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Formation of Di-Isodityrosine and Loss of Isodityrosine in the Cell Walls of Tomato Cell-Suspension Cultures Treated with Fungal Elicitors or H_2O_2[1]

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About 84% of the hydroxyproline residues in a cell culture of tomato (*Lycopersicon esculentum* × *Lycopersicon peruvianum*) were present in phenol-inextractable (i.e. covalently wall-bound) material. Treatment of the cells with any of three fungal elicitors (wall fragments from *Phytophthora megasperma* and *Pythium aphanidermatum* and xylanase from *Aureobasidium pullulans*) or with 1 mM H_2O_2 had little effect on the quantity of phenol-inextractable hydroxyproline per milligram of freeze-dried cells. However, each treatment induced a decrease in the content of phenol-inextractable isodityrosine (Idt) residues. Each treatment, except with the *P. megasperma* fragments, also induced an increase in phenol-inextractable di- (Di-Idt). The increase in Di-Idt partly accounted for the loss of Idt. We conclude that the elicitors and H_2O_2 acted to reinforce the existing cross-linking of cell wall (glyco)proteins by evoking oxidative coupling reactions to convert Idt to Di-Idt plus unidentified products. The promotion of cross-linking by elicitor treatment is proposed to be a defensive response that restricts the penetration of pathogens.

The structural proteins of the primary cell wall are divided into five major classes (Showalter, 1993), of which the extensin family is the most extensively studied. Extensins are HRGPs and are characterized by their high pI, their rod-like structure formed from the diagnostic Ser-Hyp-Hyp-Hyp-Hyp sequence, which typically appears repeatedly throughout the protein, and their high Tyr content. The Hyp-Hyp-Hyp-Hyp block, in which each Hyp residue is often decorated with an Ara-Ara-Ara-Ara oligosaccharide, forms a polyproline-II helix (van Holst, and Varner, 1984; Williamson, 1994), which gives the protein rigidity. Other common repeats in the sequence may act as recognition sites for cross-linking or for glycosylating enzymes or as domains of ionic interaction with other wall proteins and polysaccharides (Kieliszewski and Lamport, 1994; Qi et al., 1995).

There is a growing body of evidence to suggest that the covalent cross-linking of structural proteins in the primary cell wall plays an important role as a defense mechanism of the plant cell in response to pathogen attack. When extensins are secreted into the cell wall, they quickly become ionically bound to acidic polysaccharides; later, more slowly, they become covalently cross-linked within the wall, as evidenced by their inextractability in salts and SDS (Fry, 1982, 1986; Cooper and Varner, 1983). Both the synthesis and the secretion of HRGPs are accelerated upon infection (Lawton and Lamb, 1987); a subclass of Tyr-rich HRGPs may be induced specifically by infection (Kawalleck et al., 1995). The covalent cross-linking of existing cell wall proteins can be elicited rapidly by treatment with fungal cell wall fragments (Bradley et al., 1992; Brisson et al., 1994); this process appears to involve oxidative coupling, dependent on H_2O_2 or other active oxygen species (Schwacke and Hager, 1992; Vera-Estrella et al., 1992). Recent studies have pointed to an elicitor-activated signaling system that leads to the generation of active oxygen species by a membrane-bound NAD(P)H oxidase (Apostol et al., 1989; Vera-Estrella et al., 1992); the signal involves inorganic ion fluxes (Schwacke and Hager, 1992; Nürnberger et al., 1994; Hahlbrock et al., 1995) and a protein kinase/phosphatase system (Otte and Barz, 1996).

The nature of the covalent cross-links responsible for the rapid insolubilization of these proteins is still unclear. Idt, an oxidatively coupled dimer of Tyr, could be generated quickly and cause cross-linking (Fry, 1986). The oxidizing system could be H_2O_2 plus peroxidase (Fig. 1a); the formation of Idt has also been shown to occur upon treatment of Tyr peptides with hydroxyl radicals (·OH; Karam et al., 1984). Interpolypeptide Idt is therefore a strong candidate to play a role in the covalent cross-linking of extensin (Biggs and Fry, 1990). Analysis of soluble oligopeptides released from the cell wall by proteinase digestion showed that a proportion of the Idt residues formed in vivo occur as tight, intrapolypeptide loops (Epstein and Lamport, 1984), which would not render extensin inextractable. However, the intermolecularly cross-linked regions of extensin may have been more resistant to proteolysis so that any intermolecular Idt residues would have stayed with the insoluble wall residue and escaped detection. The discovery in tomato (*Lycopersicon esculentum* L.) culture filtrates of a peroxidase with a pI of 4.6 that cross-links

