Subcellular localization of peroxidase in tomato fruit skin and the possible implications for the regulation of fruit growth

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Abstract

The cessation of tomato fruit growth has been associated with the appearance of three ‘wall-bound’ peroxidase isozymes in the skin of tomato fruit. However, the presence of these isozymes in the ionically eluted ‘wall-bound’ fraction may be an artefact of either non-specific binding of symplastic peroxidase to the cell wall, or isozymes bound to membranes included in the ‘wall-bound’ fraction. Therefore, subcellular localization of peroxidase in both immature and mature tomato fruit skins was studied. Immature fruits showed intense peroxidase activity associated with the tonoplast and pro-vacuolar membranes, but little or no activity associated with the cell wall. However, the presence of peroxidase activity within the cell wall of mature green fruits was confirmed. Furthermore, peroxidase activity was also observed associated with the plasma membrane and large vesicles allied to the plasma membrane. While cross-linking in cell wall components was previously assumed to be the mechanism by which peroxidase might control fruit growth, the incorporation of ‘lignin-like’ phenolics may also play a part. Isoelectric focusing (IEF) of both symplastic and apoplastic peroxidase extracted from immature and mature tomato fruit skin showed that all peroxidase isozymes present were highly anionic. In this current study, histochemical techniques are used to demonstrate a developmental increase in ‘lignin-like’ phenolics within the sub-cuticular cell walls of the fruit skin. The localization of peroxidase within tomato fruit skin is discussed in relation to its potential role in the regulation of tomato fruit growth.

Key words: Fruit growth, localization, Lycopersicon esculentum, peroxidase, tomato.

Introduction

In tomato, as in many other horticultural crops, fruit size is one of the key determinants for both quality and yield, as fruits are size-graded (Adams et al., 2001). The regulation of tomato fruit growth has attracted much research interest, which is reflected in the extent of information available on environmental, nutritional and photoassimilate regulation of fruit growth (Monselise et al., 1978; de Koning, 1984; Ho et al., 1987; Bussières, 1993; Grange and Andrews, 1994).

The pericarp of tomato fruit flesh is composed of three distinct tissue types: the endocarp, a unicellular layer encasing the locular cavity, the mesocarp, a multicellular layer of large, thin-walled parenchyma cells (>500 μm diameter) and vascular tissue, and the exocarp (fruit skin) which is composed of an outer epidermis with an ingressing thick waxy cuticle (Osman et al., 1999) and two to three layers of thick-walled hypodermal cells (Ho and Hewitt, 1986). Thompson et al. (1998) demonstrated that turgor-driven expansion of parenchyma cells within the mesocarp creates tissue pressure, which is expressed on the exocarp layer as tissue tension. They suggested that the mechanical properties of the exocarp were important in controlling fruit growth, and linked the cessation of fruit growth to a developmental increase in peroxidase activity associated with the exocarp. Andrews et al. (2000) demonstrated the developmental appearance of three new ‘wall-bound’ peroxidase isozymes that may be responsible for this increase in peroxidase activity.

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Peroxidase (EC 1.11.1.7) is an oxidoreductase that is known to catalyse the oxidation of numerous substrates through the associated reduction of hydrogen peroxide (Dawson, 1988; Wallace and Fry, 1999). Given the ubiquitous presence of peroxidase throughout nature (Vámos-Vigyázó, 1981) and the number of substrates that may potentially be oxidized in the presence of peroxidase, it seems unlikely that a single peroxidase will catalyse a single specific reaction in vivo. In plants, the main roles of peroxidase are attributed to lignification and suberization (Espelie et al., 1986; Roberts et al., 1988; Mäder et al., 1997; Quiroga et al., 2000), cutin deposition in outer aerial epidermal layers (Fry, 1986; Brownleader et al., 1999; Hatfield et al., 1999), Peroxidase catalysed cross-linking in cell walls is thought to result from the formation of diferuloyl bridges between pectin residues, and isodityrosine bridges between hydroxyproline-rich extensin molecules (Fry, 1986; Brownleader et al., 1999; Hatfield et al., 1999).

