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PULCHEROSINE, AN OXIDATIVELY COUPLED TRIMER OF TYROSINE IN PLANT CELL WALLS: ITS ROLE IN CROSS-LINK FORMATION

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Key Word Index—*Lycopersicon* sp.; Solanaceae; tomato; cell cultures; isodityrosine; pulcherosine; di-isodityrosine; extensin; oxidative phenolic coupling; tyrosine; inter-polypeptide cross-linking; plant cell walls.

Abstract—An oxidatively coupled trimer of tyrosine has been isolated from hydrolysates of primary cell walls of a tomato cell culture. UV-absorption, fluorescence and ^{1}H NMR spectra showed that the trimer was pulcherosine, composed of isodityrosine and tyrosine oxidatively coupled via a biphenyl linkage such that the aromatic core is 2,2′-dihydroxy-3-phenoxybiphenyl. Pulcherosine could act as an intermediate in the conversion of isodityrosine to the tetramer, di-isodityrosine. Steric considerations show that the three tyrosine units of pulcherosine could not be near-neighbour residues within a single polypeptide chain. Pulcherosine therefore forms inter-polypeptide cross-links and/or wide intra-polypeptide loops.

INTRODUCTION

Extensins, proline-rich proteins and glycine-rich proteins are structural proteins of the plant cell wall, and are thought to play a strengthening and/or defensive role, especially in response to disease and wounding [1, 2]. Extensins, which are hydroxyproline-rich glycoproteins, are secreted into the cell wall, where they rapidly become ionically bound to the acidic polysaccharides. This is followed by a more gradual process in which the extensin becomes inextractable in salts, SDS, phenol–acetic acid–water (2:1:1) (PAW), and even anhydrous HF [3–5], presumably owing the formation of covalent cross-links [4, 6]. Infection or the introduction of fungal elicitors can effect a rapid decrease in the extractability of proline-rich proteins and extensins, probably attributable to an oxidative burst in which H_2O_2 and oxygen radicals are formed [7–10].

Although the nature of the covalent cross-links formed is unknown, it seems probable that the oxidative coupling of tyrosine residues plays an important role in the insolubilisation of wall proteins. Isodityrosine [4] [Fig. 1(a)], the only oxidatively coupled dimer of tyrosine known in plants, may form both intra-polypeptide loops [11] and inter-polypeptide cross-links [12]. In addition, we have shown that a tetramer of tyrosine (di-isodityrosine) exists in plant cell wall proteins [13] [Fig. 1(c)]. This tetramer is sterically incapable of forming a 'tight' intra-polypeptide loop (i.e., one in which all four tyrosine units are near-neighbour residues within a single polypeptide chain) and is concluded to participate in inter-polypeptide cross-linking. Proteins cross-linked in this way would be less easily extractable and could form a defensive barrier that restricts penetration by phytopathogens [14]. Moreover, we have shown that the conversion of isodityrosine to di-isodityrosine occurs in plant cells challenged with H_2O_2 or fungal elicitors [15], treatments that had been shown to reduce the extractability of extensins and proline-rich proteins [10, 14, 16, 17].

Three oxidatively coupled trimers of tyrosine have been reported from animal structural proteins: trityrosine [Fig. 1(e)], which has two biphenyl linkages and occurs in the insect protein resilin [18] and in nematode egg shell proteins [19]; isotrityrosine [Fig. 1(f)], which has one biphenyl linkage and one diphenyl ether linkage and occurs in the collagen of tapeworm cuticles [20]; and pulcherosine [Fig. 1(b)], which also has one biphenyl linkage and one diphenyl ether linkage and occurs in the fertilization envelope of sea urchin embryos [21]. In this paper we present evidence for the existence in plant cell wall proteins of pul-

