Invertase protein, but not activity, is present throughout development of *Lycopersicon esculentum and L. pimpinellifolium* fruit

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Received: *5 September 2000*
Accepted: *24 October 2000*

Summary

• The presence and distribution of invertase is reported in two red fruited and hexose accumulating species of tomato, *Lycopersicon esculentum* and *L. pimpinellifolium*, which differ markedly in fruit invertase activity.

• Using antibodies raised against a highly conserved invertase peptide, all invertase isoforms in green and red tomato fruit were detected and visualized by chemi luminescence and western blotting techniques. Interacellular localization of invertase was examined by electron and light microscopy.

• Invertase activities were much lower in green fruit than in red fruit from all cultivars. However, invertase protein was evenly distributed between all tissues of green and red fruit in tissue blots that had been extensively treated with preimmune serum that eliminated all non-specific binding. Immuno-gold labelling showed that invertase protein was present in the cell wall and vacuole, but not in the cytosol, of epidermal tissue from both species.

• Invertase protein is present throughout fruit development in tomato. However, the increase in activity during ripening is due to changes in the activation state of the protein rather than the amount of protein present.

Key words: invertase protein, *Lycopersicon esculentum*, *Lycopersicon pimpinellifolium*, tomato invertase isoforms, immuno-gold labelling, invertase antibodies, fruit development.

Introduction

The sucrose-cleaving enzymes of sink tissues, invertase and sucrose synthase, are essential determinants of cell division, growth, development and storage. Hence, the relative importance of each enzyme in the regulation of sucrose import and metabolism in sink tissues has been intensively studied. In general, acid invertases have been found to be more important in utilization sinks while sucrose synthase is the major enzyme of sucrose cleavage in storage sinks (Sturm, 1999; Tang *et al.*, 1999, D'Aoust *et al.*, 1999). Sucrose synthase has been shown to control fruit set and development in tomato by regulating sucrose import into young fruit (Chengappa *et al.*, 1999; D'Aoust *et al.*, 1999). In contrast the role of invertase in tomato fruit development is less well understood.

The temporal and spatial regulation of invertase activity is mediated both by transcriptional controls (Godt & Roitsch, 1997; Sturm, 1999) and by post-transcriptional regulation involving association with tight-binding protein inhibitors (Pressey, 1994; Greiner *et al.*, 1998, 1999). While the abundance of soluble acid invertase transcripts (TIV1) has been shown to increase during tomato fruit development (Klann *et al.*, 1993) no relationships between acid invertase transcripts and protein contents have been demonstrated.

Vacuole isolation techniques have been used to localize soluble acid invertase in this compartment in tomato fruit

(Konno *et al.*, 1993). Similarly, immunolocalization techniques have been used to demonstrate the presence of both cell-wall-bound and soluble forms of acid invertase in tomato roots (Benhamou *et al.*, 1991) and fruit (Iki *et al.*, 1978). However, the possibility of redistribution of soluble invertase forms during the experimental procedures complicates the interpretation of the results of such studies in terms of intracellular compartmentation. In the present study an invertase antibody was raised against a synthetic highly conserved invertase peptide and was used to localize protein within individual fruit cells and also within the whole fruit during development in order to determine the distribution of invertase protein. This antibody was previously used to determine the intercellular partitioning of invertase in leaves of a large number of species (Kingston-Smith & Pollock, 1996). The presence and distribution of invertase were studied in two red-fruited and hexose accumulating species, *L. esculentum* and *L. pimpinellifolium*, which differ markedly in fruit invertase activity. Fruit from the latter species has greater soluble acid invertase activity and sugar accumulation than the former (Manning & Maw, 1975).

This study reports that the apparent increase in invertase activity in ripening tomato fruit is not related to changes in invertase protein. The increase in activity during fruit ripening is therefore due to post-translational regulation of invertase activity. It is probable that this is achieved by alterations in the amount of the invertase inhibitor protein as described previously for tomato and other species (Godt & Roitsch, 1997; Greiner *et al.*, 1999, 2000).

Materials and Methods

Growth of plant material

Seeds of *L. esculentum* FM 6203, *L. pimpinellifolium* LA 722 and *L. pimpinellifolium* PI 126436 were planted in humax compost in seed trays and grown in a growth room under a 12-h photoperiod at 20°C for approx. 4 wk. Plants were watered every 2 d and the compost kept moist. They were then transferred to pots in a glasshouse under a 16-h photoperiod at 20–25°C and grown until fruit were fully ripe and red. The plants were watered twice a day and fed from the appearance of the first truss with Tomorite® (Levingtons Horticulture, Ipswich Suffolk, UK) tomato feed every 2 d. Ripe and unripe fruit were used for tissue printing and nondenaturing polyacrylamide gel electrophoresis (PAGE); only ripe fruit were used for the electron microscopy studies.

Tissue printing

Inter-cellular compartmentation of acid invertase within green and red fruit was studied using a modification of the technique described by Kingston-Smith & Pollock (1996) for leaves. Nitrocellulose filters (Amersham Pharmacia Biotech Little Chalfont, Bucks., UK) were soaked in 0.2 M $CaCl_2 \cdot 6H_2O$ for 30 min and air-dried. Red and green tomato fruit were cut either transversely or longitudinally, placed on the filter and pressed gently by hand. The filter was air-dried and placed in boiling 10 mM sodium ethylenediaminetetraacetic acid (EDTA) for 15 min. The filter was then placed in boiling 1% (w/v) sodium dodecyl sulphate (SDS) and incubated for 30 min. The filter was either placed in Tris-buffered saline (TBS) (pH 7.4; TBS – comprising of 20 mM tris(hydroxymethyl)aminomethane (Tris) (HCl; pH 7.4; and 0.137 M NaCl) for 10 min prior to further analysis, or used for estimation of total protein. For protein detection, the filter was incubated in amido black stain (1% (w/v) amido black in 7% (v/v) acetic acid) for 5 min. It was then de-stained in 7% acetic acid until a clear image was detectable.

