4b). The embryo is the smaller of the two tissues: an estimation of volumes at 20 dpa from images such as that in Fig. 3 suggest that the embryo volume is approximately half that of the endosperm.

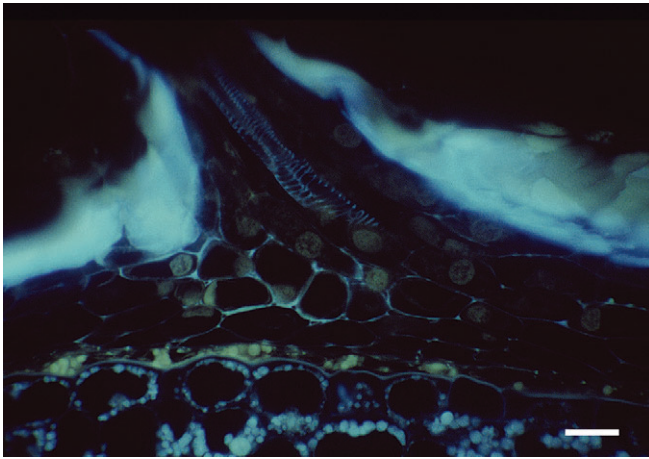
### *Tobacco seed growth and storage product accumulation*

Fresh weight accumulation, representing seed expansion, occurs primarily between 4 and 8 d post-anthesis (Fig. 5a). At this stage, hexose and sucrose are high, on a fresh weight basis, and hexose predominates over sucrose (Fig. 5c). Accumulation of other dry matter (dry matter excluding fatty acids, protein, and simple sugars, i.e. predominantly cell wall material), is maximal during and immediately after this stage. Total dry matter increases further until 20 dpa, while fresh weight is relatively constant. The storage products of tobacco seeds are predominantly fatty acids and protein. These account for most of the dry matter accumulation during mid-development (Fig. 5b) and account for approximately 40% and 20% of DW at maturity. At none of the stages analysed was any starch detectable by microscopy.

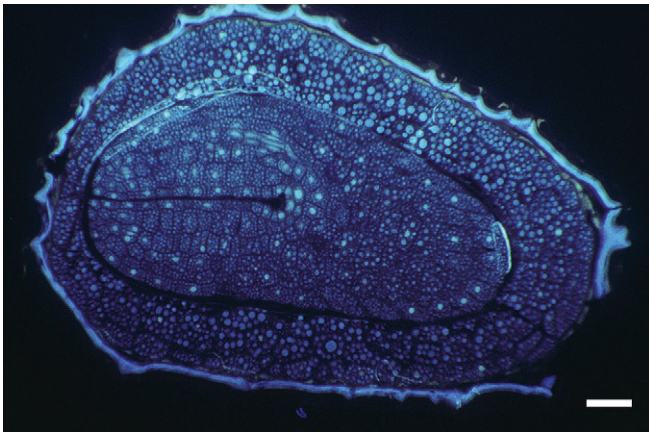
Lipid accumulation begins around 8 dpa, when the overall concentration of simple sugars has declined, but hexose still predominates. This period corresponds to the visible accumulation of oil bodies in the seed coat and endosperm. Accumulation declines between 12 dpa and 16 dpa, but then increases again, visible as oil body



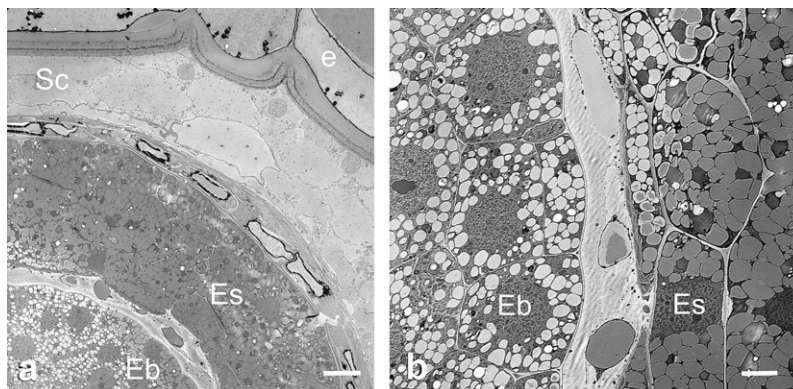
**Fig. 1.** Anatomy of developing tobacco seed coats. Toluidine-blue stained sections of 8 dpa seeds showing thin-walled parenchyma cells (inner) and a single layer of epidermal cells (outer) initiating lignin deposition on their inner cell wall (a); 10 dpa seeds showing accumulation of lignin on the inner wall of the epidermal cells (b); and 14 dpa seeds showing compression of the thin-walled parenchyma cells below the lignified epidermal cell wall. Below these cells, the innermost layer of the seed coat shows the accumulation of storage bodies above the cellular endosperm (of which three cell layers are visible) (c). Bars represent 20  $\mu$ m.



**Fig. 2.** Autofluorescence of glutaraldehyde-fixed 18 dpa seeds showing the vascular connection (upper, central) to the thin-walled parenchyma cell layer of the seed coat (upper, transverse). Bar represents 10  $\mu\text{m}$ .