The use of high ionic strength buffers to elute ‘wall-bound’ peroxidases is a standard procedure used to examine ionically bound cell wall enzymes (Ferrer et al., 1991; Andrews et al., 2000; Quiroga et al., 2000). However, this procedure may not distinguish between peroxidases bound to the cell wall and those bound to other membranes such as the plasma membrane or tonoplast, or symplastic peroxidases that may bind non-specifically to exposed cell walls when released from the cytosol during sample maceration. The in vivo function of a particular peroxidase is likely to be determined by its location in a particular tissue, cellular or subcellular compartment, which, in turn, may depend in part on its ionic nature (Chibbar and van Huystee, 1984; Mäder et al., 1986; García-Florenciano et al., 1991; Ros Barceló et al., 1991; Carpin et al., 2001). Here, using transmission electron microscopy (TEM), the subcellular localization of peroxidase in the exocarp from both immature and mature green tomato fruit is shown, together with the characterization of the isoelectric point (pI) of peroxidase isozymes from a developmental range of fruit. The ionic nature of individual peroxidase isozymes may indicate possible binding sites within the cell wall, whilst also allowing comparison with peroxidase isozymes characterized in other systems (Espelie et al., 1986). Furthermore, fluorescent histochemical techniques are used to highlight the presence of ‘lignin-like’ phenolics in the tomato fruit exocarp and their possible roles in defence and the rheological properties of the cell wall are discussed (Lulai and Morgan, 1992; Schopfer et al., 2001).

Materials and methods
Plant material
Plants of Lycopersicon esculentum Mill. cv. Espero were grown as a conventional long-season glasshouse crop following normal commercial practice (Adams et al., 2001).

Peroxidase cytochemistry
To establish the subcellular origin of the ‘soluble’ and ‘wall-bound’ peroxidase fractions from tomato fruit exocarp (Andrews et al., 2000), 3,3-diamino benzidine (DAB) an electron-dense peroxidase stain was employed (Bestwick et al., 1998). Fruit exocarp samples (2x4 mm) from three replicate fruits at each of two developmental stages, 14 d and 45 d post-anthesis (dpa), were excised directly into fixative, 1% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in buffer A (50 mM sodium cacodylate buffer pH 7.0) at room temperature for 60 min. This was followed by two subsequent 10 min washes in buffer A, and a 30 min rinse in buffer B (50 mM potassium phosphate buffer containing 20 mM 3-amino-1, 2, 4-triazole (ATZ)). ATZ, was included in all subsequent buffers to inhibit peroxidative artefacts resulting from any in vivo catalase activity (EC 1.11.1.6 H2O2:H2O2 oxidoreductase). Fixed samples were transferred to a peroxidase activity staining solution containing buffer B, with 0.5 mg ml−1 DAB and 5 mM hydrogen peroxide or one of two-control solutions (I) buffer B, without DAB, (II) buffer B, with 0.5 mg ml−1 DAB. While potassium cyanide (KCN) is commonly used as an inhibitor in DAB-stained peroxidase localization studies, it is not a specific peroxidase inhibitor and specific peroxidases may vary in their sensitivity to KCN (Barceló et al., 1991). Therefore, KCN was not used as a control in the current study. Preliminary studies showed 45 min to be an optimal staining time; however, a 15 min staining time was included as a precaution against over-staining. All staining procedures were conducted in the dark under mild vacuum conditions to exclude auto oxidation of DAB and improve penetration into the tissue. Staining was followed by two 10 min washes in buffer B, prior to fixation in 1% osmium tetroxide in buffer A, for 45 min. Samples were then washed for 10 min, twice in buffer B, and twice in double distilled water. They were then sequentially dehydrated for 15 min in each of 30%, 50%, 70%, 80%, and 90% ethanol, followed by three 20 min washes in 100% ethanol.