Abbreviations: Di-Idt, di-isodityrosine; HRGP, Hyp-rich glycoprotein; Idt, isodityrosine; MeCN, acetonitrile; PAW, phenol:acetic acid:water (2:1:1, [w/v/v]); PRP, Pro-rich protein.
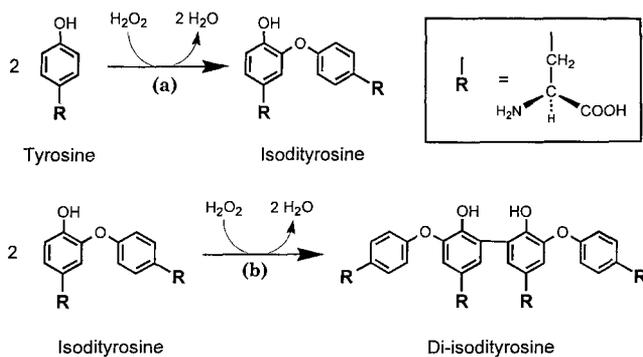
Figure 1. Chemical structures and possible in vivo mechanism of formation of Idt and Di-Idt.

soluble extensin in vitro but fails to produce Idt (Schnabelrauch et al., 1996) does not address the question of whether Idt is a natural cross-link. Idt clearly is produced in vivo.

Brady et al. (1996) reported the existence of a tetrameric derivative of Tyr, Di-Idt, which is formed by the oxidation of Idt (either the direct coupling of two Idt residues, as shown in Fig. 1b, or the sequential coupling of one Idt with two Tyr residues via an intermediary trimer). Di-Idt is sterically unable to form tight, intrapolypeptide loops and is thus very likely to form intermolecular cross-links in extensin (Brady et al., 1996), perhaps by building on initially formed intramolecular Idt loops.

In this paper we report the use of a rapid and sensitive assay to measure concentrations of both Idt and Di-Idt after elicitation of cultured tomato cells with the following previously used systems: cell wall fragments from the fungi *Phytophthora megasperma* and *Pythium aphanidermatum* (Ayers et al., 1976; Templeton and Lamb, 1988; Bradley et al., 1992; Kawalleck et al., 1995), fungal xylanase (Moreau et al., 1994), and H_2O_2.

MATERIALS AND METHODS

Analytical grade chemicals and HPLC-grade solvents were from BDH Chemicals (Poole, Dorset, UK). Xylanase (specific activity approximately 33 nkat mg^{-1} protein; from *Aureobasidium pullulans*) was from Sigma. Fungal wall fragments (from *Phytophthora megasperma* and *Pythium aphanidermatum*) were prepared as described by Ayers et al. (1976).

Elicitation of Cultured Cells

Suspension-cultured cells of tomato (*Lycopersicon esculentum* × *Lycopersicon peruvianum*), maintained as described previously (Aldington and Fry, 1994) and subcultured every 2 weeks, were inoculated into 10 250-mL conical flasks, each containing 100 mL of the medium described by Aldington and Fry (1994). Six days later, duplicate cultures were treated with 180 μg mL^{-1} *P. megasperma* wall fragments, 180 μg mL^{-1} *P. aphanidermatum* wall fragments, 17 nkat mL^{-1} xylanase (5 μg protein mL^{-1}), or 1 mM H_2O_2 or H_2O. Immediately before addition of the elicitor, and at intervals thereafter, cells were removed (approximately 500

mg fresh weight per sampling), rinsed with distilled H_2O, and frozen in liquid N_2.

Preparation and Hydrolysis of Wall Samples

The frozen cells were freeze-dried (average yield approximately 30 mg dry weight), suspended in 2 mL of PAW, stirred at room temperature for 2 h, resuspended in fresh PAW for 16 h, and finally washed free of PAW with ethanol and acetone. To the PAW solution was added 0.05 volume of 10% (w/v) ammonium formate followed by 5 volumes of acetone, and the precipitated (glyco)proteins were collected by centrifugation, washed in acetone, and dried.