**Fig. 3.** Autofluorescence of glutaraldehyde-fixed 20 dpa seeds showing the organization of the seed organs: the compressed seed coat outer to the endosperm (persistent throughout development), which surrounds the embryo. Bar represents 50  $\mu\text{m}$ .



**Fig. 4.** Electron micrographs of 12 dpa seeds showing the composition of the seed organs, the embryo (Eb), endosperm (Es), seedcoat (Sc), and epidermis (e) (a); the differences between the developing embryo (left) and endosperm (right) separated by a large apoplastic space (b). Bars represent 15  $\mu\text{m}$  (a) and 5  $\mu\text{m}$  (b).

accumulation in the embryo. Sucrose and hexose both accumulate immediately prior to this second phase of oil synthesis, which occurs between 16 dpa and 20 dpa. Sugars also change in this time: suc increases and hexoses decrease dramatically, resulting in a switch from high hex to suc to high suc to hex. Maturation processes also occur after 16 dpa: raffinose accumulates, and water content drops dramatically, from 45% to 27% of fresh weight and from approximately 63  $\mu\text{g}$  per seed to 37  $\mu\text{g}$  per seed. Maturation continues after 20 dpa until 30 dpa, during which time high sugars are maintained (representing  $\sim 2\%$  DW at maturity), hexose content declines to that of raffinose and there is significant loss of dry matter including oil and protein (Fig. 5). Hex, suc, and raffinose were the major sugars in the seed; other sugars were below the limit of detection.

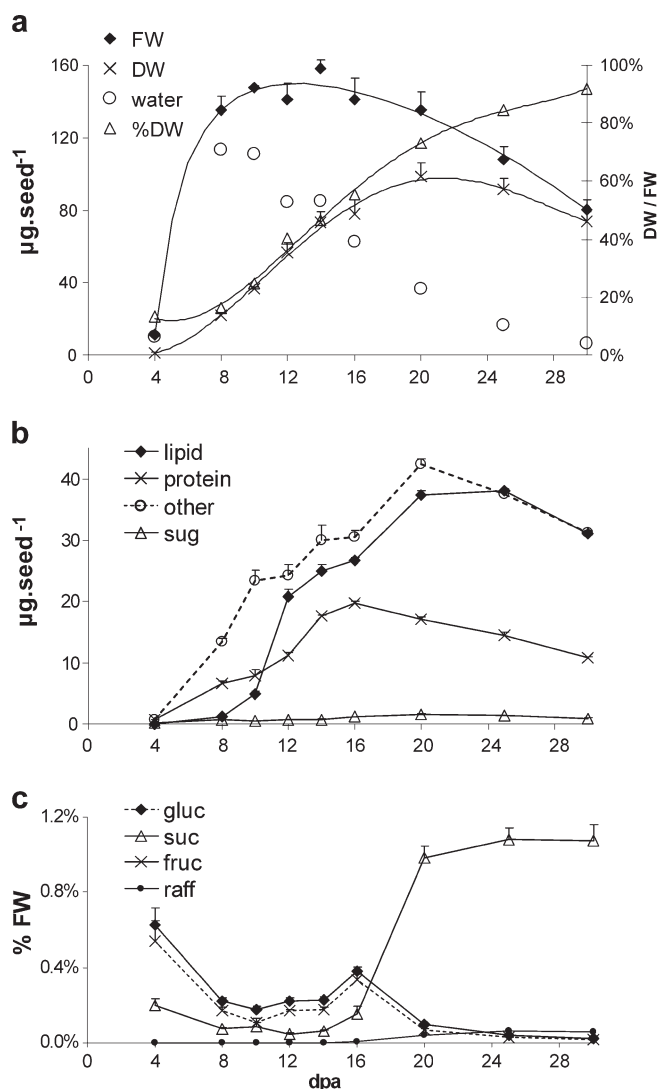
#### *Fatty acid composition*

The proportion of each major fatty acid type, as  $\text{mg g}^{-1}$  of total fatty acid, was determined during development (Table 1). Composition shifted significantly during the first half of fatty acid accumulation, and little thereafter.

#### *Carbon supply for storage product synthesis*

Entry of sucrose into plant metabolism is initiated by either sucrose synthase (SuSy) ( $\text{suc} + \text{UDP} \rightarrow \text{fruc} + \text{UDPG}$ ) or INV ( $\text{suc} \rightarrow \text{fruc} + \text{gluc}$ ). Phosphorylation of the resulting hexose requires fructokinase (SuSy pathway) or fructokinase and/or hexokinase (INV pathway). To investigate the roles of these enzymes in supply and regulation of storage product synthesis, their activities were measured over development, and compared them with the carbon demand of oil synthesis. Carbon demands during intervals in oil accumulation were calculated from the changes in oil per seed per day between the data points on Fig. 6b. The rates were converted from oil accumulation to hexose consumption on the basis that (i) oil synthesis requires approximately 1.4 carbon units from hexose per carbon in end-product (determined empirically and theoretically; Cannell and





**Fig. 5.** Wild-type seed composition during development. Fresh (FW) and dry (DW) weights per seed (a); weights per seed of the major components, lipid, protein, sugar, and other unidentified DW (b); content, as a percentage of FW, of the major sugars (c). No other sugars were above the limit of detection. Components were quantified as described in the Materials and methods. Error bars represent standard errors, of 3–5 replicate samples; dpa, days post anthesis.