Spurr’s resin embedding
Spurr’s embedding requires complete tissue dehydration (Spurr, 1969). Therefore, two further changes of 100% ethanol were conducted, each for 24 h. Samples were infiltrated through an ascending concentration of resin diluted in ethanol: 25%, 50%, 75%, 100%. The first three steps took 1.5 d the final step lasted 2–3 d with a change into fresh resin after ~8 h. The samples were embedded in freshly prepared 100% Spurr’s resin, and polymerized at 80 °C for 8 h. Ultrathin sections (~70 nm) were cut on a Reichert Ultracut E ultramicrotome, stained with uranyl acetate followed by Reynolds’ lead citrate (Reynolds, 1963) and viewed in a JEOL 100CX TEM at 80 kV. Spurr sections (~2 μm) were also prepared for light microscopy, and were stained with aqueous 0.5% toluidine blue.

Histochemical staining of ‘lignin-like’ phenolics within tomato fruit exocarp
Strips of pericarp tissue were excised from each of two green stages of tomato fruit development (14 dpa and 30 dpa). The strips were blotted on filter paper to remove excess moisture and mounted in a cryostat embedding compound (Tissue-Tek OCT; Agar Scientific, Stansted, UK) and sectioned at ±30 °C using a cryostat microtome (Bright Ltd, Huntingdon, Cambridgehire, UK). Sections were mounted on glass slides and air-dried prior to staining with 0.1% berberine which stains ‘lignin-like’ phenolics, 0.02% ruthenium red.
was employed as a counter stain (Lulai and Morgan, 1992). Sections were visualized using an Leitz Dialux 20 fluorescence microscope (with a leitz filter block ‘G’) and images were captured and analysed using an image analyser (Optimas version 6.1, Optimas Corporation, Seattle, Washington, USA).

**Ionic elution of peroxidase from cell walls**

Exocarp strips were excised from the equatorial region of a developmental range of proximal fruit 14, 21, 28, 35, 42, 49, and 56 dpa, using a razor blade. Approximately 50 mg was taken from each fruit. This, together with an equal weight of acid-washed quartz silica sand (Sigma, Poole, Dorset, UK), was ground thoroughly in a pestle and mortar in the presence of liquid nitrogen. The macerated sample was suspended in ice-cold 10 mM sodium acetate/citric acid buffer, pH 6.0, using 100 μl mg⁻¹ of the original fresh weight (OFW) of tissue. The suspension was centrifuged at 3000 g for 15 min at 3 °C. The resulting supernatant represented the soluble (symplastic) fraction. The pellet was resuspended in the same buffer and again thoroughly mixed prior to centrifugation. This process was repeated eight times to ensure all the soluble peroxidase activity had been eluted from the sample. Supernatant from the final wash was assayed for residual peroxidase activity using 3,3’,5,5’-tetramethylbenzidine (TMB) as previously described (Andrews et al., 2000).

The washed pellet was resuspended in ice-cold 100 mM sodium acetate/citric acid buffer, pH 6.0, containing 1 M NaCl (100 μl mg⁻¹ OFW). The suspension was mixed thoroughly and incubated on ice for 60 min with periodic mixing. The resulting supernatant contained the salt-extractable peroxidase which may represent in vivo for 60 min with periodic mixing. The resulting supernatant represented the soluble (symplastic) fraction. The pellet was resuspended in the same buffer and again thoroughly mixed prior to centrifugation. This process was repeated eight times to ensure all the soluble peroxidase activity had been eluted from the sample. Supernatant from the final wash was assayed for residual peroxidase activity using 3,3’,5,5’-tetramethylbenzidine (TMB) as previously described (Andrews et al., 2000).

**Isoelectric focusing**

Preliminary isoelectric focusing (pI range 3.5–9.3) showed that all symplastic and apoplastic peroxidase isozymes extracted from a developmental range of tomato fruit exocarp are highly anionic with pI values less than 3.5. However, pI values could not be determined accurately as they were below the resolution of the pre-cast gel (Amersham Pharmacia Biotech pH 3.5–9.5). Therefore, an IEF gel was prepared using Pharmalyte ampholytes ranging from pI 2.5–5.0 (Sigma, Poole, Dorset, UK), as per the manufacturer’s instructions (Amersham Pharmacia Biotech, Bucks, UK). Dialysed ‘wall-bound’ extracts were prepared (as above) from seven developmental stages of tomato fruit (exocarp 14, 21, 28, 35, 42, 49, and 56 dpa). To confirm the developmental appearance of three new peroxidase isozymes, 10 μl samples of each extract were run on native PAGE as previously reported (Andrews et al., 2000), prior to application on the IEF gel. Resolution of the isoelectric points of four peroxidase isozymes was conducted at 7.5 °C on a Multiphor II flatbed electrophoresis unit, using a low range (pI 2.8–6.5) pI marker kit as per the manufacturer’s instructions (Amersham Pharmacia Biotech, Bucks, UK). Peroxidase activity and the low range pI markers were visualized using freshly prepared chloronaphthol followed by 0.1% Coomassie blue R-250, respectively (Andrews et al., 2000).