Immunodetection of invertase

Detection of invertase proteins on the tissue blots was performed using a modification of the method of Kingston-Smith & Pollock (1996). The filter was first incubated for 1 h with 10% (w/v) bovine serum albumin (BSA) in TBS at room temperature with shaking. It was then incubated for 2 h and up to 8 times with TBS containing 0.1% (v/v) polyoxyethylenesorbitan (monolaurate) (Tween-20) and 5% (w/v) BSA (TBS-T-B).

The antibodies used for the detection of invertase were donated by Alison Kingston-Smith, who worked in our laboratory at IGER-Aberystwyth (see Kingston-Smith & Pollock, 1996, for details). This antiserum, which had been raised (in rabbit) against a synthetic highly conserved invertase peptide (Y-24-A) based on complete homology with the *Arabidopsis* and carrot genes. While only 17 out of the 24 amino acids are homologous with the soluble acid invertase gene from tomato (Elliot *et al.*, 1993) and 17 out of the 20 with the insoluble acid invertase gene (Godt & Roitsch, 1997), our studies show that these antibodies specifically recognize both the insoluble and soluble forms of invertase from tomato leaf (Kingston-Smith *et al.*, 1998) and fruit (data not shown). This antibody was therefore used to detect invertase protein on the filters containing the tissue blots.

Due to the high sensitivity of the detection method, a high degree of cross-reactivity is also observed with the preimmune serum, which was obtained prior to the production of the antiserum raised against the synthetic invertase peptide. To overcome this problem, a preliminary step involving extensive washing of the filters with the preimmune serum (Husain, 1999) prior to the addition of the antibody was added. This ensured that any nonspecific background reaction was effectively eliminated (Husain, 1999).

After incubation with BSA, the filters containing the tissue blots were incubated with preimmune serum for 1 h. Filters were then washed for 2 h and up to eight times with TBS-T-B. The filters were next incubated for 1 h at room temperature with an unconjugated anti-rabbit antiserum (a) raised in goat

(DAKO Ltd, High Wycombe, Bucks., UK) and then rinsed for 1 h and up to four times with TBS-T-B. Procedures (a) and (b) were repeated except that the incubation period for step (b) was 2 h. This provided an effective means of eliminating nonspecific antibody protein reactions from the detection system. The filters were then incubated with the antiserum raised against the invertase synthetic peptide for 1 h and then washed for 2 h and up to eight times with TBS-T-B. All antisera were diluted (1 : 1000) in TBS containing 5% (w/v) BSA and all further incubations took place at room temperature with shaking. Finally, the filters were incubated with antirabbit antiserum, raised in swine, and conjugated to horseradish peroxidase (DAKO Ltd, High Wycombe, Bucks., UK). Following this procedure, the filter was finally incubated for 2 h and up to eight times with TBS-T-B followed by incubation in TBS for 1 h.

The presence of invertase isoforms was then visualized using a chemiluminescence detection system as described by Kingston-Smith & Pollock (1996). This involved use of an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK). For this analysis, the filters prepared as mentioned earlier were dried and incubated with the reagent mixture (as directed by the manufacturer) for 1 min at room temperature. Filters were placed in saranwrap®, with hyperfilm (Amersham Pharmacia Biotech) and images were allowed to develop in the dark for 1–10 min. The hyperfilm was developed with photosol RG fixer and developer (Fuji Film UK).

As shown in Fig. 1, three sets of controls were performed. In the first control, all antibodies were omitted to show the level of reaction obtained in the presence of native tomato fruit peroxidases only (Fig. 1; control 1). In the second control, the antiserum, raised against the synthetic peptide (step 3 in Fig. 1), was omitted to determine the degree of chemiluminescence arizing from nonspecific binding (Fig. 1; control 2). In the third control, the filters were incubated with the preimmune serum instead of the antiserum raised against the synthetic peptide to confirm that all nonspecific antigenic binding sites were saturated (Fig. 1; control 3). Only the samples that were incubated with the invertase antiserum following the procedure detailed earlier yielded antibody-specific patterns of chemiluminescence arizing from the presence of invertase protein (Fig. 1; experimental).

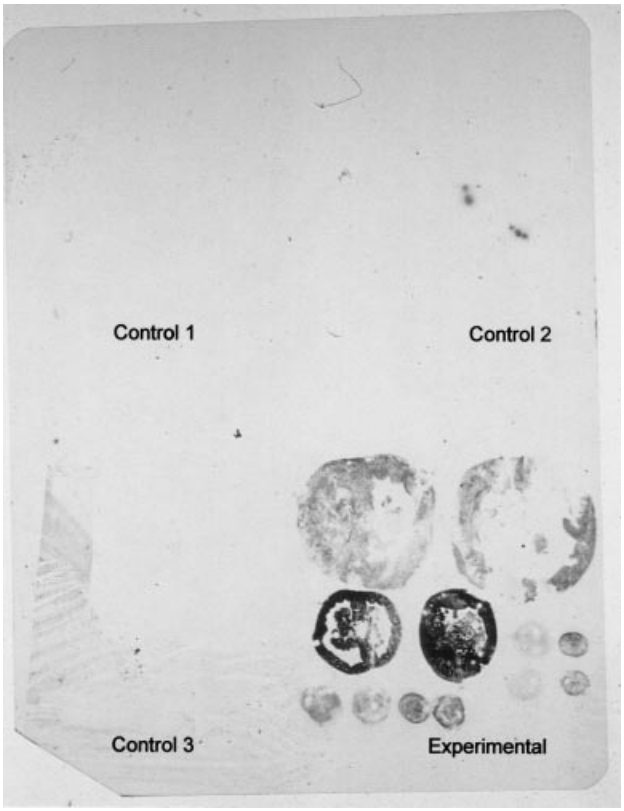

Fig. 1 Localization of invertase protein in developing tomato fruit. Fruit at all stages of development from *Lycopersicon esculentum* FM 6203, *Lycopersicon pimpinellifolium* PI 126436 and *Lycopersicon pimpinellfolium* LA 722 were blotted on to nitrocelluose and detected with specific antibodies (Experimental). Controls 1–3: perfomed as described in the Materials and Methods section.

