Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: a new potential cross-link

Jeffrey D. BRADY*†, Ian H. SADLER† and Stephen C. FRY*

* Institute of Cell and Molecular Biology and † Department of Chemistry, University of Edinburgh, The King's Buildings, Edinburgh EH9 3JH, Scotland, U.K.

A novel amino acid, di-isodityrosine, has been isolated from hydrolysates of cell walls of tomato cell culture. Analysis by UV spectrometry, partial derivatization with 2,4-dinitrofluorobenzene and mass and NMR spectrometry show that the compound is composed to two molecules of isodityrosine, joined by a biphenyl linkage. The possible reactions involved in the formation of this molecule *in vivo* are discussed, as is the possibility that it could form an interpolypeptide linkage between cell wall proteins such as extensin, and hence aid in the insolubilization of the protein in the wall.

INTRODUCTION

The structural proteins of the plant cell wall have been divided into five classes (reviewed in [1]), and of these the extensin family is the most extensively studied. Extensins are characterized by their high pI and rod-like structure formed from the diagnostic Ser-Hyp-Hyp-Hyp-Hyp sequence, which typically appears repeatedly throughout the protein. The tetrahydroxyproline block, in which each hydroxyproline is often decorated with a tetra-arabinose block which stabilizes it in the polyproline II helix [2,3], gives the protein rigidity. Other common repeats in the sequence may act as recognition sites for cross-linking, for glycosylating enzymes or for areas of ionic interaction with other protein molecules and polysaccharides [4]. Transcription of extensin genes is regulated in a tissue-specific manner; extensins are more highly expressed in primary xylem, epidermis, cambium cells and phloem [5] as well as callus tissue [6]. Higher expression of extensins has also been observed on mechanical stimulation [7], wounding [8] and infection [9].

These highly basic glycoproteins rapidly become ionically bound to the network of polysaccharide material present in the cell wall and, over a period of hours, they become covalently bound and inextractable from the wall [10]. This cross-linking process is accelerated on infection-mediated elicitation, before increased extensin expression [11,12]. Early suggestions that the extensin covalently binds to the polysaccharide network appear to be unlikely, as removal of the polysaccharide material with anhydrous HF leaves the extensin network intact [13]. A probable cause of the covalent insolubilization of extensin is the formation of isodityrosine (Idt), an oxidatively coupled dimer of tyrosine (see Figure 4, compound a) [14]. It was suggested that the peroxidase-mediated coupling of tyrosine residues, to form Idt, would serve to cross-link different extensin polypeptides, forming an extensin network [15]. This hypothesis was supported by the fact that acidified sodium chlorite in the presence of BSA can break the ether linkage of Idt without cleaving peptide bonds and can release intact extensin molecules from the wall [16]. The hypothesis was further supported by the finding that the covalent insolubilization of the glycoprotein can be delayed by treatments likely to block oxidative coupling, e.g. with dithiothreitol or ascorbate [10,14,17]. A model has been proposed based on the extensin molecules being woven between the polysaccharide network [18] and thus adding to the overall strength of the wall. It has been suggested that the proline-rich and glycine-rich families of proteins, which both contain appreciable quantities of tyrosine, may also become insolubilized in the wall *via* tyrosine-derived cross-links [1,12].

However, studies involving the analysis of trypsin-derived peptides from the cell wall revealed the presence of an intrapolypeptide loop [19], with Idt formation from the two tyrosines in the sequence Tyr-Lys-Tyr. Idt in this form is not a cross-link between different polypeptides and could not directly contribute to the formation of an extensin network. No interpolypeptide Idt was isolated in this study although, as the authors state, peptides cross-linked by Idt may be difficult to extract by tryptic digestion. It is not known at present what proportion of Idt is in the form of intrapolypeptide loops.

The possibility of a trimeric tyrosine-based molecule has been raised in studies of cell wall hydrolysis [20,21]. It is likely that a trimer (or higher oligomer) of tyrosine could cross-link different extensin chains, even if the original dimer from which it formed were intrapolypeptide. In this study we investigate the possibility that other tyrosine-based molecules could serve as interpolypeptide cross-links.

MATERIALS AND METHODS

Materials

Analytical-grade chemicals were from BDH chemicals (Poole, Dorset, U.K.) as were the HPLC-grade solvents. Lys-Lys dipeptide was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) TLC plates were Merck F_{60} silica with fluorescent indicator purchased from BDH. UV spectra were recorded on a Beckman Du-64 spectrophotometer.