Freeze-dried whole cells, wall-rich residues, and PAW extracts were hydrolyzed in 6 N HCl under N_2 at 116°C for 16 h. The hydrolyzates were centrifuged to remove any solid material, and the supernatants were dried under a vacuum. The dried material was redissolved in 1 mL of H_2O and a 10-μL portion of this was assayed for Hyp by the direct acid method (Firschein and Shill, 1966) with the modification that 2-methoxyethanol replaced propan-2-ol.

The equivalent of 300 nmol of Hyp from each HCl-hydrolyzate was made up to 1 mL with H_2O. This sample was applied to a 1.4-mL bed-volume column of phosphocellulose (P11, Whatman; prepared according to the manufacturer's instructions) and washed with 3 mL of 5 mN HCl to remove compounds containing one or no amino group; the Idt and Di-Idt were then eluted with 5 mL of 0.5 N HCl, dried under a vacuum, redissolved in 500 μL of H_2O, and applied to a 100-mg C_{18} Bond-Elut column (Varian, Sunnyvale, CA) preconditioned in MeCN and washed with H_2O. The column was washed with 500 μL of H_2O to remove nonaromatic compounds, which were rejected, and then the Idt plus Di-Idt were eluted with 10 and 20% (v/v) MeCN (500 μL each) and 40% MeCN (1 mL). The combined MeCN eluates were dried under a vacuum and redissolved in 100 μL of H_2O.

HPLC of Samples

The Idt/Di-Idt sample (equivalent to 150 nmol of Hyp) was chromatographed on a C_{18} reversed-phase HPLC column (15 × 0.4 cm, Spherisorb S5ODS2, Hichrom Ltd., Reading, UK), eluted at 1 mL min^{-1} with a 5 to 35% (v/v) MeCN gradient over 20 min. The column was then washed with 100% MeCN for 5 min and re-equilibrated in 5% MeCN for 15 min. The eluate was monitored by an on-line UV spectrophotometer (A_{280}, Shimadzu, Tokyo) and an on-line fluorimeter (excitation 280 nm, emission 420 nm, Shimadzu). Authentic Tyr, Idt (Fry, 1984), and Di-Idt (Brady et al., 1996) had retention times of 8.0, 14.5, and 12.5 min, respectively. The fluorimeter reading was used to estimate the Di-Idt and the A_{280} was used to estimate the Idt.

RESULTS

The tomato cells contained a total of 65 nmol of Hyp residues mg^{-1} (freeze-dried cells). If the Hyp residues were mainly present in extensin, this figure indicates that ap-

proximately 4% of the dry weight of the cells was extensin. About 84% of the Hyp residues was PAW-inextractable and thus regarded as covalently cross-linked, presumably within the cell wall. Only 3.5% of the Hyp residues was found in the culture medium, and approximately 13% was PAW-extractable from the cells and precipitable with acetone (Table I).

The content of PAW-inextractable Hyp residues per milligram of freeze-dried cells was little affected by any of the elicitor treatments used (Fig. 2; the scatter in the data was mainly due to variable loss of solid material during PAW washing). In the controls and all treatments there appeared to be a small increase in Hyp. Thus, the large, elicitor-induced decreases in the proportion of the HRGPs that were extractable with salt, previously reported (Brisson et al., 1994; Wojtaszek et al., 1995; Otte and Barz, 1996), did not occur in these tomato cells.

In view of the above finding, PAW-inextractable Hyp residues were taken as the basis for normalizing covalently wall-bound Idt and Di-Idt contents. On this basis, in control cultures covalently wall-bound Idt and Di-Idt underwent little change over the 24-h period of observation (Fig. 3). However, the elicitors did influence the Idt and/or Di-Idt content (Figs. 3–6). The scatter in the data for Figures 3 through 6 was probably due to the multistep nature of the procedure needed to isolate and assay Idt and Di-Idt after normalization against Hyp.