Detection of invertase activity and protein on nondenaturing gels

Nondenaturing polyacrylamide gel electrophoresis (native-PAGE) was performed on mini-gel apparatus (Biorad, Hemel Hempstead, Herts., UK). Gels were run for 45 min until the solvent front had reached the end of the gel (70 mm). All procedures were based on the method of Laemmli (1970) except that SDS and 2-mercaptoethanol were omitted from all the buffers. Samples were not boiled before loading and were maintained at 4°C.

Invertase was extracted according to the method of Stommel (1992) with minor modifications. Aliquots of frozen tomato powder (20–25 mg) were extracted 3 times in 100 µl of an extraction buffer containing 50 mM Hepes (NaOH) (pH 7.5) 5 mM $MgCl_2$, 1 M NaCl, 1 mM sodium EDTA, 5 mM dithiothreitol (DTT) and 0.1% (v/v) t-octylphenoxypolyethoxyethanol (Triton x-100). Extracts were microfuged at 10000 ***g*** for 10 min at 4°C and the supernatants were then desalted and concentrated using microconcentrators (Amicon, Millipore, Watford, Herts., UK). The concentrates were diluted with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulophonic acid) (Hepes) (NaOH) (pH 7.5) 5 mM $MgCl_2$ and 5 mM DTT to 100 µl. Samples were mixed in a 4 : 1 ratio with buffer consisting of 0.3 M Tris (HCl) pH 7.5 and 25% (v/v) glycerol, and loaded on to the gel. Duplicate sets of samples were run on the same gel at a concentration of 20–40 µg protein per well with running buffer of 25 mM Tris and 192 mM glycine. Electrophoresis was allowed to proceed for 45 min at 4°C and 200 V. After separation, the electrophoresis apparatus was disassembled and the gel cut into two halves. Each half contained one set of samples. One half was stained for invertase activity and the second half was used for Western blotting.

Invertase activity

Invertase activity was extracted from frozen tomato fruit tissue according to the methods of Stommel (1992), as described by Husain *et al.* (1999).

After electrophoresis, invertase was detected as described by Cairns & Ashton (1991) with minor modification as follows. The gel was first immersed in citrate/phosphate buffer (pH 4.4) at 37°C for 10 min and then in this buffer containing 0.120 M sucrose for 30–60 min at 37°C. The solution was decanted and replaced by boiling 0.2% (w/v) 2,3,5-triphenyltetrazolium chloride in 4% (w/v) NaOH. The gel was incubated until bright red bands of invertase activity had developed (5–10 min) due to the formation of a red formazan product. Gels were then stored in 5% (v/v) acetic acid prior to photography. Gels with samples, which were subjected to the staining procedure for glucose and fructose without sucrose, did not show any red colouration (data not shown).

Western blotting

Western blotting was carried out as described by Walker & Pollock (1993) with minor modifications. Immediately after electrophoresis, the gel was incubated in transfer buffer, consisting of 20 mM Tris, 154 mM glycine and 20% (v/v) methanol, for at least 15 min. Western blotting was then performed using mini trans-blot electrophoresis transfer cell equipment (Biorad, Hemel Hempstead, Herts., UK). The proteins were transferred to nitrocellulose filters that had been equilibrated in transfer buffer. Proteins were transferred from the gel to the nitrocellulose filter over a 1.5-h transfer period at 4°C under conditions of 110 V. After transfer, the filter was boiled in 1% SDS for 30 min and incubated in TBS. Invertase proteins were detected as for the tissue blots.

Electron microscopy

All procedures were optimized to obtain minimum redistribution of soluble forms during preparation of the sections. Sections from fresh ripe red *L. esculentum* FM 6203 and *L. pimpinellifolium* PI 126436 fruit were prepared at 4°C. Pieces of tomato fruit tissue (1–2 mm^3) were placed into a fixative solution (Karnovsky, 1965), prepared with glass distilled reverse-osmosis deionized water and containing 67 mM sodium cacodylate (pH 7.4) 4% (w/v) *para*-formaldehyde, 1% (w/v) glutaraldehyde and 1% (w/v) tannic acid. Air was removed from the intercellular spaces in the tissue by gentle evacuation, the initial solution replaced with fresh fixative and the samples held at 4°C for approx. 18 h. The tissue was incubated at 4°C for 1 h in sodium cacodylate (pH 7.4). The tissue was then incubated at 4°C for 1 h in distilled H_2O. It was then transferred to distilled H_2O at 4°C in a modified 6 compartment biopsy cassette (Microsette®; MERCK Limited, B.D.H., Poole, Dorset, UK) in which the lid plastic lattice had been replaced with 200 μm^2 nylon mesh. The cassette was placed in a wide mouth glass bottle (120 ml) for further solution changes (approx. 30 ml) and incubations.

After 30 min in distilled H_2O at 3°C, the tissue was dehydrated in a graded ethanol series. This consisted of: (1) 30% (v/v) ethanol at 3°C for 2 h during which the temperature was reduced to −10°C; (2) 50% (v/v) ethanol at −10°C for 2 h, reducing the temperature to −20°C; and (3) 70% (v/v) ethanol at −20°C for approx. 18 h reducing the temperature to −25°C. All subsequent ethano l and resin solutions were prechilled to −25°C and all incubations were at −25°C. The 70% solution was replaced with 95% (v/v) ethanol for 2 h, followed by three incubations of 2, 2 and approx. 18 h with 100% (v/v) dry ethanol.

