Di-isodityrosine Is the Intermolecular Cross-link of Isodityrosine-rich Extensin Analogs Cross-linked in Vitro

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Extensins are cell wall hydroxyproline-rich glycoproteins that form covalent networks putatively involving tyrosyl and lysyl residues in cross-links catalyzed by one or more extensin peroxidases. The precise cross-links remain to be chemically identified both as network components in muro and as enzymic products generated in vitro with native extensin monomers as substrates. However, some extensin monomers contain variations within their putative cross-linking motifs that complicate cross-link identification. Other simpler extensins are recalcitrant to isolation including the ubiquitous P3-type extensin whose major repetitive motif, Ser-(Hyp)_4-Ser-Hyp-Ser-(Hyp)_4-Tyr-Tyr-Tyr-Lys, is of particular interest, not least because its Tyr-Tyr-Tyr-Tyr tetra-tyrosine intermolecular isodityrosine cross-link motifs are also putative candidates for further intermolecular cross-linking to form di-isodityrosine. Therefore, we designed a set of extensin analogs encoding tandem repeats of the P3 motif, including Tyr → Phe and Lys → Leu variations. Expression of these P3 analogs in Nicotiana tabacum cells yielded glycoproteins with virtually all Pro residues hydroxylated and subsequently arabinosylated and with likely galactosylated Ser residues. This was consistent with earlier analyses of P3 glycopeptides isolated from cell wall digest and the predictions of the Hyp contiguity hypothesis. The tyrosine-rich P3 analogs also contained isodityrosine, formed in vivo. Significantly, these isodityrosine-containing analogs were further cross-linked in vitro by an extensin peroxidase to form the tetra-tyrosine intermolecular cross-link amino acid di-isodityrosine. This is the first identification of an intermolecular cross-link amino acid in an extensin module and corroborates earlier suggestions that di-isodityrosine represents one mechanism for cross-linking extensins in muro.

Hydroxyproline-rich glycoproteins (HRGPs), which include the extensins, proline-rich proteins, and arabinogalactan proteins (AGPs), contribute to extracellular matrix architecture throughout the plant kingdom and the chlorophycean green alga (1–3). HRGPs are involved in all aspects of plant growth and development, including wall assembly during embryogenesis (4), and responses to biotic and abiotic stress that include mechanical stress (5), physical wounding (6), pathogenesis (7), and symbiosis (8–10).

HRGPs are extended macromolecules consisting of small repetitive peptide and glycopeptide motifs that form peptide modules and glycomodules of functional significance, as in “mix-and-match” mode they define the molecular properties of the overall macromolecule. The glycomodules result from a combination of post-translational modifications unique to plants, namely proline hydroxylation (11) and its subsequent glycosylation (12) that leads either to short arabino-oligosaccharide or larger arabinogalactan polysaccharide addition to the Hyp residues. A sequence-dependent O-Hyp glycosylation code directs the addition of oligosaccharide and polysaccharides (13), and it is likely that other sequence-dependent codes direct serine O-galactosylation and inter- and intramolecular covalent cross-linking of HRGPs. Cross-linked HRGPs, most notably extensins, contribute to wall architecture and defense responses by forming interpenetrating cross-linked networks in the wall; however, the precise identity of the intermolecular cross-link or cross-links has remained elusive.

Three major types of monomeric extensin precursors to the insoluble extensin wall network are widespread in the dicots (14, 15) as follows: the P1-type (precursor 1) extensins that are characterized by the repetitive motif Ser-Hyp-Hyp-Tyr-Thr-Hyp-Val-Tyr-Lys; the P2-type (precursor 2) extensins that contain repeats of the motif Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys-Tyr-Lys; and finally the P3 (precursor 3) type extensins that contain variations of the repeat Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys (15). In addition to intermolecular cross-linking, both P2 and P3 type extensins undergo intramolecular cross-linking of Tyr residues within the cross-linking module Tyr-X-Y (underlined in the sequences above) (1, 16) to form the diphenyl ether cross-linking amino acid, isodityrosine (IDT) (17).
Tyrosine Is Required for P3 Extensin Cross-linking in Vitro