Thornley, 2000) and (ii) the molecular weight per carbon in hexose is 2.1-fold greater than that in fatty acid. A similar approach has been taken to estimate fluxes in oilseed rape (Hill *et al.*, 2003). The two peaks of oil accumulation at  $\sim 10$  dpa and 16 dpa are estimated to consume  $\sim 6$  and 2 nmol of hexose  $\text{min}^{-1}$  seed $^{-1}$ . For direct comparison with enzyme activities, the rate estimates are shown converted to rates  $\text{g}^{-1}$  FW (Fig. 6).

Insoluble acid invertase (CW INV) and SuSy activities (Fig. 6a) far exceeded those of soluble acid and alkaline invertase (Fig. 6b) and were both in excess of the sucrose demand from oil synthesis (Fig. 6a). CW INV and SuSy had similar activities at 8 dpa, when the first phase of oil synthesis was underway. Later, CW INV exceeded SuSy

**Table 1.** Fatty acid composition during wild-type seed development

Fatty acid composition was determined as described in the Materials and methods.

Age (dpa)	Fatty acid composition ( $\text{mg g}^{-1}$ FA)					
	16:0	18:0	18:1	18:2	18:3	Others <sup>a</sup>
4	206	21	42	518	143	71
8	121	23	82	679	68	27
10	66	23	88	796	23	4
12	64	26	101	795	11	3
14	69	26	103	792	9	1
16	67	26	117	780	8	3
20	65	26	116	782	8	4
25	68	28	118	775	7	4
30	68	28	115	778	7	4

<sup>a</sup> Others are mostly 22:0 (75%) and 20:0 (25%).

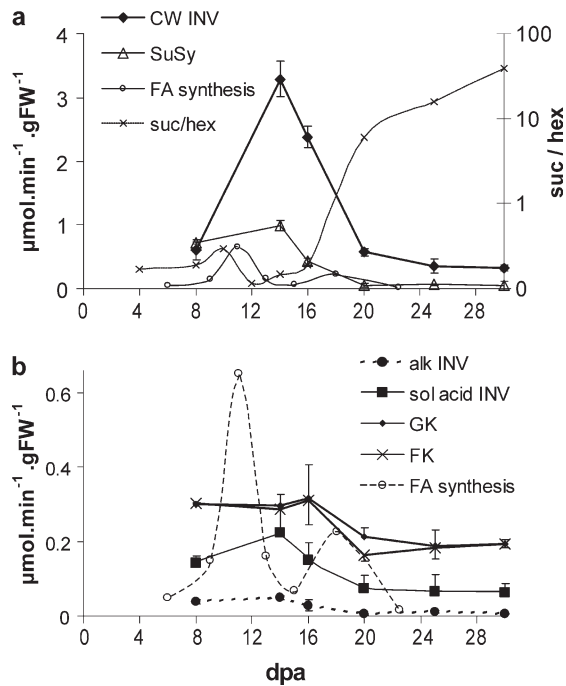
more than 4-fold, rising more by 14 dpa and decreasing less thereafter.

The high insolubility of acid INV is good evidence that it is predominantly apoplastic. The soluble activity (6–20% of the total) could be catalysed by solubilized CW INV or vacuolar INV. The pH optima of the insoluble and soluble fractions differed slightly (pH 4.8 and 4.3 respectively) suggesting that vacuolar invertase made a significant contribution to the 0.15–0.22  $\mu\text{mol min}^{-1}$   $\text{g}^{-1}$  FW soluble acid activity.

Soluble alkaline INV activity was  $\sim 15\%$  and  $\sim 6\%$  that of acidic activity at mid- and late-development, respectively. This could be due to the cytosolic isoform(s), with an alkaline pH optimum, or to other INV isoforms with residual activity at alkaline pH. The pH profiles of soluble and insoluble INV suggested that the former contains an isoform with pH optimum around pH 8.5: the pH profile of sol INV had major and minor peaks at pH 4.3 and 8.5, respectively, whereas insoluble INV had only a single, acidic peak. The data (not shown) suggest that 50–75% of the soluble activity at alkaline pH is due to the cytosolic isoform, i.e. 14–38  $\text{nmol min}^{-1}$   $\text{g}^{-1}$  FW between 8 dpa and 16 dpa. This is 30–40 times less than SuSy, and significantly lower than the demand from fatty acid synthesis.

Hexose phosphorylating activity was similar with either glucose or fructose substrate (Fig. 6b). Compared with the sucrolytic enzymes, the activity was much lower and relatively constant over development, being closer to the demand from fatty acid synthesis. Activity was maximal between pH 7 and 8.5 (data not shown).