The tissue pieces were infiltrated with graded solutions of L. R. Gold resin (London Resin Company Ltd, Reading, Berks, UK) initially diluted with 100% (v/v) dry ethanol. The 100% (v/v) ethanol solution was replaced with L. R. Gold resin 5% (v/v) 2 h; 10% 2 h; 20% 2 h; 40% approx. 18 h; 60% 2 h; 75% 4 h; 90% 2 h; and 100% approx. 18 h. After a further incubation in fresh 100% (v/v) L. R. Gold resin for 9 h, the final incubation was in L. R. Gold resin containing 0.1% (w/v) benzoin ethyl ether for 24 h.

The tissue samples, under the resin mixture at –20°C, were transferred to the BEEM® polyethylene flat embedding moulds (Agar Scientific, Stanstead, Essex, UK). The moulds were covered by Parafilm® B.D.H., Poole, Dorset, UK, the air bubbles removed and the resin polymerized under uv light (365 nm at 6 W) for 24 h at −20°C and for a further 24 h whilst the temperature was allowed to increase to +10°C.

Thin sections (approx. 100 nm thick with silver/gold interference colours) for electron microscopy were cut with a Reichert ULTRACUT ultramicrotome (Leica, Milton Keynes, Bucks., UK) using a diamond knife (Diatome, Beil, Switzerland) onto 5% (v/v) ethanol. The sections were collected on either 200 mesh nickel grids with a support film of formvar coated with carbon, or picked up using nickel slot grids and allowed to dry down on formvar films supported on steel mesh bridges (Carter Rowley & Moran, 1975).

Antigenic detection of invertase in the sections was performed using a modification of the method of Gordon *et al.* (1992). Except where indicated, grids were incubated at room temperature on 10–20 µl of incubating solution on sealing film on moist filter paper. They were first incubated with 15 mM tri-sodium citrate (pH 7.2) 75 mM NaCl and 0.015% (v/v) Tween-20 (SSC-T; 30 min) and then equilibrated with CTM buffer (10 mM Tris (HCl; pH 7.6) 154 mM NaCl, 0.5% (w/v) casein hammarsten, 0.02% (w/v) thimeorsal (Kenna *et al.*, 1985) containing 0.015% (v/v) Tween-20 (CTM-T; 1 h).

The antibodies used for the detection of invertase were as for the tissue blotting. After equilibration, the grids were either incubated with preimmune serum or the antiserum raised against the invertase synthetic peptide diluted 1 : 50 in CTM-T (1–2 h). The grids were washed three times with 2 ml of distilled water. They were then dried by touching to

filter paper, equilibrated a second time with CTM-T for 30 min and incubated with 15 nm colloidal gold conjugated to antirabbit antiserum raised in goat (IgG; Brisith Biocell International), diluted 1 : 50 with CTM-T (1–2 h). They were then washed by sequential transfer to 5 drops of SSC-T (3 min each) and then to 5 drops of distilled water (3 min each) and dried by touching to filter paper.

Sections were poststained with 2% (w/v) aqueous uranyl acetate for 15 min, and lead citrate solution (Reynolds, 1963) for 3 min. Grids were viewed on a JEOL (Tokyo, Japan) model JEM 1010 electron microscope operated at 80 V. Electron micrographs were photocopied and each micrograph was divided into cytoplasm, cell wall and vacuolar compartments. The compartments were cut out and their areas determined from the weight of a known area of the same paper. The numbers of gold particles in each area were counted. Values were calculated for both preimmune and immune treatments in order to quantify invertase specific labelling.

Three sets of controls were performed. In the first control, the primary antibody was omitted to show the level of reaction obtained in the presence of secondary antibody only. In this control, no gold particles were found (data not shown). In the second control, the secondary antibody was replaced with gold-linked BSA to confirm that all nonspecific antigenic binding sites were saturated. Again, no gold particles were found (data not shown). In the third control, the secondary antibody was replaced by gold-labelled antirat antibody to determine the number of gold particles arizing from nonspecific binding. As with controls 1 and 2, gold particles were also absent from control 3 (data not shown). Only the samples that were incubated with the preimmune serum or with invertase antiserum, followed by gold-labelled antirabbit antibodies yielded gold particles detectable under electron microscopy.

Light microscopy

Fresh ripe pericarp and epidermal tissue from *L. esculentum* FM 6203 and *L. pimpinellifolium* PI 126436 fruit were prepared and embedded as for electron microscopy. Sections (approx. 0.1 μm thick) for light microscopy, were cut with a Reichert ULTRACUT ultramicrotome (Thermometric Ltd., Northwich, Cheshire, UK), using a glass knife. Sections were transferred to water drops on glass microscope slides, dried at 60°C and stained using equal parts of 1% (w/v) methylene blue in 1% (w/v) sodium borate and 1% (w/v) Azure II for 2 min. The sections were rinsed with water, dried and covered with Eukitt® (Merck Limited, B.D.H., Poole, Dorset, UK) mounting medium before viewing under a light microscope.

Results

Invertase activity

Soluble and insoluble invertase activities were much greater in *L. pimpinellifolium* PI 126436 than in fruit from the other two lines at any equivalent stage in development (Table 1). In all cases, invertase activity was lowest in green fruit, increasing up to 100-fold in *L. esculentum*, 10-fold in *L. pimpinellifolium* LA 722 and fourfold in *L. pimpinellifolium* PI 126436 (Table 1).

Antibody specificity

The antibody used in the intra- and intercellular localization of invertase was raised against a synthetic highly conserved invertase peptide (Y-24-A). It has been previously used in the detection of invertase protein in *L. esculentum* leaves (Kingston-Smith & Pollock, 1996). This antibody had not previously been used in studies on fruit but a series of experiments in our laboratories indicated that the antibody was specific to invertase in tomato fruit. Fig. 2 shows that the

Table 1 Invertase activities in green and red fruit from *Lycopersicon esculentum* and *Lycopersicon pimpinellifoluim* PI 126436 and LA 722

Tomato fruit	Invertase activity Soluble	Insoluble
L. esculentum FM 6203		
(a) Green	148 ± 43	14 ± 3
(b) Red	964 ± 166	48 ± 11
L. pimpinellifolium PI 126436		
(a) Green	558 ± 56	26 ± 5
(b) Red	2630 ± 108	80 ± 4
L. pimpinellifolium LA 722		
(a) Green	89 ± 15	11 ± 3
(b) Red	541 ± 60	30 ± 4

Soluble and insoluble invertase activities were determined in green fruit (10–30 d after anthesis) and red fruit (30–50 d after anthesis) from *L. esculentum* and *L. pimpinellifoluim* PI 126436 and LA 722 as described in 'Materials and Methods'. Invertase activities are given in μmol hexoses produced/g fr.wt./h and values represent the mean of between 50 and 150 samples ± standard error.