IDT, first observed as an unknown tyrosine derivative in extensin peptides (18) and identified in cell wall hydrolysates (17), was initially assumed to be an intermolecular cross-link responsible for transforming the soluble extensin monomeric precursors, P1, P2, and P3, into an insoluble extensin network in muro (17, 19). But IDT was identified only as an intramolecular cross-link in P2- and P3-derived extensin peptides purified from enzymic digests of cell walls (16). Furthermore, extensin peptides cross-linked by intramolecular IDT have never been isolated. Indeed, Lamport and co-workers (20) found no IDT in extensin P1 after cross-linkage in vitro by the p 4.6 extensin peroxidase, although the abundance of Val-Tyr-Lys motifs in several cross-linking extensins, including P1, suggested intermolecular cross-links involving tyrosine and/or lysine (20).

More recently, Fry and co-workers (21, 22) identified the tetrameric tyrosine derivative, di-isodityrosine (di-IDT), and the trimeric tyrosine derivative, pulcherosine, in wall hydrolysates. They speculated that IDT-containing extensins could be insolubilized into the cell wall network through intermolecular cross-linking of IDT and Tyr residues to form di-IDT and pulcherosine. This suggests a significant role in wall assembly and defense responses for IDT-rich extensins, which is supported by the recent discovery that RSH, an extensin containing 14 intramolecular IDT motifs, is crucially involved in positioning the cell plate during the earliest stages of embryogenesis in Arabidopsis (4). However, to date we lack direct demonstration of an extensin intermolecular cross-link. The results of extensin in vitro cross-linking assays have been difficult to interpret as the cross-linking substrates contained both Tyr and Lys in more than one type of repetitive motif, and the amino acids formed by cross-linking were not identified (20, 23, 24). Other approaches involving the isolation of intermolecularly cross-linked peptides from the cell wall itself have also proven intractable.

To simplify the results of in vitro cross-linking assays and to test for the involvement of Tyr and Lys in cross-linking, we created a series of extensin mutants using a synthetic gene approach, described earlier (25–27). P3-type extensin was of particular interest because it is the most widespread extensin in the plant kingdom and therefore functionally significant, although frustratingly recalcitrant to isolation as a soluble monomeric precursor of network extensin; furthermore, its major repetitive motif possesses Tyr and Lys and a repetitive intramolecular IDT motif. The synthetic gene approach allowed us to explore the roles of IDT, Tyr, and Lys in cross-linking through the design of a series of simple synthetic genes, one encoding 20 and the other 8 repeats of the P3 repetitive motif. Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Lys, as well as variants containing Tyr → Phe and Lys → Leu mutations. Expression of these genes as enhanced green fluorescent protein (EGFP) fusion proteins in Bright Yellow-2 (BY-2) tobacco cells (Nicotiana tabacum) yielded secreted transgenic extensins in bulk for biochemical characterization and in vitro cross-linking reactions. They were glycosylated as predicted by the Hyp contiguity hypothesis and contained IDT similar to native P3-type extensins. Furthermore, the p 4.6 extensin peroxidase characterized earlier (EC 1.11.1.7) (20) catalyzed intermolecular cross-linking only between those P3 extensin analogs which contained Tyr and formed the intermolecular cross-link, di-IDT.