Hexose phosphates supply fatty acid synthesis via glycolysis, beginning with their phosphorylation by ATP-dependent phosphofructokinase (PFK) or PPI-dependent phosphofructokinase (PFP) in the cytosol, and/or PFK in the plastid. The activities of PFK and PFP during mid-development were 0.2–0.44 and 2.1–3.1  $\mu\text{mol min}^{-1}$   $\text{g}^{-1}$  FW, respectively. PFK and PFP declined by 20 dpa, as did the other enzymes assayed. It was tested whether the overall



**Fig. 6.** Wild-type seed carbohydrate metabolism during development. Major sucrolytic activities, and the weight ratio of sucrose to hexose (glucose+fructose) (a); minor sucrolytic activities and hexose phosphorylating activities (b). Sucrolytic activities are per hexose produced. The estimated consumption of sucrose, as hexose equivalents, for fatty acid production (FA synthesis) is given on both graphs for comparison against the enzyme activities. Error bars represent standard errors, of three replicate samples; dpa, days post-anthesis.

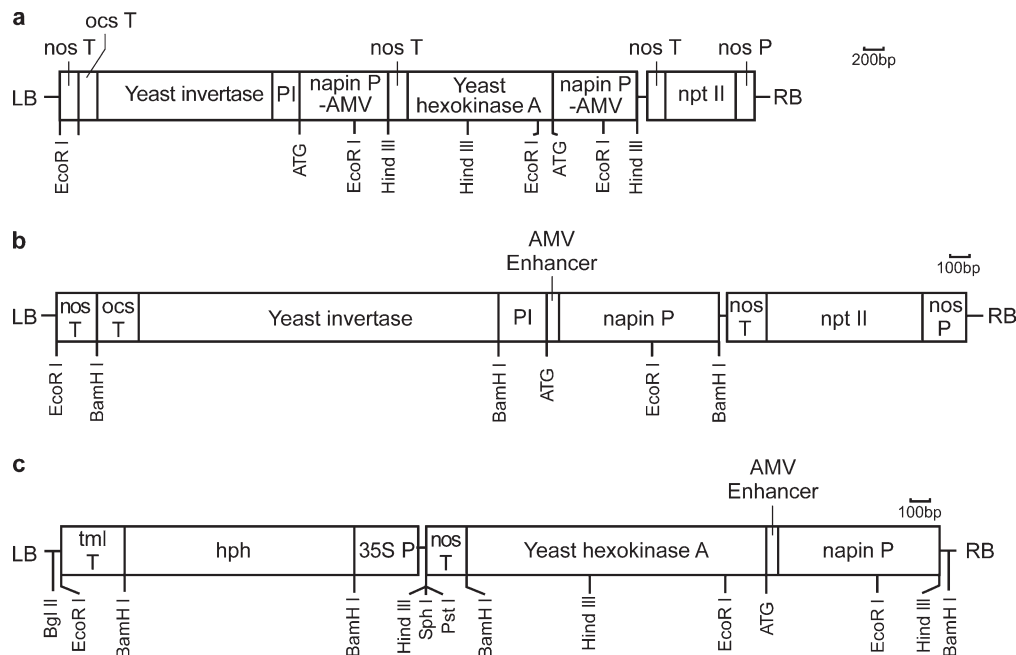
decline in activities was an artefact arising from interference in extracts of older seeds, by performing mixing experiments between young and old seed. For all enzymes, the activity in mixed extracts was within 12% of the sum of the parts, suggesting a true decline in activity *in vivo*.

#### Manipulation of INV and HK activity

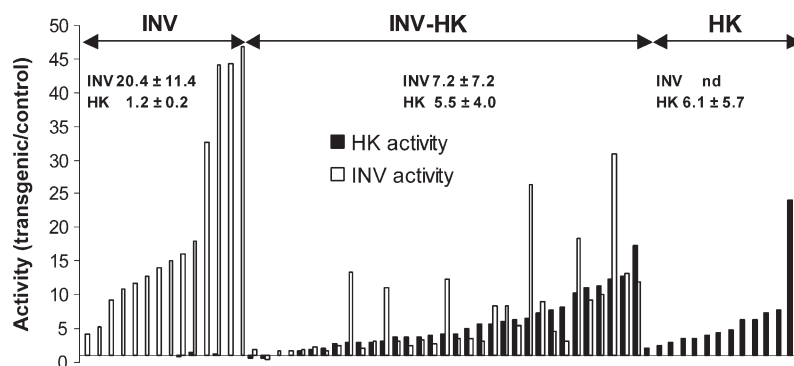
To investigate the role of INV and HK in tobacco seed development, genes were expressed for these enzymes from yeast, under a seed-specific promoter, to introduce additional INV and HK activity into the apoplast and cytosol, respectively (see Materials and methods).

As shown in Fig. 7, the napin promoter was used for seed-specific expression. In tobacco the promoter is active in the endosperm and embryo throughout the storage phase: napin-driven GUS activity rises up to 16 dpa and declines after 25 dpa (Demoor, 1992). At 10 dpa and 15 dpa, the GUS activity was approximately 20% and 50% of the activity at 16 dpa, respectively. The rise in napin-driven activity occurs over the stage when endogenous hexokinase and invertase are declining (Fig. 6). Expression has also been reported in the innermost layer of the tobacco seed-coat (Stalberg *et al.*, 1993). The AMV translational enhancer was used to ensure high protein levels from both chimaeric gene constructs.