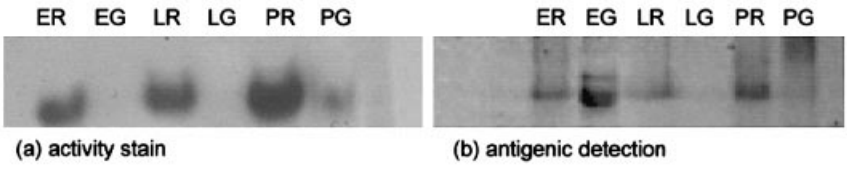

Fig. 2 Relationships between invertase activity and protein as detected by specific antibodies. Extracts of red (R) and green (G) fruit from *Lycopersicon esculentum* FM 6203 (E), *Lycopersicon pimpinellifolium* LA 722 (L) and *Lycopersicon pimpinellifolium* PI 126436 (P) were subjected to native PAGE. (a) Gel stained for invertase activity; (b) gel shows antigenic detection using the specific antibody.

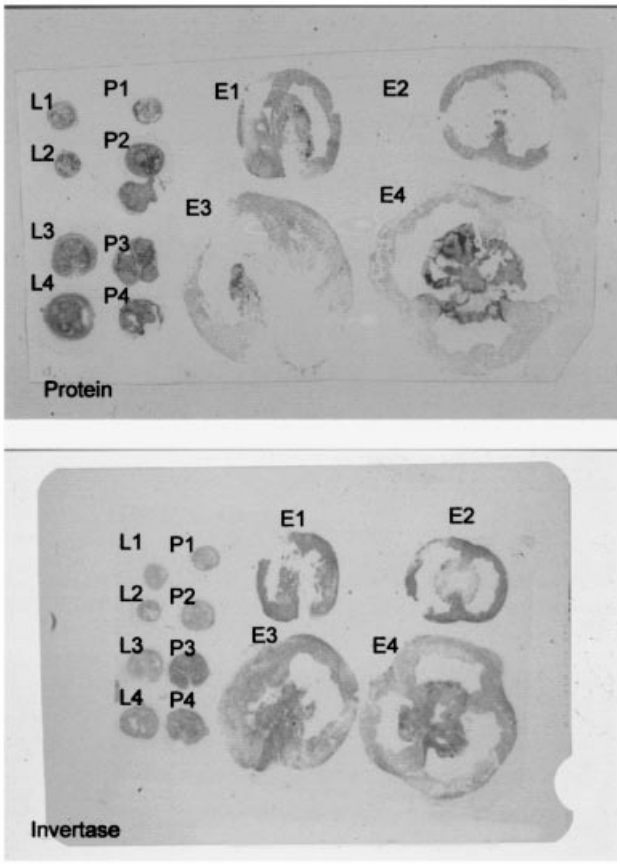

Fig. 3 Intercellular distribution of invertase protein. Green (1 and 2) and red (3 and 4) fruit from *Lycopersicon esculentum* (E), *Lycopersicon pimpinellifolium* LA 722 (L) and *Lycopersicon pimpinellifolium* PI 126436 (P) were blotted onto nitrocellulose filters and either stained for total protein content (top panel) or subjected to antigenic detection of invertase protein (bottom panel).

major band of invertase activity was detected in red fruit from all lines and this coincided with the presence of the major band detected by the antibody on the western blots. It is noticeable, however, that invertase protein was detectable in the green fruit from *L. esculentum* but not green fruit from the *L. pimpinellifolium* lines. Only one band of invertase activity (Fig. 2a) and one major band of invertase protein (Fig. 2b) were detected on the gels. Other bands detected on the western blot, which were obtained from a native gel, also arise from invertase, but they had no activity. Multiple bands are typical on western blot analyses of invertase and it is generally agreed that these result from post-translational modifications of the invertase protein (Faye *et al.*, 1986). The number of visible bands increases with the degree of development of the antigen/antibody reaction. Work in our laboratory has shown that these largely arise from different glycosylation states of the invertase protein (data not shown) but the presence of tightly bound inhibitors in some bands cannot be discounted.

The intercellular location of acid invertase

The intercellular location of acid invertase was investigated using tissue blotting of green and red fruit from each cultivar (Fig. 3). The upper blots on Fig. 3 show that protein was evenly transferred from the whole of the cut surface of the fruit to the blot. To ensure the specificity of the detection method and that only invertase protein was detected on the tissue blots, the filters were extensively washed with preimmune serum prior to the addition of the invertase antibody. The lower blots on Fig. 3 thus confirm that invertase protein was present in both the green and red fruit from all lines. Furthermore, they indicate that invertase protein was also uniformly distributed across the different tissues of the fruit. Invertase protein was located in the epidermis, pericarp and gelatinous central core tissue. This was true for all tomato fruit studied, regardless of age. The pericarp tissue of the *L. pimpinellifolium* fruit was less well defined than that for *L. esculentum*, due to the high liquid content of the fruit. Green fruit were found to contain qualitatively as much invertase protein (per unit surface area) as red fruit and none of the tissue appeared to have greater concentrations of invertase protein. It should be noted, however, that the antibody is likely to identify both active and inactive forms of invertase protein.