Preparation of tomato cell wall hydrolysate

Suspension-cultured tomato cells, a *Lycopersicon esculentum* × *Lycopersicon peruvianum* hybrid, maintained in Murashige and

Abbreviations used: Idt, isodityrosine; PAW, phenol/acetic acid/water (2:1:1, by vol.); BAW, butanol/acetic acid/water (12:3:5, by vol.); DNFB, 2,4-dinitrofluorobenzene; FC, Folin & Ciocalteu reagent; HVPE, high-voltage paper electrophoresis.

† To whom correspondence should be addressed.

Skoog medium (Sigma Chemical Co.) (subcultured weekly), were harvested by filtering through muslin, washed with distilled water and lyophilized. To remove non-covalently bound material from the dried cells (10 g), they were suspended in 800 ml of phenol/acetic acid/water (2:1:1, by vol) (PAW) and stirred at room temperature for 48 h. The cells were resuspended in fresh PAW every 48 h until the extract contained no protein detectable by an acetone precipitation test. The cell-wall-rich residue was collected on glass fibre and washed free of PAW with ethanol and finally, to aid drying, acetone. The dried cell walls (3.3 g) were hydrolysed in 6 M HCl containing 1 mM phenol (800 ml) by boiling under reflex for 18 h, with constant N_2 infusion. The hydrolysate was then filtered and dried under vacuum to remove the HCl and the phenol.

Fractionation of the hydrolysate by phosphocellulose chromatography

The dried hydrolysate was redissolved in water (250 ml), and 7 kBq of [^{3}H]Idt [22] was added as a marker. The hydrolysate was applied to a phosphocellulose cation-exchange column (Whatman P11; 80 ml; 2.4 cm internal diameter) previously equilibrated with water after precycling according to the manufacturer's instructions. The column was then washed with three 100 ml aliquots of 0.05 M HCl, three 100 ml aliquots of 0.1 M HCl, three 100 ml aliquots of 0.5 M HCl, one 100 ml aliquot of water and finally one 100 ml aliquot of 3.5% (w/v) ammonia solution. The eluates from these washes were collected as fractions 1 to 11. A small portion (100 μl) of each was assayed for ^{3}H by liquid-scintillation counting to find the [^{3}H]Idt marker (fractions 4 and 5).

Analysis of fractions and preparation of samples by paper chromatography and high-voltage paper electrophoresis (HVPE) at pH 2

Aliquots (500 μl) from each of the fractions 1–11 and the material that failed to bind to the phosphocellulose (fraction 0) were dried under vacuum, resuspended in water (10 μl) and chromatographed on Whatman 3MM paper in butanol/acetic acid/water (12:3:5, by vol.) (BAW). Alternatively, samples were subjected to HVPE at pH 2 [water/formic acid/acetic acid [45:1:4, by vol.)] [15] or at pH 6.5 [water/acetic acid/pyridine (450:2:50, by vol.)] at 2 kV for 50 min.

The chromatograms and electrophoretograms were examined under short-wavelength (254 mm) UV light and either stained in 0.5% ninhydrin or sprayed with Folin & Ciocalteu reagent for phenolics (FC) followed by exposure to ammonia vapour. For the preparative chromatography and HVPE the paper was dried and the sample eluted from the paper and dried under vacuum.

Purification by HPLC

HPLC was performed on a preparative reverse-phase C_{18} column (10 mm × 25 cm; Spherisorb ODS2; 5 μm). Solvent A was water and solvent B was methanol/dichloromethane (9:1 v/v). Solvent A was run at 100% for 5 min followed by a gradient to 35% B over 25 min at 4 ml/min. The eluate was monitored with an on-line UV detector at 280 nm.

TLC

TLC was performed on silica F_{60} plates, using propan-1-ol/27% ammonia (2:1, v/v).