About 15 transgenic lines were generated with each of the genes separately and about 45 plants were generated with both genes, using the vectors described in Fig. 7. INV and HK activity were determined at 16 dpa. There were



**Fig. 7.** T-DNA structure of (a) pHEXINV; (b) pSMAPOINV; (c) pHEX:hph. Napin P, napin promoter from BngNAP1 (Baszczynski and Fallis, 1990); 35S P, cauliflower mosaic virus 35S promoter; AMV- alfalfa mosaic virus translational enhancer (Datla *et al.*, 1993); PI, 0.23 kb of the potato proteinase inhibitor II gene (von Schaewen *et al.*, 1990); nos T, nopaline synthase transcriptional terminator; ocs T, octopine synthase transcriptional terminator; tml T, tumour morphology large gene transcriptional terminator; npt II- neomycin phosphotransferase II gene coding sequence; hph, hygromycin phosphotransferase gene coding sequence; RB- T-DNA right border; LB- T-DNA left border.



**Fig. 8.** INV and HK activity in transgenic lines. Activities  $\text{g}^{-1}$  FW were determined at 16 dpa for controls (two wild type and two empty vector control transgenics), and plants transformed with the INV gene (INV), INV and HK genes (INV-HK), or the HK gene alone (HK), as described in the Materials and methods. Activities are expressed relative to the average activity in the control lines, 2.5 and  $0.2 \mu\text{mol min}^{-1} \text{g}^{-1}$  FW for INV and HK, respectively: the y intercept (1) represents the control activity. Inset, the average and SD for each of the three groups. Where bars are absent the activity was not determined (nd).

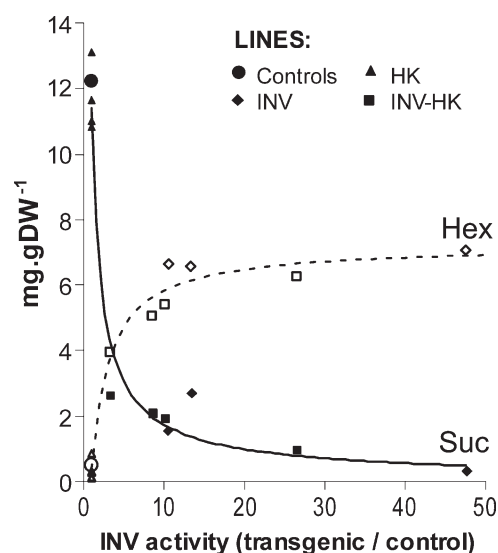
dramatic increases in activity for both enzymes, whether expressed independently (INV or HK) or together (INV-HK) (Fig. 8). INV activity at 16 dpa was, on average, increased 20-fold and 7-fold in the INV and INV-HK lines, respectively, with 30-fold and greater increases in  $\sim 6$  lines. As discussed above, these increases would be predicted to be more modest when oil accumulation initiates ( $\sim 10$  dpa), but to persist over the latter phase of oil accumulation (16–20 dpa) and the maturation phase (upto 28 dpa).

The difference in INV activity between the two groups (INV and INV-HK) was highly significant ( $P < 1\%$ ). By contrast, the HK overexpression was equally effective with either construct: activity was increased on average by  $\sim 6$ -fold in both HK and INV-HK groups, with over 10-fold increases in seven lines. In the INV-HK group, although a few lines had a far greater increase in one activity than the other, overall there was significant correlation between the INV and HK activities ( $P < 5\%$ ).

#### Mature seed composition in INV and HK transgenic seed

INV expression had a clear impact on the sugar composition of mature seed, dramatically increasing hexose and decreasing sucrose. The relationship between INV activity and each sugar was curvilinear; the changes are near-maximal by 10-fold increase in INV (Fig. 9). The suc-to-hex ratio in mature INV seed (between 0.04 and 0.4), was 50–500 times lower than that in mature WT seed, and was notably similar to the ratio in immature WT seed over 6–16 dpa (0.11–0.24, Fig. 6).

The total weight of hexose plus sucrose was lower in the INV lines, ranging between 52% to 75% of that in the control. By contrast, the molar content of hexose plus sucrose was unaffected (79–120% of the control, average 101%). Together, these data suggest homeostatic regulation of sugar levels to maintain osmotic potential, rather than sugar content per se.