The intracellular localization of invertase

Sections were removed from both epidermis and pericarp tissues as shown in Fig. 4. The pericarp tissue of *L. pimpinellifolium* PI 126436 showed a high degree of cellular disruption, such that the cytoplasm could not be distinguished from the vacuole (see Fig. 5b). In this case, therefore, the localization of the invertase protein could not be determined beyond the limits of a 'vacuole + cytoplasm' fraction (Table 2). The degree of immuno-gold label present with following incubation with antibody was significantly ($P < 0.1$) higher than that following treatment with preimmune serum, in the vacuole of the pericarp tissue from *L. esculentum* fruit (Table 2). There was no labelling difference in the cytoplasm with antiserum compared to the preimmune serum. The presence of cell wall invertase could not be detected in either *L. esculentum* or *L. pimpinellifolium* pericarp tissue.

The intracellular localization of acid invertase was investigated using ripe epidermal tissue from fruit of *L. esculentum* FM 6203 and *L. pimpinellifolium* PI 126436 (Fig. 5). Immunogold labelling of acid invertase protein was significantly ($P < 0.1$) higher in the cell wall and vacuole of the epidermal tissue from both *L. esculentum* and *L. pimpinellifolium* PI 126436 than in comparable experiments using preimmune serum instead of the invertase antiserum (Table 2). There was no significant increase in label in the cytoplasm compared to the preimmune serum incubations (Table 2).

In *L. esculentum*, significantly more ($P < 0.01$) invertase protein was located in the vacuole than in the cell wall of the

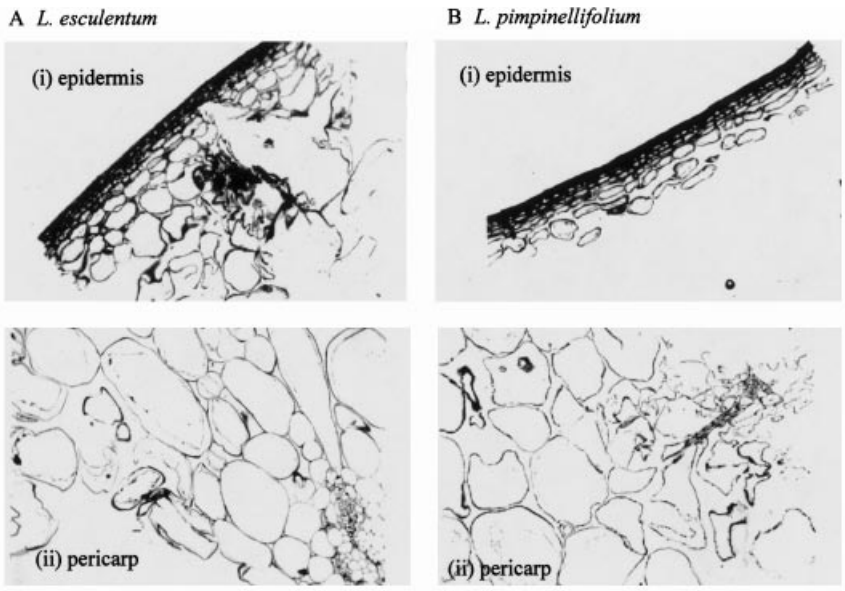

Fig. 4 Light micrographs of tomato fruit tissue. Light microscope sections of epidermal (i) and pericarp tissue (ii) from *Lycopersicon esculentum* FM 6203 and *Lycopersicon pimpinellifolium* PI 123436 (magnification × 100).

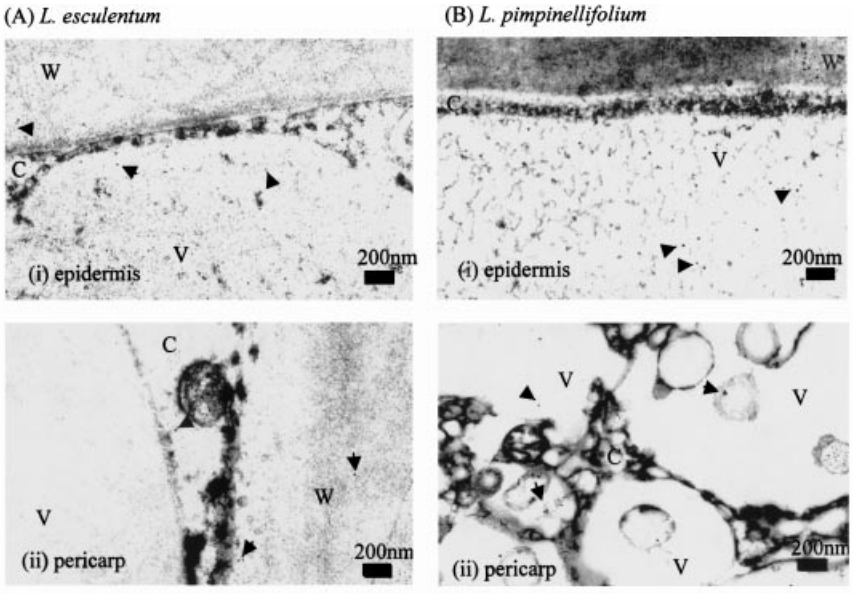

Fig. 5 Immuno-gold labelling of invertase protein in tomato fruit tissue. Invertase protein was detected in epidermal (i) and pericarp tissue (ii) from *Lycopersicon esculentum* FM 6203 and *Lycopersicon pimpinellifolium* PI 123436 using gold particles, indicated by arrowheads. Abbreviations: C, cytoplasm; W, cell wall; V, vacuole.