Cells treated with 1 mM H₂O₂ (Fig. 3) showed a rapid and pronounced decrease in covalently wall-bound Idt. The levels reached within 10 min (60–70% of controls) were approximately maintained for the next 24 h. Covalently wall-bound Di-Idt levels increased more gradually than the Idt levels decreased, suggesting a conversion of the dimer to the tetramer via an intermediary trimer of Tyr.

Cells elicited with P. aphanidermatum fragments (Fig. 4) showed a gradual decline in covalently wall-bound Idt between 20 and 440 min. After 1440 min, Idt had decreased to less than one-half of the original value. Covalently wall-bound Di-Idt increased after 60 min but soon reached a plateau. The final Di-Idt level approached double that of the control cells.

Cells elicited with xylanase showed somewhat similar trends (Fig. 5). Covalently wall-bound Idt did, however, show a brief increase at 10 and 20 min before decreasing to less than 30% of the control values. Covalently wall-bound Di-Idt showed an appreciable increase after 40 min and this was maintained until the end of the experiment.

P. megasperma fragments decreased the covalently wall-bound Idt levels (to 60–70% of control values within 40
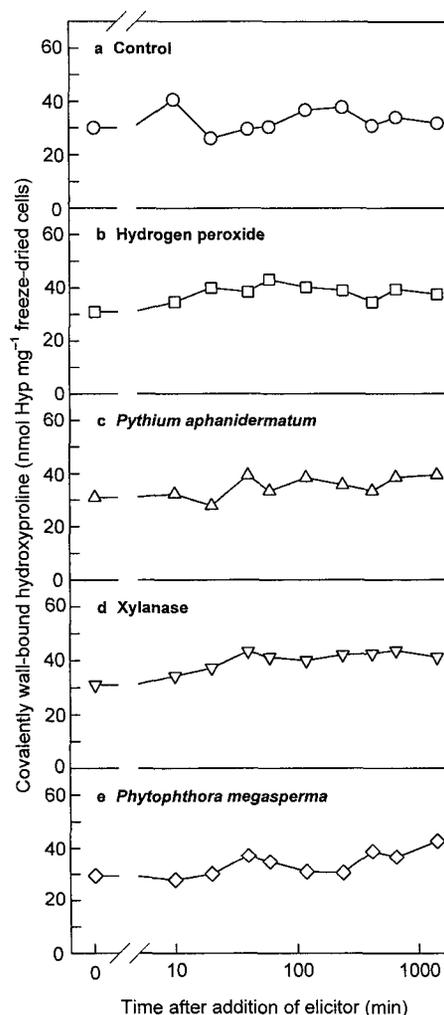

Figure 2. Effects of four potential elicitors on the quantity of covalently wall-bound (PAW-insoluble) Hyp residues in cultured tomato cells. a, Control (without elicitor); b, 1 mM H₂O₂; c, 180 μg mL^{-1} P. aphanidermatum wall fragments; d, 17 nkat mL^{-1} xylanase; and e, 180 μg mL^{-1} P. megasperma wall fragments.

min) without noticeably affecting the amount of covalently wall-bound Di-Idt (Fig. 6). However, in one experiment (data not shown), P. megasperma wall fragments induced a 5-fold increase in Di-Idt within 7 h.

DISCUSSION

Other workers have reported that the amount of HRGP that is salt-extractable from the wall rapidly decreases

Table I. Distribution of Hyp residues in fractions of a 6-d-old cell-suspension culture of tomato