**Results**

**Peroxidase cytochemistry**

TEM observations from immature tomato fruit exocarp showed a lack of peroxidase activity associated with the cell wall (Fig. 1A, B). However, intense peroxidase activity was associated with the tonoplast, pro-vacuolar membrane and a few particulates within the vacuole (Fig. 1A, B). Mature fruit sections confirmed the presence of peroxidase activity within the inner edge of the cell wall (Fig. 2) and occasionally near the middle lamella (Fig. 3). No activity was observed in the cuticle of the outer epidermal layer. By contrast to immature fruit, mature fruit exocarp cells have proportionally larger vacuoles with numerous particulates, less cytoplasm, no pro-vacuolar vesicles, and little or no activity associated with the tonoplast (Fig. 3). Mature fruit also had intense peroxidase activity staining associated with the plasma membrane, particulates and ‘vesicle-like’ structures with membranes that sometimes appeared continuous with the plasma membrane (Fig. 3). Endoplasmic reticulum was allied to the majority of ‘vesicle-like’ structures at the plasma membrane (Fig. 3). Image analysis of TEM photomicrographs showed that the ‘vesicle-like’ structures had diameters ranging from 1–2 μm. Neither peroxidase staining of the plasma membrane nor the presence of ‘vesicle-like’ structures were observed in sections from...
immature tomato fruits (Fig. 1A, B). Control sections with and without DAB showed no electron-dense staining due to auto-oxidation of DAB (images not presented).

**Histochemical localization of ‘lignin-like’ phenolics within tomato fruit exocarp**

Light microscope sections prepared from mature tomato fruit exocarp were stained with 0.5% toluidine blue (Fig. 4). The section illustrates the cellular organization of the exocarp and provides a reference point for the fluorescence observed in subsequent sections (Fig. 5A, B). Furthermore, the section demonstrates the extent of cuticular ingress from the outer epidermis into the hypodermal layers of the fruit exocarp. Fruit exocarp

![Fig. 2. Mature fruit exocarp cell (45 dpa), showing intense DAB staining of the inner edge of the cell wall (CW), and the plasma membrane (PM). The cytoplasm, shows little or no DAB staining, with no activity associated with organelles such as mitochondria (M), chromoplasts (CH) and the nucleus (N). The tonoplast (T) bordering the vacuole (V) shows little or no DAB staining compared with the tonoplast of immature tomato fruit (Fig. 1A, B). Calibration bar=1 μm.](image1)

![Fig. 3. Mature fruit exocarp cells (45 dpa). DAB staining of membranes of ‘vesicle-like’ structures (VE) associated with the plasma membrane (PM) and the vacuole (V). DAB staining is also located in areas of the plasma membrane (PM), cell wall (CW) allied to the ‘vesicle-like’ structures (VE) and tentatively in the middle lamella (ML). The vacuole (V) also contains partially degraded vesicle-like structures (PVE), and particulates (PA), which show residual amounts of DAB staining. Little or no DAB staining was observed in organelles such as the chromoplasts (CH). Calibration bar=1 μm.](image2)

![Fig. 4. Mature fruit exocarp (45 dpa) stained with both DAB and 0.1% toluidine blue and viewed under a light microscope. The ingressing cuticle (CU) as distinct from the cell wall (CW) can clearly be seen within both the outer epidermis (EP) and the hypodermal layer (HY). Calibration bar=10 μm.](image3)