Partial dinitrophenylation

To determine the number of amino groups of compound (**1**), an aliquot was derivatized with 2,4-dinitrofluorobenzene (DNFB) [23]. The sample (2.5 μg) was dissolved in 1% triethylamine (10 μl) and allowed to react with 10% (v/v) DNFB in acetone (2.5 μl). The reaction was stopped at 0.1, 1, 5 and 25 min by the addition of ice-cold diethyl ether (1 ml) to extract the changed DNFB. The ether phase was removed, 20 μl of water added to the aqueous phase and the extraction with diethyl ether repeated. The final aqueous phase was then dried and electrophoresed on Whatman (no. 1) in pH 0.6 buffer [90% formic acid/acetic acid/water (134:100:268, by vol)] on L-20 Shandon flat-bed electrophoresis equipment at 2 kV for 55 min. The sheet was then dried and examined for yellow spots from the dinitrophenylated amino groups and stained with ninhydrin to detect unchanged amino groups.

Analysis by electrospray mass spectrometry

Mass spectrometry was carried out at the Complex Carbohydrate Research Centre, Athens, Georgia, U.S.A., on a Sciex mass spectrometer by the electrospray method. The sample was injected in a solution in methanol/water (1:1, v/v).

RESULTS

Purification of compound (1)

An acid hydrolysate of tomato cell walls was passed through phosphocellulose with a stepwise [HCl] gradient and fractions (0–11) were analysed by paper chromatography in BAW (Table 1). Molecules with one or no amino groups, e.g. phenolic material and neutral and acidic amino acids, failed to bind to the column in water. Histidine was eluted in the last 0.05 M fraction. As the HCl concentration increased, lysine and Idt were eluted (fractions 4 and 5), binding more tightly as they contain two primary amino groups. Amino acids eluted after fraction 5 may contain more than two amino groups, for example the main constituent of fraction 7, compound (**1**) (R_F 0.03). Electrophoresis of a further portion of fraction 7 at pH 2 revealed a fluorescent FC-positive ninhydrin-positive spot (mobility relative to tyrosine, m_{Tyr}, 1.24), presumably the same compound as observed by paper chromatography. Compound (**1**) was partially purified from the bulk of fraction 7 by preparative paper chromatography followed by HVPE.

Compound (**1**) was then further purified by preparative HPLC, being eluted as a major UV-absorbing peak at 16 min. The homogeneity of the compound was further tested by TLC, giving a single fluorescent FC-positive spot with an R_F value of 0.18. The yield of the compound from 3.3 g of cell walls was 160 μg (estimated by peak area from the HPLC relative to known tyrosine samples) as compared with a yield of 240 μg of Idt from the same preparation (results not shown).

Compound (**1**) would appear not to be an artifact of the treatment as addition of [^{3}H]Idt to the cell wall material (on a smaller scale) before hydrolysis showed that none of the ^{3}H appeared with compound (**1**) (results not shown).

UV absorbance and fluorescence properties

The UV spectra at several pH values were recorded; below pH 6 the λ_{max} was 275 nm and at pH 7 and above the maximum shifted to 313 nm. This bathochromic shift is similar to that observed in dityrosine (see Figure 4, compound b; 280 nm to 312 nm [23]).

Table 1 Analysis of fractions from phosphocellulose chromatography by paper chromatography in BAW

Fraction	R_F of main spots	FC reaction*	Ninhydrin reaction	Fluorescence†	Tentative identification
0–2	Many	+	−	+	Phenolic material
	Many	−	+	−	Neutral and acidic amino acids
3	0.19	−	+	−	Histidine
4–5	0.18	−	+	−	Lysine
	0.34	+	+	−	Idt
6	None detected				
7	0.03	+	+	+	Tyrosine derivative (> 2 amino groups) (**1**)
8–10	None detected				

* The reaction with Folin and Ciocalteu's reagent for phenols.
† A vivid blue fluorescence was observed when viewed under short-wave (254 nm) UV light.

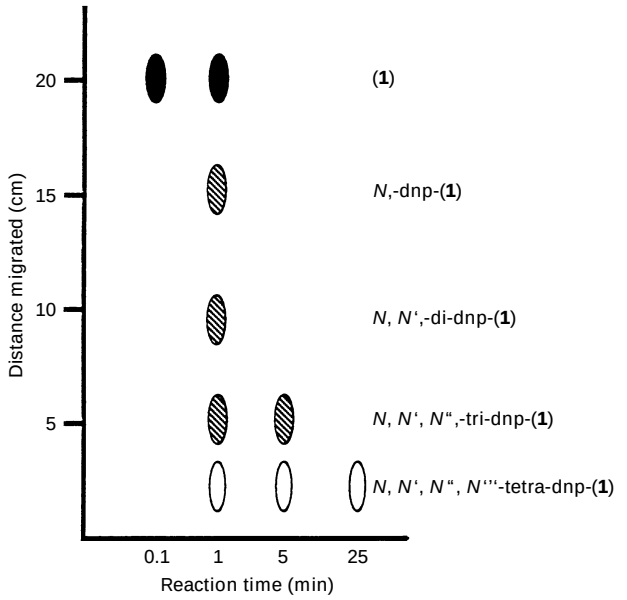

Figure 1 Schematic representation of the electrophoresis of the DNFB-treated compound (1)