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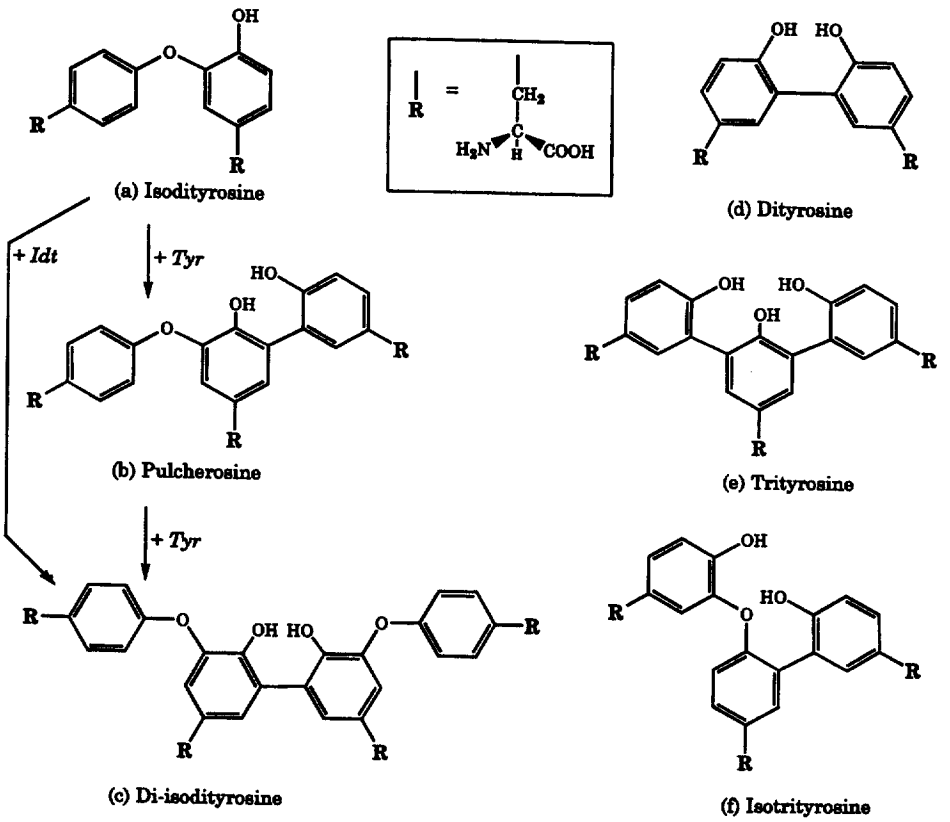

Fig. 1. The proposed structure of pulcherosine compared with other oxidative coupling products of tyrosine. Possible routes for the conversion of isodityrosine (a) to di-isodityrosine (c), either by direct coupling of two molecules of isodityrosine (Idt) or *via* pulcherosine (b) by the addition of single tyrosine residues. Also shown are related compounds not yet found in plants: (d), dityrosine; (e), trityrosine; (f) isotrityrosine.

cherosine, which could act as the natural intermediary between isodityrosine and di-isodityrosine.

RESULTS

Purification of pulcherosine (1)

Phosphocellulose column chromatography of a hydrolysate of tomato cell walls gave an elution profile, monitored by analytical paper chromatography and TLC, similar to that previously described (Table 1 of ref. [13]). Isodityrosine (PC R_F 0.32, TLC R_F 0.27; Folin–Ciocalteu (FC)- and ninhydrin-positive; not visibly fluorescent) was found in phosphocellulose fractions 5 and 6. Di-isodityrosine (PC R_F 0.05, TLC R_F 0.09; FC- and ninhydrin-positive; the unstained compound showed a strong blue fluorescence) was in fraction 9. A new compound (**1**: PC R_F 0.11, TLC R_F 0.13; FC- and ninhydrin-positive; unstained compound showed blue fluorescence) eluted in fractions 8 and 9.

On preparative HPLC of fractions 8 + 9, **1** eluted as a major UV-absorbing and fluorescing peak with R_t 12 min. The yield of **1** from 21 g of dried cells was 110 μg (estimated by A_{280} peak area relative to known tyrosine samples) as compared to 1230 μg isodityrosine and 820 μg di-isodityrosine.