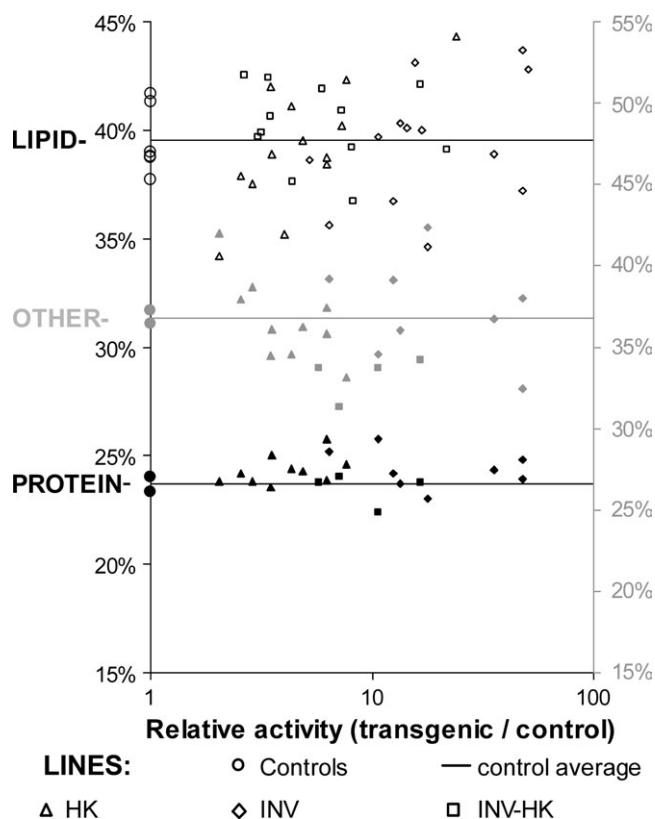
**Fig. 9.** Impact of transgenic activities on mature seed sugar contents. Sucrose (suc) and hexose (glucose+fructose, hex) contents  $\text{g}^{-1}$  DW of mature seed. Three to four lines were chosen, on the basis of transgene activity, for each of the groups shown in Fig. 8. Sugar content is compared against INV activity for all lines. HK activity was 2–24-fold higher and 7–11-fold higher than the control in the HK lines and INV-HK lines, respectively.

There was no effect of increased HK activity on sugar level or type, either in the wild-type or INV-background (Fig. 9; data not shown).

Neither INV nor HK overexpression, alone or in combination, had any effect on the major components of mature seeds (Fig. 10). There were no significant changes in oil, protein, or summed other components as a percentage of total DW.

The lack of impact on oil content was also reflected in oil quality: fatty acid composition was very similar amongst control and high activity lines (Table 2). The proportion of each fatty acid type, as  $\text{mg g}^{-1}$  of total fatty acid, differed no more than 12% between the control and each of the other





**Fig. 10.** Impact of transgenic activities on DW components. Lines (12 to 13 per group) were chosen at random from each of the transgene groups shown in Fig. 8, and analysed for lipid content  $\text{g}^{-1}$  DW (open symbols). A random subset of these lines were also analysed for protein content (solid black symbols), for which the content of other DW was also determined, as the DW not attributed to protein or lipid (solid grey symbols). The activities are given relative to the control as in Fig. 8, for INV activity for the INV and INV-HK lines, and for HK activity for the HK lines.

groups. Only the proportion of 16:0 fatty acid differed significantly, being approximately 3, 10, and 11% lower than the control average for HK, INV, and INV-HK groups, respectively. Within each group, there was no correlation between enzyme activity and the proportion of any of the fatty acid types.

#### Gross morphology of transgenic seed

To determine whether the overexpression of INV or HK had a major impact on seed morphology, anatomy and size were studied in seed from several transgenic lines, including the highest activity lines. Light microscopy showed that there was no gross change in anatomy, size, or storage product accumulation of the seed coat, endosperm, or embryo, and there was no significant impact on mature seed dry weight. There was also no effect on seed viability (data not shown).

## Discussion

#### Wild-type seed architecture and development

The persistence of a cellular endosperm in tobacco, seen here and in previous studies (Scarafoni *et al.*, 2001) contrasts with

beans, rape, and *Arabidopsis*, in which the embryo is surrounded by liquid endosperm during development. In other oilseeds such as those of the Umbelliferae family and castor bean, the endosperm far exceeds the embryo in final weight (Greenwood and Bewley, 1982; Ross and Murphy, 1993). Tobacco lies between these, with the endosperm being approximately twice the size of the embryo, and both being significant stores for protein and oil.

The collapse of thin-walled parenchyma cells in the seed coat, beginning around 14 dpa and complete by 20 dpa (Figs 1c, 3) coincides with the drop in CW INV (Fig. 6a). In other seeds it has been shown that the bulk of seed CW INV activity is in the seed coat, and its decline after seed establishment is due to the collapse of seed coat cells. Thus it appears likely that this also occurs in tobacco.

#### Wild-type storage product accumulation

Starch accumulation during seed development varies widely across oilseeds. Oilseeds such as *Arabidopsis*, oilseed rape, and *Sinapis alba* accumulate starch transiently prior to and during the early phase of storage lipid accumulation (Norton and Harris, 1975; Fischer *et al.*, 1988; da Silva *et al.*, 1997; Eastmond and Rawsthorne, 2000; Baud *et al.*, 2002). It is not clear what purpose the

**Table 2.** Fatty acid composition in mature seed of transgenic lines

Fatty acid composition was determined as described in the Materials and methods. Data are presented as the average  $\pm$ SE of the 4–5 lines shown individually below. Controls include wild type and empty vector control lines.