Black symbols, ninhydrin-positive, fluorescent; hatched symbol, ninhydrin-positive, yellow; white symbol, yellow. dnp, Dinitrophenyl.

The fluorescence properties of the molecule were also similar to those of the biphenyl linkage group of dityrosine. The emission wavelength at both of the UV-absorption maxima of compound (**1**) discussed above was 420 nm. It is likely that the compound contains a biphenyl linkage *ortho* to each of two phenolic hydroxy groups, as in dityrosine.

Determination of the number of amino groups

Amino groups can be 'counted' by partial *N*-dinitrophenylation with DNFB followed by HVPE at pH 0.6 [23]. Unchanged compound (**1**) migrated rapidly towards the cathode as a colourless ninhydrin-reactive spot, e.g. as seen after 0.1 min incubation with DNFB (Figure 1). After 25 min, the only product was an immobile (allowing for electroendo-osmosis) yellow spot that did not stain with ninhydrin (Figure 1), indicating complete *N*-dinitrophenylation. At intermediate times, e.g. 1.0 min, there were three additional spots of intermediate mobility; these were yellow and also stained with ninhydrin, indicating that they were partially *N*-dinitrophenylated. *O*-Dinitrophenylation of the phenolic hydroxy groups of compound (**1**) probably occurred simultaneously, but this would not contribute to the yellow colour of the products or affect their ninhydrin reactivity, and would have little effect on electrophoretic mobility. The fact that progressive dinitrophenylation of compound (**1**) led to four discrete shifts in electrophoretic mobility indicates the presence of four amino groups. The movement of the N,N',N'',N'''-tetradinotrophenylated derivative, which is almost uncharged at this pH, was due to electroendo-osmosis and was the same as for a fully derivatized Lys-Lys standard that was run on the same sheet for comparison. As compound (**1**) was electrophoretically immobile at pH 6 (results not shown), it would seem likely that there are also four carboxy groups on the molecule and therefore that it is composed of four covalently bound amino acids.

Analysis by electrospray mass spectrometry

Electrospray mass spectrometry of an aliquot of compound (**1**) (40 μg) gave a major signal at m/z 719 (Figure 2), which was attributed to the $[M+H]^+$ of the molecule, accompanied by sodium adducts at m/z 741, 763, 785 and 807. The doubly charged signal was observed at m/z 360 $[M+2H]^{2+}$. This results suggests that compound (**1**) consists of four oxidatively coupled tyrosines (M_r 718).

Analysis by proton NMR spectroscopy

A proton NMR spectrum was obtained from a solution of the sample in deuterium oxide using a Varian Unity spectrometer operating at 600 MHz for protons. The spectrum shows a pair of mutually coupled ($J = 2.0$ Hz; *meta* coupling) aromatic resonances at 7.00d and 7.30d, each integrating for one proton equivalent, another pair of mutually coupled ($J = 8.4$ Hz; *ortho* coupling) aromatic resonances to 6.96d and 7.24d each integrating for two proton equivalents, two overlapping doublet of doublet resonances ($J = 5.0$, 8.1 Hz) centred at 3.93d and 3.95d corresponding to tyrosine-type α-protons (two proton equivalents), two overlapping doublet of doublet resonances ($J = 5.0$, 14.5 Hz) centred at 3.24d and 3.27d and two doublet of doublet resonances at 3.04d and 3.08d ($J = 8.1$, 14.5 Hz) corresponding to tyrosine-type β-protons (four proton equivalents). This spectrum is consistent only with a molecule having equal numbers of tyrosine units (Figure 3a) and asymmetrically *meta*-disubstituted tyrosine units (Figure 3b) symmetrically arranged in the entire molecule.