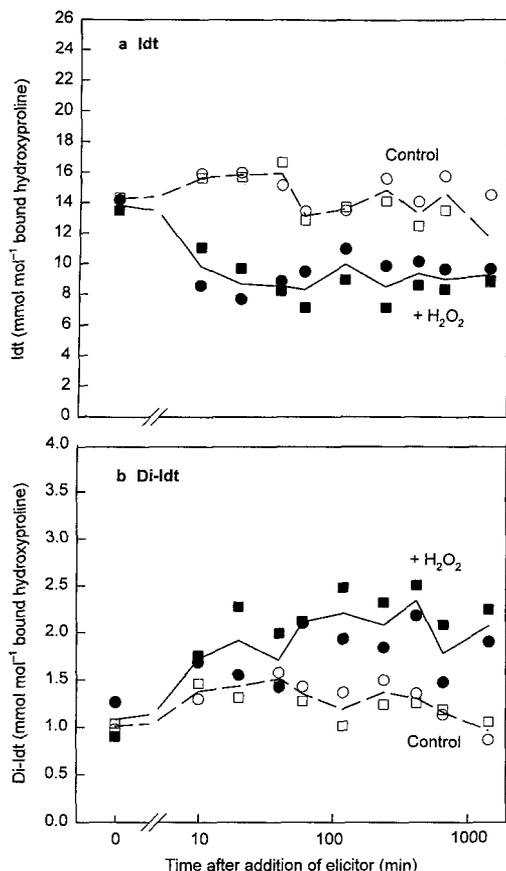| Fraction	Hyp Content	Proportion of Total Hyp
	nmol mg^{-1} freeze-dried cells	% of (b + c + d)
(a) Whole freeze-dried cells	68.8	106
(b) Soluble in culture medium	2.28	3.5
(c) PAW-soluble and acetone precipitable	8.28	12.7
(d) PAW-insoluble	54.5	83.8
(b) + (c) + (d)	65.0	100

Figure 3. Effect of treatment with 1 mM H_2O_2 on the quantity of covalently wall-bound Idt (a) and Di-Idt (b) residues per unit of covalently wall-bound Hyp residues in tomato cell cultures. ● and ■, Two replicate treated cultures; and ○ and □, two replicate control cultures.

upon treatment with a variety of elicitors (Brisson et al., 1994; Wojtaszek et al., 1995; Otte and Barz, 1996). Wall glycoproteins are not known to undergo rapid turnover; therefore, by implication, the amount of salt-inextractable HRGP in the walls of elicited cells must increase, presumably owing to covalent cross-linking. It has been hypothesized that this involves oxidative cross-linking, possibly requiring the generation of active oxygen species by an NAD(P)H oxidase (Apostol et al., 1989; Vera-Estrella et al., 1992; Otte and Barz, 1996).

We have measured elicitor-induced changes in quantities of Idt and Di-Idt, two potential cross-links (Fry, 1982; Brady et al., 1996), in the covalently bound material of the walls of cultured tomato cells. Rather than salt extraction, we carried out PAW extraction, which solubilizes (glyco)-proteins from cultured plant cells more effectively than either salt or SDS (Fry, 1978). Despite this, about 84% of the Hyp residues in these cells was present in proteins that, at the time of elicitor treatment, were already PAW-inextractable (covalently cross-linked). The data (Fig. 2) thus show that any further insolubilization of extensin was negligible and suggest that elicitation did not appreciably increase the de novo synthesis of extensins.

It is not known whether the Idt and Di-Idt were in HRGPs, PRPs, or both. However, we assume that Di-Idt is formed by the reaction of Idt, either with one additional Idt residue (Fig. 1) or with two separate Tyr residues (i.e. via an intermediary trimer). The only plausible alternative precursor would be dityrosine, but this is unlikely because it has not been detected in plants. Intramolecular Idt loops occur in extensin peptides (Epstein and Lamport, 1984); it is therefore reasonable to suggest that the formation of Di-Idt would involve extensin molecules.