	Group	Activity in transgenics		Fatty acid composition (mg g <sup>-1</sup> FA) <sup>a</sup>					
		INV/control	HK/control	16:0	18:0	18:1	18:2	18:3	others
Averages	Control			65.3 $\pm$ 0.9	27.0 $\pm$ 0.4	127 $\pm$ 4	771 $\pm$ 4	7.0 $\pm$ 0.4	4 $\pm$ 0
	HK		9 $\pm$ 4	63.4 $\pm$ 0.2*	28.4 $\pm$ 1.5	118 $\pm$ 4	778 $\pm$ 5	7.6 $\pm$ 0.2	4 $\pm$ 0.3
	INV	24 $\pm$ 9		59.0 $\pm$ 0.4*	26.3 $\pm$ 0.6	132 $\pm$ 1	772 $\pm$ 1	7.3 $\pm$ 0.3	3.8 $\pm$ 0.6
	Hex-INV	12 $\pm$ 4	8 $\pm$ 1	58.0 $\pm$ 0.4*	26.5 $\pm$ 0.3	121 $\pm$ 2	783 $\pm$ 2	7.5 $\pm$ 0.3	3.8 $\pm$ 0.3
Individual lines	Con			63	27	120	779	7	4
	Con			65	27	121	776	8	4
	Con			67	28	127	767	6	4
	Con			66	26	138	760	7	4
	HK		2.0	64	25	113	786	8	4
	HK		4.3	64	28	107	790	8	3
	HK		4.9	63	27	123	775	7	4
	HK		7.6	63	34	129	761	7	5
	HK		24.1	63	28	119	779	8	4
	INV	nd		59	26	133	770	7	4
	INV	10.6		60	26	132	770	7	4
	INV	13.3		58	28	130	776	7	2
	INV	47.6		59	25	133	771	8	5
	Hex-INV	3.3	8.2	57	26	117	789	8	3
	Hex-INV	8.6	5.7	58	27	122	782	8	4
	Hex-INV	10.1	11.4	58	27	120	784	7	4
Hex-INV	26.6	6.6	59	26	126	778	7	4	

<sup>a</sup> Asterisk indicates significant difference from the control group (Students *t*-test, *P* <5%).

transient starch accumulation serves. It is clearly not essential for oilseed development, as tobacco and castor bean (Canvin, 1963) lack detectable starch accumulation throughout development yet accumulate high levels of storage lipid.

Tobacco is typical in that triacylglycerol (TAG) makes up the vast majority (97%) of its oil (Batchelder *et al.*, 1994). The FA composition found in this study agrees with that in a previous study, which found that tobacco seed oil may be an appropriate substitute for diesel fuel and could provide an alternative future use for tobacco agriculture (Giannelos *et al.*, 2002).

Tobacco is similar to other oilseeds in that it also accumulates storage protein, and the two products accumulate over similar time periods. It is unusual, however, in that protein accumulation stops prior to that of oil. Other studies have shown the end of protein accumulation to coincide with that of oil, for example, castor endosperm (Greenwood *et al.*, 1984) and rape seed (Norton and Harris, 1975) or to occur later, for example, castor embryo (Greenwood *et al.*, 1984) and *Arabidopsis* seed (Baud *et al.*, 2002). Thus, the co-ordination between regulation of oil and protein synthesis may vary between species and/or seed types.

#### Wild-type sucrose metabolism

As in other seeds, CW INV is high in young tobacco seeds and declines during development. Unlike starchy beans (Borisjuk *et al.*, 2003) and oilseed rape (Hill *et al.*, 2003),

the drop in INV occurs after the bulk of storage product accumulation, and there is no overall switch from INV to SuSy. In both rape and tobacco, however, there is an excess of INV activity relative to the demand for hexose for storage product synthesis. The most detailed analysis available of carbohydrate metabolism in an oilseed (rape) shows that the pathway from suc to oil could involve INV or SuSy, or possibly both via sucrose recycling (Hill *et al.*, 2003). As discussed below, in tobacco, high INV is clearly compatible with high oil accumulation. This is in agreement with work on cereals, involving the spatial resolution of enzymes and storage products during maize endosperm development. This suggests that while suc utilization for starch synthesis occurs via SuSy, the INV pathway predominates for oil synthesis (Doehlert and Felker, 1987; Doehlert, 1990). Although these data are consistent with a model for INV-mediated oil synthesis, a role for the SuSy pathway cannot be ruled out given the lack of spatial resolution in this study. It is also possible that sucrose is resynthesized in some part(s) of the seed via sucrose phosphate synthase.

#### Sugars as signal molecules in plant seed development

The most strongly supported and ubiquitous feature of the model for sugars as signal molecules in plant seed development (Wobus and Weber, 1999) is the role of INV in seed establishment. INV, particularly cell-wall INV, and hex are a consistent feature of early seed development, not just for starch-storing organs, for example, rice (Hirose

*et al.*, 2002, and references within), but also oilseeds (this work, plus references above). INV facilitates sucrose unloading, and the resultant hexose levels appear to regulate mitotic activity (Borisjuk *et al.*, 1998; Cheng and Chourey, 1999). Tobacco seed development is entirely consistent with this. After seed establishment, all seeds appear to undergo sugar changes, and reducing hex-to-suc ratios, but the proposal that this triggers storage function is less well supported and may apply less generally. Decreases in the hex-to-suc ratio as seeds move from cell division and towards storage product synthesis may result from changes in metabolism rather than triggering those changes (for a discussion see Hill *et al.*, 2003). In certain cases it is clear that sugar changes lag behind the onset of the storage function: the onset of starch accumulation often occurs before the suc-to-hex ratio declines, for example, in barley (Weschke *et al.*, 2003) and fava bean (Weber *et al.*, 1995); and in this study oil and protein accumulation begin several days before the switch from hex to suc. There are two alternative explanations for these observations: sugars are regulating the storage function in a way which is not detectable at the whole seed level (e.g. suc accumulation in a minority of cells), or other signals control a switch to the storage function in tissues with an overall high ratio of hex-to-suc. This is discussed further below.