UV absorption and fluorescence properties of pulcherosine

The UV-absorption and fluorescence-emission spectra of **1** in acid and in alkali were similar to those

Table 1. Chemical shifts and coupling constants for the aromatic protons of pulcherosine obtained from a 2D-COSY spectrum

System	Chemical shift (ppm)			Coupling constant (Hz)		
	δ_α	δ_β	$\delta_{\beta'}$	$J_{\alpha\beta}$	$J_{\alpha\beta'}$	$J_{\beta\beta'}$
1	4.036	3.341	3.159	5.0	8.0	14.8
2	3.995	3.293	3.097	5.0	8.4	14.9
3	3.990	3.321	3.100	5.0	8.1	14.7

of di-isodityrosine and dityrosine. At pH < 6 the λ_{max} was 279 nm whereas at pH > 7 it was 318 nm, a bathochromic shift characteristic of the 2,2′-dihydroxybiphenyl group. The fluorescence-emission wavelength was 422 nm, regardless of pH, similar to the behaviour of the biphenyl linkage group in dityrosine [18], isotrityrosine [20] and di-isodityrosine [13], again indicating that **1** also contains a 2,2′-dihydroxybiphenyl group.

Analysis by ^{1}H-NMR spectroscopy

The ^{1}H NMR spectrum (Fig. 2) of **1** shows resonances from nine aromatic and nine aliphatic protons. Even with the signal overlaps around δ 7.25 and 7.03, the three tyrosine fragments (Fig. 3) are identified as follows: (A) three mutually coupled single-proton aromatic resonances centred at δ 6.967 (J = 8.2 Hz, *ortho*), δ 7.251 (J = 2.2 Hz, *meta*; 8.2 Hz, *ortho*) and δ 7.429 (J = 2.2 Hz, *meta*) comprise an AMX system corresponding to the protons in a 1,2,4-trisubstituted benzene ring; (B) two mutually coupled single-proton doublet resonances (J = 2.0 Hz, *meta*) at δ 7.032 and 7.254 correspond to a 1,2,3,5-tetrasubstituted benzene ring; (C) two mutually coupled two-proton aromatic resonances centred at δ 7.033 and 7.313 with patterns that characterise an $[AX]_2$ system of a *para*-disubstituted benzene ring.

These couplings were confirmed by a 2D-^{1}H-COSY spectrum from which it was also possible to identify and distinguish the three aliphatic AMX proton systems arising from the α- and β-protons of each tyrosine fragment (Table 1). It was not possible to assign these aliphatic protons to the appropriate tyrosine fragments as the quantity of material was insufficient to carry out the appropriate NOE measurements.

DISCUSSION

The results show that **1** was pulcherosine, an oxidatively coupled trimer of tyrosine [Fig. 1(b)]. The differences between the 600 MHz ^{1}H NMR spectrum of **1** (Fig. 2) and the 400 MHz spectrum of pulcherosine shown by Nomura *et al.* [21] may be due to differences in the pH of the solution. The first phenolic pK_a of dityrosine is ~ 6.7 [4]; pulcherosine also has a 2,2′-dihydroxybiphenyl group and thus probably has a similar pK_a, so two independently prepared, unbuffered solutions of pulcherosine may differ in ionic form.