The PAW inextractability of the Hyp at the time of elicitor treatment suggests that HRGPs already contained cross-links such as Idt and Di-Idt. Untreated tomato cultures contained relatively high levels of Di-Idt (approximately 1 mmol Di-Idt mol^{-1} covalently bound Hyp). For comparison, the apical 2 cm of the stems of 2-week-old tomato seedlings contained only approximately 0.04 mmol Di-Idt mol^{-1} Hyp (data not shown). A typical extensin contains approximately 120 Hyp residues (Memelink, 1988); therefore, if we assume that most of the PAW-inextractable Hyp in the cultured cells was in extensins, there were approximately 0.12 Di-Idt residues per molecule of extensin. If each Di-Idt residue cross-links four extensin molecules, then on average approximately 50% (= 4 × 0.12) of the wall's extensins carried one ¼-Di-Idt residue; if
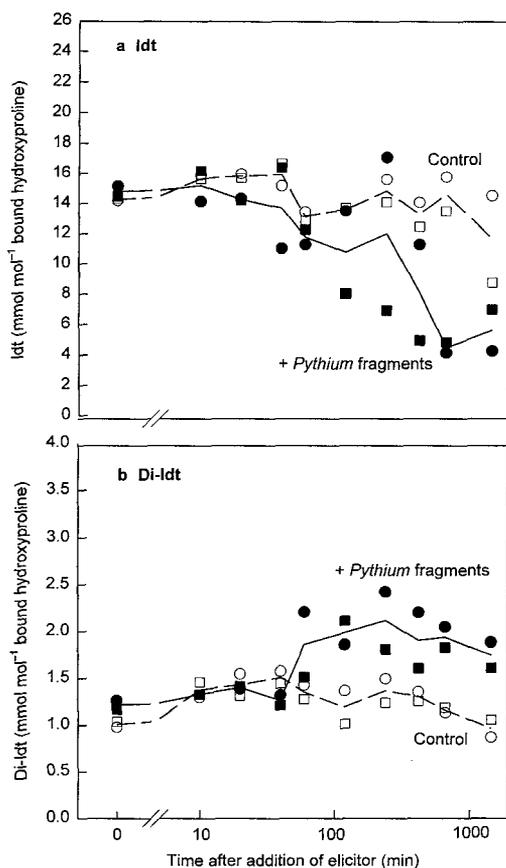

Figure 4. Effect of treatment with *P. aphanidermatum* wall fragments on Idt and Di-Idt in tomato cultures. ● and ■, Two replicate treated cultures; and ○ and □, two replicate control cultures.
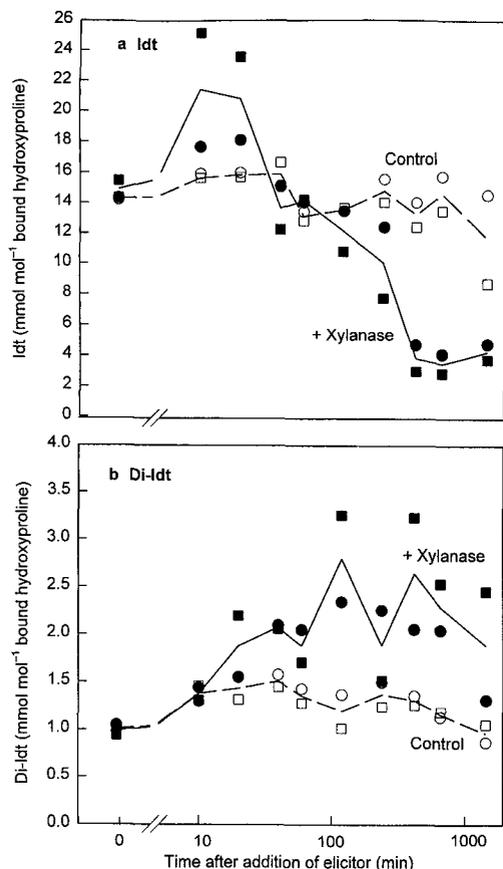
Figure 5. Effect of treatment with xylanase on Idt and Di-Idt in tomato cultures. ● and ■, Two replicate treated cultures; and ○ and □, two replicate control cultures.

Di-Idt mainly cross-links pairs of extensin molecules, then on average approximately 25% of the extensins possessed two ¼-Di-Idt residues. (A "¼-Di-Idt residue" is defined as a Tyr residue that is a component of a Di-Idt residue.) Prior to elicitor treatment the cells also contained Idt (approximately 14 mmol Idt mol⁻¹ Hyp, equivalent to approximately 3.4 residues of ½-Idt per molecule of extensin). Some Idt residues occur as intrapolypeptide loops (Epstein and Lamport, 1984); others are likely to be interpolypeptide cross-links (Biggs and Fry, 1990). Even if only 30% of the Idt residues were interpolypeptide, this would still signify an average of one (½-Idt) bridge per extensin molecule. The wall also contains other Tyr derivatives (including an oxidatively coupled trimer; Brady et al., 1997), not quantified in the present work. Taken together, the Idt, Di-Idt, and other Tyr-based coupling products seem adequate to account for the PAW inextractability of the majority of the tomato cells' extensin (Table I). However, this does not eliminate the possibility that other cross-links remain to be discovered, e.g. cross-links to pectin (Qi et al., 1995) and the unidentified cross-links that are created in extensins by the action of a specific peroxidase in vitro (Schnabelrauch et al., 1996).