#### *INV and HK effects: implications for the control of seed storage function*

In this study there was no inhibitory effect (upon oil and protein accumulation) of introducing very high INV and HK activities into tobacco seeds. Expression under the napin promoter is known to give activity throughout the storage tissues during the storage phase, and the 50–500-fold increase in hex-to-suc ratio in the mature INV seed indicates that the apoplastically-targeted activity had a strong influence on overall sucrose metabolism. Persistence of the normal storage function in the high INV transgenics is consistent with the fact that, in the wild type, most oil accumulation and all protein accumulation occurs while the seed has high INV activity and a high hex-to-suc ratio. An environment of low INV and high suc-to-hex ratio is clearly not required for the onset of the tobacco seed storage function, or for the majority of accumulation. The results strongly suggest that the model for sugars as signal molecules in plant seed development (Wobus and Weber, 1999) does not apply universally, and is consistent with the observed correlations between INV and oil synthesis (discussed above).

In wild-type tobacco, the second phase of oil accumulation does coincide with decreasing INV and increasing suc-to-hex ratio, and it remains possible that a causal relationship between changes in suc metabolism and storage function exists in this phase. This appears to be unlikely, however. As found in castor bean (Greenwood *et al.*, 1984), oil accumulation in the embryo lags behind

that in the endosperm, occurring mostly in the second phase. If this were dependent upon the changes in INV and the hex-to-suc ratios, oil accumulation in transgenic embryos would be strongly disrupted by the effects of high INV, which is predicted to act throughout the seed apoplast and throughout the storage phase. Thus, the visible persistence of normal oil accumulation in the transgenic embryos strongly suggests that although the second phase of oil synthesis coincides with changes in INV and hex, it is not dependent on these changes. In *Arabidopsis* there is also a second phase of oil accumulation which coincides with a decreasing hex-to-suc ratio (Baud *et al.*, 2002), and the authors note a striking correlation between peaks in the hex-to-suc ratio and transition phases during embryogenesis. Whether this reflects the sugar regulation of the oilseed storage function is not yet clear.

The failure of altered apoplastic INV to perturb oil accumulation in tobacco seeds does not preclude a role for sugar metabolism in the control of oil synthesis: the impact of manipulating sucrose metabolism in the cytosol of an oilseed is not yet determined. For example, the link between suc levels and starch synthesis has been both supported and challenged by INV and HK transgenic plants. The most detailed work is on potato, in which starch synthesis appears to be regulated via suc supply in potato tubers (Geigenberger, 2003). Consistent with this, apoplastic or cytosolic INV overexpression in tubers lowers starch content (Sonnewald *et al.*, 1997; Trethewey *et al.*, 1998; Fernie *et al.*, 2000). But intriguingly, wild-type starch levels are restored to the tubers with high apoplastic invertase by overexpression of HK, despite the fact that the dramatic decrease in sucrose and increase in hexose content is unaltered (Fernie *et al.*, 2000). Thus it is possible that neither oil nor starch synthesis are directly controlled by the large developmental changes in sugar levels: sugar metabolism could have more subtle roles in controlling both processes.

While the role(s) of sugars and sugar metabolism in the regulation of storage function are not yet clear, it is clear that sugar-sensing pathways interact with hormonal signalling (Leon and Sheen, 2003). This includes ABA-mediated signalling (Nambara and Marion-Poll, 2003), which is essential to processes such as storage protein accumulation and maturation. For example, tobacco with seed-specific immunoreduction of free ABA produces less storage protein and oil (Koornneef *et al.*, 1989). The role(s) of sugar and its interaction with ABA must vary between seeds and/or storage product types. This variation should provide clues in defining the regulation of seed metabolism and development, complementing approaches to identify genes specific to sugar-signalling pathways (Rook and Bevan, 2003).

#### *INV and HK effects: implications for control of oil synthesis*

In this study, many-fold increases in CW INV and HK activity during oil accumulation had little impact on seed



weight or oil content: if suc metabolism controls sink strength or oil accumulation in tobacco, little of this control appears to lie at HK or apoplasmic INV. Thus, either most control over oil synthesis lies within the committed pathway, particularly fatty acid synthesis (Ramli *et al.*, 2002) or in supply to the committed pathway: sugar uptake into the cell; glycolysis; metabolite uptake into the plastid (Eastmond and Rawsthorne, 2000; Fox *et al.*, 2001); and provision of energy supply and reductant (Rawsthorne, 2002).

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