EXPERIMENTAL PROCEDURES

Synthesis of Plasmid pBI121-SS<sup>ω</sup>-FK9-EGFP—Gene synthesis of the P3 extensin analog, FK, encoding repeats of the consensus sequence SPFPSPSPPPSPPPPFFKR (supplemental Fig. 8A) was accomplished through a modified version of the head-to-tail method described earlier by using oligonucleotide primers and 5′- and 3′-DNA linkers (25, 28). Polymerized FK synthetic gene constructs of variable lengths were subcloned into pUC18 as BamHI-EcoRI fragments to form plasmids pUC18-FK<sub>x</sub>. Plasmids were screened for insert size by XmaI-NcoI restriction followed by 1% agarose gel electrophoresis. A plasmid harboring 9 internal repeats of FK (pUC18-FK9) was selected and sequenced to verify reading frame integrity. The FK9 synthetic gene was then subcloned between a tobacco extension signal sequence (SS<sup>ω</sup>) (29) and the EGFP gene (Clontech) in vector pUC18 as an XmaI-NcoI fragment to form pUC18-SS<sup>ω</sup>-FK9-EGFP. The SS<sup>ω</sup>-FK9-EGFP gene was subcloned as a BamHI-SacI fragment into the binary plant transformation vector pBI121 (30) in place of the β-glucuronidase reporter gene to form the plasmid pBI121-SS<sup>ω</sup>-FK9-EGFP. All primers were designed using Primer Premier software (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Integrated DNA Technologies, Inc. (Corvalle, IA).

Synthesis of pBI121-SS<sup>ω</sup>-YK20-EGFP, pBI121-SS<sup>ω</sup>-YK8-EGFP, and pBI121-SS<sup>ω</sup>-YL8-EGFP—Gene synthesis of tomato P3 extensin analog encoding repeats of the consensus sequence SPPSPPSPPPPYYYK (YK) and SPPPPSPSSPPPYYYLY (YL) was accomplished using a modified version of a method described earlier (31). Briefly, mutually priming sense and antisense oligonucleotide pairs (supplemental Fig. 8, B and C) were heat-denatured (94 °C for 7 min) and then annealed (60 °C for 5 min and then 45 °C for 10 min). Primer extension with DNA polymerase 1 Klenow fragment (Promega, Madison, WI) followed by separation in 200 microspin column purification (Amersham Biosciences) yielded double-stranded YK and YL DNA constructs (supplemental Fig. 9). Constructs YK and YL were each digested with the restriction enzymes XmaI and SacI, followed by Sephacryl S-400 microspin column purification (Amersham Biosciences). The YK and YL XmaI-SacI fragments were subcloned into pUC18 forming the plasmids pUC18-YK and pUC18-YL. We verified the sequences by DNA sequence analysis using unique BssM and BamFI (New England Biolabs, Beverly, MA) restriction sites in YL and YK were used to create larger genes, as depicted in the flow chart (supplemental Fig. 9). Nonrecombinable, complementary sticky ends were produced by separate BsmFI-SacI and BssM-SacI restrictions of plasmid preparations harboring one repeat of the synthetic genes. Ligation of the insert containing the BsmFI-SacI fragment with the insert containing the BssM-SacI fragment produced (32). Tobacco BY-2 cell cultures were transformed by Agrobacterium (strain LBA4404) transformed with pBI121-SS<sup>ω</sup>-FK9-EGFP, pBI121-SS<sup>ω</sup>-YK8-EGFP, and pBI121-SS<sup>ω</sup>-YL8-EGFP. These genes were also subcloned into pBI121 as BamHI-SacI fragments forming the plasmids pBI121-SS<sup>ω</sup>-FK9-EGFP, pBI121-SS<sup>ω</sup>-YK8-EGFP, and pBI121-SS<sup>ω</sup>-YL8-EGFP.