![Fig. 5. (A) Immature green fruit (14 dpa) exocarp stained for ‘lignin-like’ phenolics with berberine/ruthenium red. The intensity of fluorescent staining in the outer epidermis is low and specific detail cannot be observed. (B) Mature green fruit (50 dpa) exocarp stained for ‘lignin-like’ phenolics with berberine/ruthenium red. The fluorescent staining is intense within the outer epidermal cell wall (CW), but not in the cuticle (CU). Little or no fluorescent staining was observed in the underlying hypodermal layer (HY). Calibration bars=10 μm.](image4)
sections stained for the aromatic (lignin) domain of suberin using berberine and counter-stained with ruthenium red, showed fluorescence in the epidermal layer of the exocarp (Fig. 5A, B). The degree and intensity of fluorescence from berberine staining was greater in exocarp sections from mature fruit (Fig. 5B) compared with those from immature fruit (Fig. 5A). In mature fruits, staining was largely associated with the sub-cuticular cell walls of the epidermis, in comparison to the limited staining of the cuticle (Figs 4, 5A). The low intensity of staining within the exocarp of immature fruit (Fig. 5A) made further localization of stain speculative.

**Isoelectric focusing**

Figure 6A illustrates isoelectric focusing of ‘wall-bound’ peroxidase isozymes extracted from seven developmental stages of tomato fruit exocarp on an IEF gel pH range 2.5–5.0. Samples from immature fruit (14–21 dpa) show a single peroxidase isozyme with an apparent pI value of 3.1. At 28 dpa a further peroxidase isozyme is detected, with an apparent pI value of 3.9. Further peroxidase isozymes are detected at 35 dpa and 49 dpa with pI values of 2.9 and 3.5, respectively. Native PAGE of the same developmental range of wall-bound peroxidase (Fig. 6B) also illustrates the appearance of four peroxidase isozymes at fruit maturation. By correlating the age of fruit at which these bands first appeared with those detected on the IEF gel, it seems likely that bands A, B, C, and D (Fig. 6B) have isoelectric points of 3.1, 3.9, 2.9, and 3.5 (Fig. 6A), respectively.

**Discussion**

‘Soluble’ and ‘wall-bound’ peroxidase activity was previously reported in the skin of immature tomato fruits (Andrews et al., 2000), both fractions contained a single isozyme (58 kDa). However, these localization studies have shown that peroxidase activity was associated with the tonoplast and pro-vacuolar membranes in the exocarp of immature fruits, with little or no obvious ‘wall-bound’ activity. This apparent contradiction may be due to either non-specific binding of liberated symplastic peroxidase to exposed cell walls during sample preparation, which appears unlikely (Andrews et al., 2000), or peroxidase binding to the tonoplast or pro-vacuolar vesicle membrane, that pelleted on centrifugation with the cell wall fraction. Peroxidase isozymes present in the ‘soluble’ fraction may represent precursors readily released from the tonoplast and cell periphery on tissue homogenization, and more strongly bound isozymes may be liberated on ionic elution and may represent the ‘wall-bound’ fraction. Similar observations and conclusions were drawn for peroxidase found in maize root tips. Furthermore, peroxidase activity associated with the tonoplast and pro-vacuolar membranes was thought to have a role in the formation of the tonoplast and vacuole (Parish, 1975).

Peroxidase activity was observed in the cell walls of mature tomato fruit exocarp cells. It was apparent that peroxidase activity was mainly located in the inner regions of the cell wall with occasional activity observed within the middle lamella. The presence of ‘wall-bound’ peroxidase activity in mature fruit confirms earlier findings and supports the notion that peroxidase mediated ‘stiffening’ of the exocarp cell walls leads to the cessation of fruit growth (Thompson et al., 1998; Andrews et al., 2000). Furthermore, the lack of peroxidase activity associated with the cuticle strengthens the hypothesis that peroxidase is not involved in cuticle formation in tomato fruit (Andrews et al., 2000). Peroxidase activity associated with particulates, ‘vesicle-like’ structures and the plasma membrane, may contribute either to the ‘soluble’ or the ‘wall-bound’ fraction, or both. The origin of both ‘soluble’ and ‘wall-bound’ activity can only be speculated upon, except when peroxidase activity is clearly identified within the cell walls of mature fruit exocarp.