Table 2 Quantitative analysis of electron microscopy

		L. esculentum FM 6203			*L. pimpinellifolium* PI 126436		
Tissue		Cell wall	Cytoplasm	Vacuole	Cell wall	Cytoplasm	Vacuole
Pericarp	Immune minus preimmune	n.d	0.12 ± 0.055	0.82 ± 0.35*	n.d		0.48 ± 0.14*
Epidermis	Immune minus preimmune	0.71 ± 0.25*	0.56 ± 0.44	2.01 ± 0.95*	1.16 ± 0.47*	0.13 ± 0.093	1.02 ± 0.41*

Fresh ripe epidermal and pericarp from *Lycopersicon esculentum* FM 6203 and *Lycopersicon pimpinellifolium* PI 126436 were fixed, embedded, sectioned, subjected to antigenic detection for the acid invertase protein as described in the text. Electron micrographs were photocopied on to paper of known weight, vacuolar, cytoplasmic and cell wall compartments cut out and weighed and the number of gold particles in each compartment counted. In the case of *Lycopersicon impinellifolium* pericarp tissue, vacuole and cytoplasmic compartments could not be separated. The concentration of invertase in each compartment was estimated from the number of gold particles per μm^2 (cut surface area) and values are the mean from between 10 and 23 values ± standard error (n.d = below the level of detection).* indicates a significant ($P < 0.1$) difference.

epidermal cells (Table 2) whereas in *L. pimpinellifolium* PI 126436 epidermis, there was no significant difference between invertase protein in the cell wall and vacuole (Table 2). Immuno-gold labelling was significantly ($P < 0.1$) higher with the antiserum than the preimmune serum in this fraction. The cell wall compartment was clearly distinct in these tissues (Fig. 5) but there no significant difference in immuno-gold labelling was detected between the immune and preimmune treatments.

Discussion

The present study provides a comprehensive description of the distribution of invertase protein in tomato fruit. To ensure the specific detection of invertase on the tissue blots with the Y-24-A antibodies, a rigorous blocking procedure with preimmune serum, was undertaken prior to addition of antibodies. Furthermore, the Y-24-A antibodies were demonstrated to detect only the multiple forms of invertase and peptides derived from invertase in both tomato leaves (Kingston-Smith & Pollock, 1996; Kingston-Smith *et al.*, 1998) and fruit (Husain, 1999). The immuno-localization studies were used to detect the presence of invertase protein only and they do not distinguish between cell wall and soluble forms. They provide a relatively unequivocal determination of the presence and intracellular localization of invertase during fruit development.

Invertase protein was found in both of the acidic compartments (vacuole and apoplast) of the epidermal and pericarp cells. As the pericarp tissue was less amenable to this type of analysis we can only discuss the data from the epidermis tissue in detail. Invertase was localized in the cell wall and vacuoles of epidermal cells in both cultivars (Fig. 5). No invertase was detected in the cytoplasmic fraction in any of the tissues analysed. Cell wall invertase has been detected previously in pericarp tissue of *L. esculentum* (Iki *et al.*, 1978) and in the vacuole in *L. esculentum* and *L. pimpinellifolium* cultivars (Konno *et al.*, 1993). Many factors may account for the apparent differences in data obtained between studies reported in the literature. These may be related, for example, either to the extraction procedures or to the specificity of the different antibodies used in each study. In the studies of *L. esculentum* invertase, Yelle *et al.* (1991) and Klann *et al.* (1996) who used partially purified invertase and antibodies either to the carrot invertase (Yelle *et al.*, 1991) or the TIV1 tomato sequence (Klann *et al.*, 1996) found that the invertase protein increased with fruit development. Significantly, Klann *et al.* (1996) and Schaffer & Petreikov (1997) used the same antibodies raised against a sequence from tomato invertase, TIV1, but obtained different results, suggesting that the extraction procedures also influence the results. In the tissue blots presented in Fig. 3(b), protein was transferred directly on to the filters and invertase protein is clearly detectable in the green fruit.

In this study, large amounts of invertase protein were present in red fruit from all three cultivars in the epidermis, pericarp and central tissue (Fig. 3). At this stage, the highest amounts of invertase activity were measured (Table 1) as discussed in Husain *et al.* (1999). Surprisingly, large amounts of invertase protein were detected in green fruit, where there was very little or no measurable invertase activity (Table 1; Husain *et al.*, 1999). While this agrees with the observations of Schaffer & Petreikov (1997) on *L. esculentum*, it differs from the results obtained by Yelle *et al.* (1991) and Klann *et al.* (1996). Using SDS-PAGE, Schaffer & Petreikov (1997) detected invertase protein in the pericarp, placental and gelatinous tissue of green fruit. Substantial amounts of invertase protein were detectable in green fruit from *L. esculentum* and *L. pimpinellifolium* cultivars. This observation implies that invertase activity is controlled at the protein level rather than gene expression.

Extractable invertase activity was lower in green fruit than in red fruit. This was not due to differential inactivation of invertase during extraction as determined by recovery experiments on both green and red fruit (Husain, 1999). The occurrence of two isoforms of invertase inhibitor have been demonstrated at early stages of fruit development (Godt & Roitsch, 1997; Greiner *et al.*, 2000), suggesting that the difference in activity between developmental stages observed in this study is due to changes in the amounts of invertase inhibitor protein. We suggest that the measured differences in invertase activity throughout development (Husain *et al.*, 1999) are due to changes in the activation state of the protein rather than changes in the absolute amount of protein. While *L. esculentum* has lower soluble acid invertase activity and lower sugar accumulation than *L. pimpinellifolium* (Husain *et al.*, 1999), the content and distribution of invertase protein is remarkably similar in fruit from these species throughout development. This suggests that rigorous control of invertase activity rather than protein content accounts for measured differences of activity throughout fruit development. Post-translational regulation of acid invertases via inhibitory proteins was postulated 40 yr ago (Schwimmer *et al.*, 1961), but it is only recently that the invertase inhibitor genes have been cloned from species such as potato, tobacco and tomato (Pressey, 1994; Greiner *et al.*, 1999, 2000). We are currently investigating the developmental changes in the production of the tomato invertase inhibitor to determine whether the presence of these proteins accounts for discrepancies between invertase protein content and activity.

Acknowledgements

This paper is dedicated to the memory of Professor Tom ap Rees, who initiated this work. S. E. Husain is deeply indebted to Tom, who died tragically in the first year of her Ph.D. studies from which this manuscript is derived. This work was supported by the BBSRC in the form of a CASE Studentship in Collaboration with the Plant Breeding Institute (Monsanto/

Unilever). The authors thank Dr Robert Shields (Plant Breeding Institute, Cambridge) for critical discussion of this manuscript.