Agrobacterium Transformation and Selection of Cell Lines—Agrobacterium tumefaciens (strain LBA4404) was transformed with pBI121-SS<sup>ω</sup>-FK9-EGFP, pBI121-SS<sup>ω</sup>-YK8-EGFP, and pBI121-SS<sup>ω</sup>-YL8-EGFP. These plasmids were transformed by electroporation A. tumefaciens (strain LBA4404) was transformed with pBI121-SS<sup>ω</sup>-FK9-EGFP, pBI121-SS<sup>ω</sup>-YK8-EGFP, and pBI121-SS<sup>ω</sup>-YL8-EGFP. These plasmids were transformed by electroporation

Fractionation of the Concentrated Medium by Hydrophobic Interaction Chromatography—Concentrated medium was adjusted to 2 m sodium chloride and then centrifuged at 20,000 x g for 20 min. Supernatants were loaded onto a butyl-Sepharose 4 Fast Flow hydrophobic interaction chromatography (HIC) column (inner diameter, 1.6 x 40 cm, Amersham Biosciences) equilibrated in 2 m aqueous sodium chloride. Elution proceeded stepwise using first 2 m aqueous sodium chloride, then 1 m aqueous sodium chloride, and finally distilled-deionized water. Visibly green fractions eluted in water were collected, dialyzed against distilled, deionized water for 36 h at 4 °C, and then lyophilized. The corresponding fractions were designated YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP.
Fractionation of YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP by Gel Permeation Chromatography—The green fluorescent peaks from HIC were dissolved at 30 mg/ml in 200 mM sodium phosphate buffer (pH 7) containing 0.005% (w/v) sodium azide (Superose buffer) and further fractionated by gel permeation chromatography on a preparative Superose-12 or a preparative Superose-6 column (Amersham Biosciences) equilibrated in Superose buffer. Eluates (flow rate, 0.5–1 ml/min) were monitored at 220 nm and by eye for green color.

Purification of YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP by Reversed-phase HPLC—Fractions from gel permeation chromatography were injected directly onto a semi-preparative C4 reversed-phase HPLC column (inner diameter, 1 × 25 cm, Vydac, Hesperia, CA), equilibrated in 0.1% (v/v) aqueous trifluoroacetic acid, and eluted with a 60-min linear gradient of 0–100% end buffer (0.1% aqueous trifluoroacetic acid/water, v/v, in 80% acetonitrile, v/v) at a flow rate of 2 ml/min. Spectrophotometric detection was at 280 nm.

Neutral Sugar Analyses—The neutral sugar compositions of YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP (100 μg each) were determined as alditol acetate derivatives (34). Glucose chromatography was performed on an HP-5890 Series II GC using an HP-5 column (cross-linked 5% PH ME Siloxane; inner diameter 3 mm × 0.32 mm; 0.25-μm film thickness; Hewlett-Packard) programmed from 130 to 177 °C at a rate of 1.5 °C/min and then from 177 to 200 °C at a rate of 10 °C/min.

Hyp-O-Glycosylation Profiles—YK20-EGFP (2.20 mg), YK8-EGFP (4.10 mg), YL8-EGFP (2.25 mg), and FK9-EGFP (2.21 mg) were each treated with HF (36) resulting in the deglycosylated fusion proteins dYK20-EGFP, dYK8-EGFP, dYL8-EGFP, and dFK9-EGFP (1 mg each) and YK20XL (2.2 mg) were treated with HF (36) resulting in the deglycosylated fusion proteins dYK20XL, dYK8-EGFP, dYL8-EGFP, and dFK9-EGFP (1 mg each) and YK20XL (2.2 mg) were hydrolyzed in 6 N HCl (aqueous) with 10 mM phenol (2 mg/ml; 110 °C; 20 h). Hydrolysates were dried under a stream of nitrogen (gas) and then redissolved at 10 mg/ml in water. The hydrolysates were fractionated by gel permeation chromatography on a poly-hydroxethyl A column (inner diameter, 9.4 × 200 mm, 10 nm pore size, Poly LC Inc., Columbia, MD) equilibrated in 50 mM formic acid and eluted isocratically at a flow rate of 0.8 ml/min. UV absorbance was monitored at 280 nm. The amounts of IDT and di-IDT in the hydrolysates were determined by comparison with authentic IDT and di-IDT standards. Response factors were determined from three level calibrations of IDT and di-IDT standards. The IDT standard was a gift from Professor Derek T. A. Lamport, University of Sussex, and the di-IDT standard was a gift from Professor Stephen Fry, University of Edinburgh.