Whilst it is generally accepted that both exocytosis and endocytosis occur within plant cells, it is not possible to distinguish between these processes through examination of TEM photomicrographs (Battey et al., 1999). However, the observation that both intact and partially degraded ‘vesicle-like’ structures appear to exist within the vacuole may indicate endocytotic trafficking from the plasma membrane into the vacuole as it seems unlikely that the vacuole will possess the ‘machinery’ for vesicle production. A similar phenomenon involving the transport of degradative enzymes by multivesicular bodies into the vacuole has previously been reviewed (Battey et al., 1999). However, the possibility that these ‘vesicle-like’ structures are an artefact of tissue preparation cannot be excluded.

Confirmation of wall-bound peroxidase activity within the cell walls of mature tomato fruit exocarp does not reveal the \textit{in vivo} function. However, the presence of
highly anionic peroxidase isozymes within the exocarp cell walls (pI 2.9–3.9) may indicate binding to positively charged components, for instance, the Ca\textsuperscript{2+} pectin matrix, which provides an integral component of the cell wall (Penel and Greppin, 1979; Ros Barceló \textit{et al}., 1989; Carpin \textit{et al}., 2001). These results contradict previous tentative measurements of a single pI value for all four peroxidase isozymes of 4.6 (Andrews \textit{et al}., 2000). The results presented here have been consistent throughout five repeats, and it is not possible to explain why an earlier preliminary measurement gave a pI value of 4.6.

The role of a highly anionic peroxidase in suberization of wounded potato tubers has been previously reported and appears to mediate deposition of aromatics in the cell wall (Espelie \textit{et al}., 1986), but is also present in wounded tomato fruits (Roberts \textit{et al}., 1988; Sherf and Kolattukudy, 1993). Two linked genes, \textit{tap1} and \textit{tap2}, were cloned and sequenced and shown to encode for this peroxidase activity (Robert \textit{et al}., 1988). However, gene expression of \textit{tap1} and \textit{tap2} has also been shown to increase 2-fold throughout development until the onset of the climacteric in tomato (Sherf and Kolattukudy, 1993). There are remarkable similarities when comparing this peroxidase (45 kDa, pI 3) with some of the isozymes reported here (pI 2.9–3.9, with bands A–D having apparent molecular weights of 43–58 kDa (Andrews \textit{et al}., 2000).

The developmental increase of aromatics associated with tomato fruit cuticle development was reported previously (Hunt and Baker, 1980). The major component of the fruit cuticle is cutin, composed of hydrophobic aliphatic fatty acids (Osman \textit{et al}., 1999). Previous studies have demonstrated the deposition of specific flavonoids into the cuticular membranes of the fruit epidermis during fruit ripening (Fernández \textit{et al}., 1999). However, this study has shown the presence of subcuticular ‘lignin-like’ phenolics within the epidermal cell walls of green tomato fruit exocarp. The presence of such an aromatic domain normally associated with lignin and suberin deposition may also provide resistance to pathogen infection (Lulai and Corsini, 1998). Relatively few pathogens affecting the fruit of tomato appear to gain entry into the fruit through the fruit skin. Those that do, appear to enter when the fruit are immature and the skin is thin and poorly developed or through wound or growth cracks in older fruit. Therefore, the protective barrier presented by the fruit skin may be fundamental to resistance against localized pathogen attack (Watterson, 1986).

In conclusion, the developmental localization of highly anionic peroxidase isozymes within the cell walls of the exocarp layer supports earlier biochemical observations on the activity of peroxidase during fruit development (Thompson \textit{et al}., 1998; Andrews \textit{et al}., 2000). Peroxidase activity was localized throughout the exocarp tissue layer, whereas ‘lignin-like’ phenolics were restricted to the epidermis. This suggests that it is unlikely that the isozymes present are involved solely in the deposition of aromatics. It is suggested that peroxidase isozymes located within the outer fruit exocarp may have a dual role in restricting fruit expansion through cross-linking of cell wall components and producing a protective barrier in the epidermis.

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