The isolation of **1** from hydrolysates of phenol-

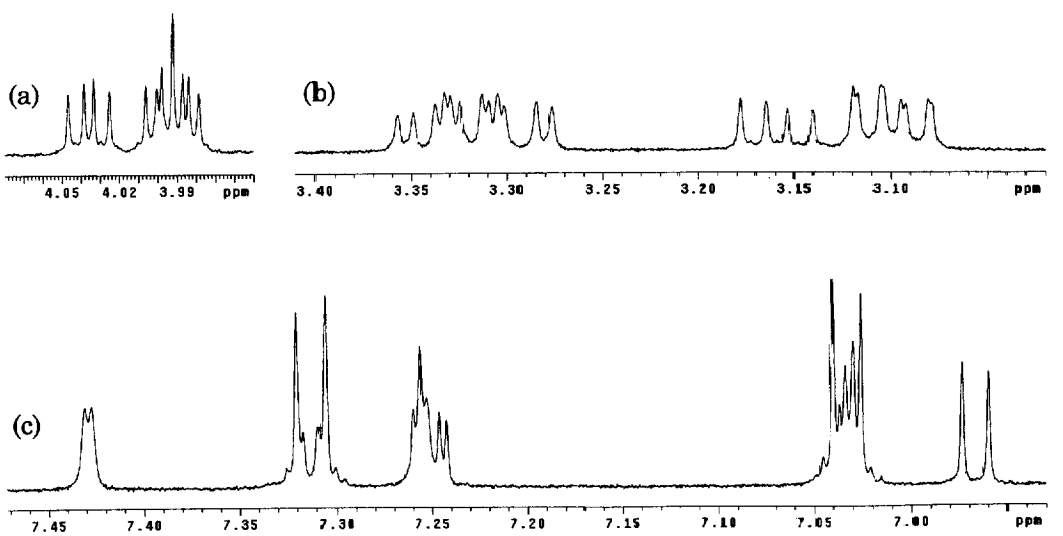

Fig. 2. 600 MHz ^{1}H NMR spectrum of pulcherosine in ^2H_2O. (a) α-Proton resonances, (b) β-proton resonances, (c) aromatic proton resonances.

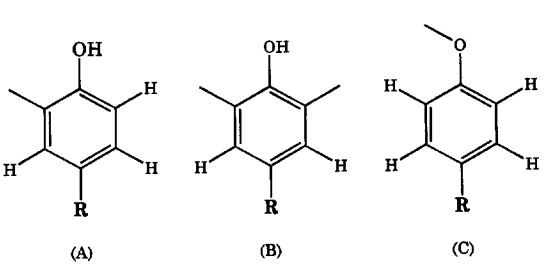

Fig. 3. Positions of the aromatic protons in pulcherosine as revealed by NMR spectroscopy. (A)–(C) are the three tyrosine fragments discussed in the text. For identity of —R see Fig. 1.

washed cell walls indicates that pulcherosine was a natural component of wall protein(s). It is not likely to have arisen artefactually by the oxidative coupling of tyrosine and isodityrosine during the hydrolysis or work-up: experiments in which a trace of [^{14}C]tyrosine was added to cell walls prior to hydrolysis yielded no [^{14}C]isodityrosine [4]. Pulcherosine could act as the biosynthetic intermediate between isodityrosine and di-isodityrosine, as part of a pathway in which the compounds are built up by the sequential addition of tyrosine residues. However, it is also possible that pulcherosine is an end-product, with the formation of di-isodityrosine occurring by the direct oxidative coupling of two isodityrosine units.

It is not known with certainty whether pulcherosine occurs in extensins, proline-rich proteins, or other cell wall (glyco)proteins. However, we assume that pulcherosine is formed from isodityrosine. The only plausible alternative precursor would be dityrosine, but this has not been detected in plants. Isodityrosine occurs in extensin peptides [11]; it is therefore reasonable to suggest that extensin is one of the glycoproteins in which pulcherosine occurs.

The discovery that plant cell walls contain pulcherosine, in the apparent absence of trityrosine and isotrityrosine whereas structural proteins of the arthropod exoskeleton contain trityrosine and isotrityrosine but apparently no pulcherosine, reinforces the view that oxidative coupling of phenolic groups *in vivo* is a highly specific process. Similarly, plants have isodityrosine but not dityrosine, whereas arthropods have dityrosine but not isodityrosine. It would thus appear that factors in the plant cell wall act to orientate the coupling of the phenolic free radicals that are the presumed precursors of the dimers and trimers of tyrosine.

It seems unlikely that pulcherosine could form a 'tight' intra-molecular loop, i.e. one in which the three participating Tyr residues were near-neighbours in a peptide sequence. Although the sequence Tyr–Tyr–Tyr is found in some extensins [22, 23], steric considerations show that this sequence could not form pulcherosine. Simulated annealing energy minimization calculations show that the tripeptide Tyr–Tyr–Tyr with all three aromatic rings linked as in pulcherosine is impossible ($\sim$ 160 kJ mol^{-1} less stable than pulcherosine itself). Pulcherosine with two of the three Tyr units directly linked by a peptide bond is highly implausible (e.g. pulcherosine with the two ether-linked units directly peptide-bonded to each other would be $\sim$ 34 kJ mol^{-1} less stable than pulcherosine). It is, however, possible for the two tyrosine residues in the peptide sequence Tyr–Xaa–Tyr, which is frequent in some extensins, to couple to form isodityrosine [11], and this could then couple to a third tyrosine unit, in a different polypeptide chain, to make pulcherosine.