Isolation of Tomato P1 Extensin—Tomato (Lycopersicon esculentum) P1 was isolated from Bonnie Best cell suspension culture medium by cation exchange chromatography as described earlier (15).

In Vitro Cross-linking of P1 Extensin and P3 Extensin Analogs by Tomato pl 4.6 Extensin Peroxidase—The pl 4.6 extensin peroxidase fraction was isolated from the culture medium of tomato cell suspension cultures by a single step of DEAE-anion exchange chromatography (20) and used for the cross-linking assays. The basic extensin peroxidase characterized earlier from tomato cell walls was also isolated by salt elution of the cells as described by Everdeen et al. (23). Peroxidase activity was quantified by 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid assay (20, 23) and by heme concentration using εmax = 100 mm-l cm⁻³ (38).

Isolated YK20, YK8, YL8, and FK9 and native tomato P1 extensin were used as substrates in in vitro cross-linking reactions. The assay involved addition in the following order: 10 μl of extensin substrate (6 mg/ml in a 5% (w/v) sodium bicarbonate buffer, 5 mM Tris-HCl, pH 7), 50 mM hydrogen peroxide (freshly prepared), 5 μl of enzyme in sodium acetate buffer (1 ng of enzyme, either the pl 4.6 peroxidase or the basic peroxidase). Cross-linking reactions (20 μl final volume) were performed at room temperature as described earlier (20); the final concentration of substrate was 3 mg/ml and the hydrogen peroxide was 60 μM. After incubation for 1–15 min, the reactions were stopped by the addition of 10 μl of 50 mM mercaptoethanol. Two-thirds of each reaction mixture (40 μg) was then injected onto an analytical Superose-6 FPLC gel permeation column equilibrated in Superose buffer (described above) and eluted at a flow rate of 0.75 ml/min; the resulting peak areas were integrated. Based on the areas of time 0 controls, response factors were determined, and the cross-linking rates of three replicates of each reaction mixture were determined by the decrease in the amount of monomer peak area after a 1-min reaction, as described earlier (23).

The results were represented as relative rates compared with P1 cross-linking rates, as the absolute rates varied from one peroxidase preparation to another.

In Vitro Cross-linking of YK20 for Isolation of Cross-linking Amino Acid—YK20 (20 mg) was cross-linked overnight as described above, and the reaction was stopped with 50 mM 2-mercaptoethanol and then dialyzed. The dried products were suspended in a minimal volume of water and centrifuged at 10,300 × g for 20 min. The insoluble YK20XL pellet was washed with water and repelleted to remove salts and soluble peroxidase until the conductivity of the supernatant was equal to that of water, and then YK20XL was lyophilized.

Plasmolysis of Transformed Tobacco Cells—Transformed tobacco cells were plasmolysed in 500 mM potassium phosphate buffer (pH 7) or 750 mM mannitol and inspected for EGFP fluorescence via fluorescence microscopy using a Zeiss LSM 510 laser-scanning confocal microscope (488 nm excitation; 510 nm emission).

Fractionation of the Unknown Isolated from the YK20XL Hydrolysate by Paper Chromatography—The unknown peak isolated by gel permeation chromatography of the YK20XL hydrolysate was further fractionated by paper chromatography on Whatman 5MM paper using butanol/acetic acid/water (BAW; 12:3:5 by volume) as described earlier (21); the amino acids di-IDT, IDT, and Tyr served as the standards.

MALDI-TOF Mass Spectrometry—Mass analysis was performed by MALDI-TOF MS using an Applied Biosystems ABI4700 TOF/TOF mass spectrometer (Applied Biosystems Inc., Framingham, MA) with an accelerating voltage of 20 kV. Samples were mixed in a 1:6 ratio of tri-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid. A 0.5-μl aliquot was applied to the sample plate.