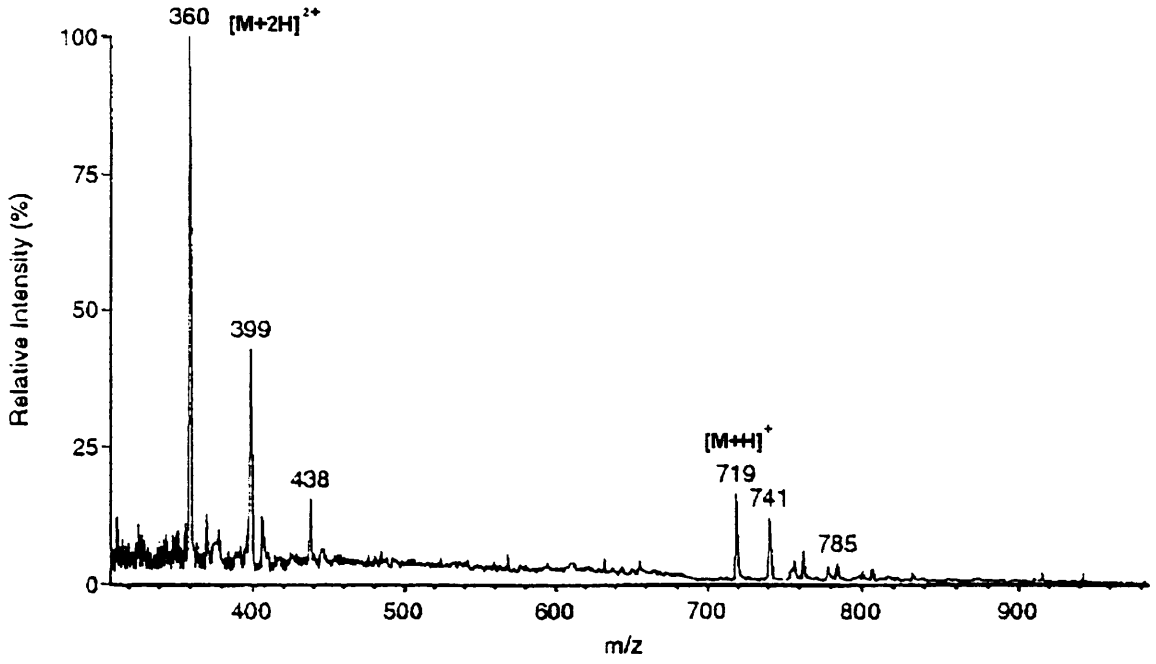

Figure 2 Electrospray mass spectrum of compound (1)

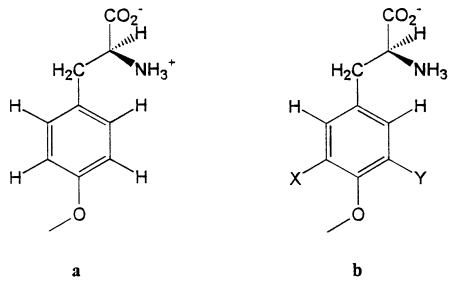

Figure 3 The positions of the aromatic protons as revealed by NMR spectroscopy

Compound (**1**) contains two aromatic rings of the type shown in (**a**) and two of the type shown in (**b**), with the four rings arranged symmetrically.

DISCUSSION

The results show that compound (**1**) is composed of four oxidatively coupled tyrosines. To deduce the further details of the structure of this isomer of tetratyrosine, we assume that: (a) a tetramer must be formed *via* an intermediate dimer; (b) the dimer involved in plants *in vivo* is Idt (Figure 4, compound a) [dityrosine (Figure 4, compound b) does not occur in plants]; (c) oxidative coupling of tyrosine derivatives can only occur through a free phenolic OH group or through a C atom *ortho* to a free (not etherified) phenolic OH group; (d) the molecule contains a biphenyl linkage *ortho* to each of two phenolic groups, as discussed with regard to the UV-absorbance and fluorescence properties. Figure 4 shows the three possible structures that a tetramer can adopt if these assumptions are correct. Compounds c and e can be excluded on the basis of NMR analysis, as there were no aromatic rings with three protons. The NMR spectrum described above is consistent only with the structure of compound d, the aromatic core of which is 2,8-dihydroxy-3,9-diphenoxybiphenyl. We propose di-isodityrosine as the trivial name for compound (**1**).