Tyrosine residues separated by relatively short hydroxyproline-rich or glycine-rich blocks (e.g. the underlined blocks within the peptide sequences

PPPPTYSSPPPPPFY

PPPPYYYKSPPPPSPSPPPPYYYSSPPPP

PPPPYVYKSPPPPSPSPPPPYVYKSPPPP

GGGGGYPGGGYPGGGGYRGGGG

which are predicted from gene sequences coding for putative cell wall proteins of tomato; most of the Pro residues are hydroxylated and arabinosylated *in vivo* [22]) would also be unable to form pulcherosine owing to the rigidity [24] of the intervening peptide. Therefore, pulcherosine is likely to form either an inter-polypeptide bridge (cross-linking two or three polypeptide chains) or a 'wide' intra-polypeptide loop (i.e., one in which at least one of the three participating Tyr residues is not a near-neighbour of the other two).

Inter-polypeptide bridges would clearly contribute to the cross-linking of polypeptide chains and thus to the assembly of a defensive network within the cell wall; wide intra-polypeptide loops could also achieve the same end, for example by concatenating with each other or by encircling unrelated wall components such as cellulosic microfibrils or pectin molecules.

The present findings emphasise that the formation of isodityrosine is only a first step in the pathway of tyrosine cross-linking. The foundation of isodityrosine is built upon by further oxidative coupling with additional Tyr or isodityrosine groups.

EXPERIMENTAL

Materials. Analytical grade chemicals, HPLC grade solvents, and Merck F_{60} silica gel TLC plates with fluorescent indicator were from BDH Chemicals (Poole, Dorset). The HPLC on-line fluorimeter and UV detector were manufactured by Shimadzu, Japan.

Preparation of tomato cell wall hydrolysate. Suspension-cultured tomato cells (a *Lycopersicon esculentum* × *L. peruvianum* hybrid) were maintained, freeze-dried (21 g), washed free of non-wall material in PAW [6], hydrolysed and fractionated as described [13]. Briefly, PAW-washed, cell wall-rich preparations were hydrolysed in 6 M HCl at 116° for 18 hr under N_2. The hydrolysate was dried under vacuum, re-dissolved in H_2O, applied to a phosphocellulose cation-exchange column to give frs 0 (non-binding) and 1–11 (each 100 ml; eluted with a 0.005–0.5 M HCl step gradient [13]).

Paper chromatography and TLC. Portions (500 μl) from each of frs 0–11 were dried under vacuum, redissolved in H_2O (10 μl) and subjected to paper chromatography on Whatman 3 MM paper in 1-BuOH–HOAc–H_2O (12:3:5 by vol.). Additional portions (equivalent to 10 μl of each phosphocellulose fr.) were analysed by TLC on silica-gel in 1-PrOH–27% NH_3 (7:3). Chromatograms were examined for fluorescence under 254 nm UV light and then stained for amino acids with 0.5% ninhydrin in Me_2CO, or for

phenolics with Folin and Ciocalteu's phenol reagent (FC) followed by exposure to NH_3 vapour [6]. For prep. chromatography the paper was dried and the fluorescent sample eluted from the paper in 1 M NH_3 and dried under vacuum.

Purification by HPLC. Prep. RP-HPLC was performed on a C_{18} column (1 × 25 cm, Spherisorb ODS2, 5 μm). The solvent a linear gradient of 5–35% MeCN in H_2O over 25 min at 4 ml min^{-1}. The eluate was monitored for A_{280} with an on-line UV detector and for fluorescence with an on-line fluorimeter (excitation 280 nm, emission 420 nm).

Analysis by ^{1}H-NMR Spectroscopy. 1D- and 2D-^{1}H NMR spectra were obtained at 40° from a soln of **1** in ^2H_2O using a Varian 'INOVA' NMR spectrometer operating at 600 MHz for protons.

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