Some of the Idt and Di-Idt seen in the tomato cells may have formed cross-links between other wall (glyco)pro-

teins, such as PRPs, that contain little or no Hyp. PRPs, a subset of the structural cell wall proteins, do become covalently cross-linked in the cell wall upon elicitation (Bradley et al., 1992). It seems likely that the active oxygen species, generated in response to elicitor treatment, would simultaneously cross-link both PRPs and HRGPs, as reported by Otte and Barz (1996).

Since we assume that Idt is a precursor of Di-Idt, the cells' responses to elicitors—increase in Di-Idt and depletion of Idt—imply net formation of Di-Idt from Idt, irrespective of any de novo formation of Idt. Di-Idt could not have been the only product formed from Idt: for example, in response to H₂O₂, the amount of Di-Idt increased relative to the control by approximately 1 mmol mol⁻¹ Hyp (Fig. 3b), which would have required an Idt loss of only 2 mmol mol⁻¹ Hyp; however, the Idt levels actually decreased by approximately 6 mmol mol⁻¹ Hyp, indicating that 60 to 70% of the lost Idt was converted to products other than Di-Idt. Also in the other systems studied (Figs. 4–6), the depletion of Idt exceeded the increases in Di-Idt; indeed, with *P. megasperma* elicitation there was no consistent increase in Di-Idt and yet there was a decrease in Idt (Fig. 6). It seems generally true, therefore, that other uncharacterized oxidation products were formed from Idt upon exposure to elicitor-induced active oxygen species.
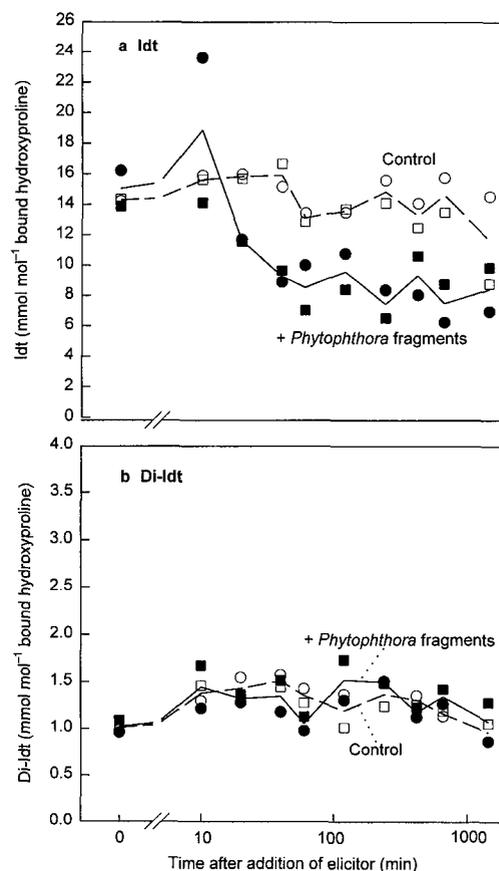

Figure 6. Effect of treatment with *P. megasperma* wall fragments on Idt and Di-Idt in tomato cultures. ● and ■, Two replicate treated cultures; and ○ and □, two replicate control cultures.

The products could include trimers of Tyr and products formed by the coupling of Idt to other wall phenolics such as polysaccharide-bound ferulate and p-coumarate.

The data indicate that Idt, the product of oxidative dimerization of Tyr residues, is itself also subject to oxidative coupling in the walls of living cells (Fig. 1). The activation of an NAD(P)H oxidase, with consequent generation of active oxygen species, would enable the oxidative coupling that we have observed. Since Di-Idt, unlike Idt, is fluorescent (excitation maximum 280 nm, emission maximum 420 nm; Brady et al., 1996), the formation of Di-Idt at a site of infection would contribute to the formation of a localized area of fluorescence, as was observed previously (Bennet et al., 1996).

Our results show that elicitation of plant cells led to increases in Tyr-based oxidative coupling in the cell wall, and we propose that this would augment the ability of the wall to act as a defensive barrier to pathogens.

ACKNOWLEDGMENTS

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