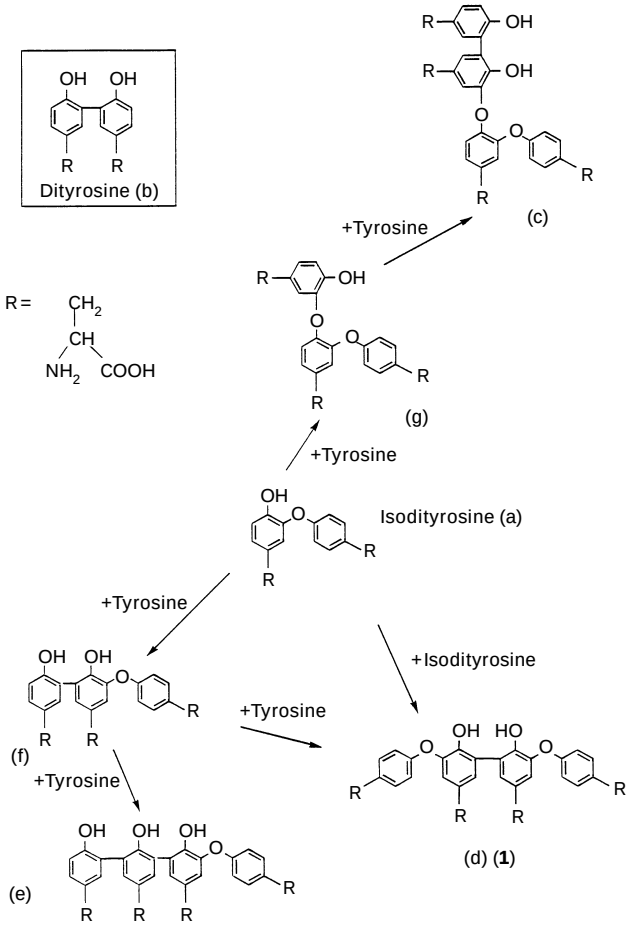

Figure 4 Possible structures of a tetrameric tyrosine-based compound if the assumptions discussed in the text are correct

As shown in Figure 4, there are two possible routes for the formation of compound (**1**), one by the sequential coupling of two molecules of tyrosine to Idt and the other by the direct coupling of two molecules of Idt. Which of these routes is taken, or whether a combination of the two occurs, is unknown. The direct coupling of two molecules of Idt would need a high degree of organization of the extensin, possibly by the interaction of regions of different hydrophobicity or carbohydrate decoration, to align the molecules so that the relatively infrequent Idt residues could become close enough to react.

Do the di-isodityrosine groups form tight intrapolypeptide loops (as demonstrated within the sequence Tyr-Lys-Tyr for at least some of the Idt groups of extensins [19])? The three major tyrosine-rich proteins of plant cell walls are the extensins and the proline-rich and glycine-rich proteins. The tightest clusters of four tyrosine residues capable of oxidative phenolic coupling (adjacent tyrosine residues cannot oxidatively couple owing to the conformation of the polypeptide backbone [19]) in known tomato extensins (Tom J-10 and Tom L-4 [24] and 'class I' extensins [8]) are YYYKSPPPPSPYKY and YVYKSPPPPSPKYVY. The four tyrosine residues in either of these sequences would be unable to form an intrapolypeptide di-isodityrosine group because the intervening SPPPP domain forms a rigid polyproline II helix. In the proline-rich proteins, the tyrosine residues occur singly and are widely spaced and also separated by rigid sequences rich in proline [1,25], which would preclude tight intrapolypeptide di-isodityrosine loops. In the glycine-rich proteins, single tyrosine residues are also well dispersed along the peptide sequence (typically spaced by a 2–8-amino acid glycine-rich region) [8,26,27].

Thus in none of the major wall proteins could di-isodityrosine form a tight intrapolypeptide loop. It could either form a wide intrapolypeptide loop or an interpolypeptide cross-link. Wide intrapolypeptide loops would be of interest in wall architecture as they could entrap other wall polymers such as pectins [28] or could permit interpolypeptide concatenation. Direct interpolypeptide cross-links formed by di-isodityrosine, either within or between the three tyrosine-rich protein families, could also play a major role in the assembly or strengthening of the cell wall.

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