References

Benhamou N, Grenier J, Chrispeels M. 1991. Accumulation of β-fructosidase in the cell walls of tomato roots following infection by a fungal wilt pathogen. *Plant Physiology* **97**: 739–750.

Cairns AJ, Ashton JE. 1991. The interpretation of *vitro* measurements of fructosyl transferases activity: and analysis of patterns of fructosyl transfer by fungal invertase. *New Phytologist* **118**: 23–24.

Carter Rowley J, Moran DT. 1975. A simple procedure for mounting wrinkle-free sections on formvar-coated slot grids. *Ultramicroscopy* **1**: 151–155.

Chengappa S, Guilleroux M, Shields R. 1999. Transgenic tomato plants with decreased sucrose synthase are unaltered in starch and sugar accumulation in the fruit. *Plant Molecular Biology* **40**: 213–221.

D'Aoust M-A, Yelle S, Nguyen-Quoc B. 1999. Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *Plant Cell* **11**: 2407–2418.

Elliot KJ, Butler WO, Dickinson CD, Konno Y, Vedvick TS, Fitzmaurice L, Mirkov TE. 1993. Isolation and characterisation of fruit vacuolar invretase genes from two tomato species and temporal deifferences in mRNA levels during fruit ripening. *Plant Molecular Biology* **21**: 515–524.

Faye L, Moutassim B, Ghorbal A. 1986. Cell wall and cytoplasmic isozymes of radish β-fructosidase have different N-linked oligosaccharides. *Plant Physiology* **80**: 27–33.

Godt DE, Roitsch T. 1997. Regulation and tissue specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. *Plant Physiology* **115**: 273–282.

Gordon AJ, Thomas BJ, Reynolds PHS. 1992. Localization of sucrose synthase in soybean root nodules. *New Phytologist* **122**: 35–44.

Greiner S, Köster Lauer K, Rosenkranz H, Vogel R, Rausch T. 2000. Plant invertase inhibitors: expression in cell culture and during plant development. *Australian Journal of Plant Physiology* **27**: 807–814.

Greiner S, Krausgrill S, Rausch T. 1998. Cloning of a tobacco apoplasmic invertase inhibitor. *Plant Physiology* **116**: 733–742.

Greiner S, Rausch T, Sonnewald Herbers K. 1999. Ectopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers. *Nature Biotech* **17**: 708–711.

Husain SE. 1999. *The role of invertase in the carbohydrate metabolism of tomato fruit.* PhD thesis, University of Cambridge, UK.

Husain SE, Harrison J, ap Rees T, Shields R, Foyer CH. 1999. The role of invertase in carbohydrate metabolism of tomato fruit. *Acta Hort* **487**: 77–84.

Iki K, Segiguchi K, Kurata K, Tada T, Nakagawa H, Ogura N, Takehana H. 1978. Immunological properties of β-fructofuron-osidase from ripening tomato fruit. *Phytochemistry* **17**: 311–312.

Karnovsky MJ. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for the use in electron microscopy. *Journal of Cell Biology* **27**: 137a–138a.

Kenna JG, Major GN, Williams RS. 1985. Methods for reducing non-specific antibody binding in enzyme-linked immunosorbent assay. *Journal of Immunological Methods* **85**: 409–419.

Kingston-Smith AH, Gattier N, Pollock CJ, Foyer CH. 1998. Soluble acid invertase activity in leaves is independent of species differences in leaf carbohydrates, diurnal sugar profiles and paths of phloem loading. *New Phytologist* **139**: 283–292.

Kingston-Smith AH, Pollock CJ. 1996b. Tissue level localisation of acid invertase in leaves: an hypothesis for the regulation of carbon export. *New Phytologist* **134**: 423–432.

Klann EM, Chetelat RT, Bennett AB. 1993. Expression of acid invertase gene controls sugar composition in tomato (*Lycopersicon*) fruit. *Plant Physiology* **103**: 863–870.

Klann EM, Hall B, Bennett AB. 1996. Antisense acid invertase (*TIV1*) gene alters soluble sugar composition and size in transgenic tomato fruit. *Plant Physiology* **112**: 1321–1330.

Konno Y, Vedvick T, Fitzmaurice L, Mirkov TE. 1993. Purification, characterisation, and subcellular localization of soluble invertase from tomato fruit. *Plant Physiology* **141**: 385–392.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685.

Manning K, Maw GA. 1975. Distribution of acid invertase in the tomato plant. *Phytochemistry* **14**: 1965–1969.

Pressey R. 1994. Invertase inhibitor in tomato fruit. *Phytochemistry* **36**: 543–546.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208–213.

Schaffer AA, Petreikov M. 1997. Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. *Plant Physiology* **113**: 739–746.

Schwimmer S, Makower RU, Rorem ES. 1961. Invertase and invertase inhibitor in potato. *Plant Physiology* **36**: 313–316.

Stommel JR. 1992. Enzymic components of sucrose accumulation in the wild tomato species *Lycopersicon peruwanium. Plant Physiology* **99**: 324–328.

Sturm A. 1999. Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiology* **121**: 1–7.

Tang G-Q, Lüscher M, Sturm A. 1999. Antisense repression of vacuolar and cell wall intertase in transgenic carrot alters early plant development and sucrose partitioning. *Plant Cell* **11**: 177–189.

Walker RP, Pollock CJ. 1993. The purification and characterization of soluble acid invertase from coleoptiles of wheat (*Triticum aestivum* L. cv. Avalon). *Journal of Experimental Botany* **44**: 1029–1037.

Yelle S, Chetelat RT, Dorais M, DeVerna JW, Bennett AB. 1991. Sink metabolism in tomato fruit: 4. Genetic and biochemical analysis of sucrose accumulation. *Plant Physiology* **95**: 1026–1035.