One-dimensional 'H and Two-dimensional GMQ-COSY NMR—One- and two-dimensional proton NMR experiments were carried out on a Varian INOVA 500-MHz spectrometer operating at a 1H frequency of 499.896 MHz. The analysis was conducted at 25 °C. The unknown sample (~75 μg) was dissolved in 700 μl of 10% deuterium oxide and 90% water.

RESULTS

Synthetic Gene Assembly—We used two methods to make our synthetic genes. The first method, involving polymerization of duplex DNA oligomer sets, gave rise to the synthetic gene.
encoding nine repeats of SPPPPSPPPPPFFFK, designated FK9 (see supplemental Fig. 8A) (28). As this method only produced relatively short genes giving little control over resulting gene sizes, a second method (31) was used to make synthetic genes encoding either 8 and 20 repeats of SPPPPSPSPPPPYYYK, designated YK8 and YK20, respectively, and 8 repeats of SPPPPSPSPPPPYYYL, designated YL8; it involved nonregenerable restriction sites (supplemental Fig. 9). DNA sequencing confirmed the in-frame assembly of the transgenes between the encoded tobacco signal sequence (SStob) and the EGFP gene (Fig. 1).

From known extensin gene sequences, we estimated that 20 synthetic gene repeats represented a “full-length” extensin, containing about 320 amino acids (39), although there is considerable variation in extensin lengths. Therefore, we made one construction, YK20, containing 20 P3 cross-linking modules. We made shorter variants, namely YK8, YL8, and FK9, to determine the amino acid requirements for intermolecular cross-linking and the effect of repeat unit number on cross-linking rates.

Selection of Transformed Cell Lines and Localization of Transgene Products—Transformation of tobacco BY-2 cells with the synthetic P3 analog genes SStob-YK8-EGFP, SStob-YK20-EGFP, SStob-YL8-EGFP, and SStob-FK9-EGFP yielded several cell lines for each construction. Lines expressing the most fusion protein into the culture medium of liquid cultures were selected for isolation and biochemical characterization of the transgene products.

Cells expressing the SStob-YK20-EGFP synthetic gene demonstrated EGFP localization in the cell walls of plasmolysed cells; however, this was only apparent at times when they were most highly expressed (Fig. 2).

Fusion Glycoprotein Purification and Yields—A combination of hydrophobic interaction chromatography (HIC) and gel permeation chromatography on Superose-6 or Superose-12 for subsequent biochemical characterization. Typical yields ranged from 3 to 27 mg liter\(^{-1}\) for YK20-EGFP, from 4 to 7 mg liter\(^{-1}\) for YK8-EGFP, from 6 to 23 mg liter\(^{-1}\) for YL8-EGFP, and from 0.1 to 3.3 mg liter\(^{-1}\) for FK9-EGFP.

Amino Acid Compositions of YK20, YK8, YL8, FK9, and Cross-linked YK20 (YK20XL)—Amino acid composition analyses of the P3 analogs after removal of EGFP (YK20, YK8, YL8, and FK9) including cross-linked YK20 (YK20XL) (Table I) indicated that most of the proline residues were hydroxylated. Fractionation of YK20, YK8, YL8, and FK9 hydrolysates on the polyhydroxyethyl A column resolved Tyr, IDT, and di-IDT and showed that YK20, YK8, YL8, and FK9 all contained IDT (Fig. 4).

For the sample analyses shown in Table I, about half of the 60 total Tyr residues in YK20 and 24 total Tyr residues in YK8 occurred as IDT (30 residues of Tyr = 15 IDT residues per YK20 molecule; 12 Tyr residues = 6 IDT residues per YK8 molecule), whereas YL8 contained less IDT (~2–3 IDT residues and 18 Tyr). However, different preparations of YK20 and YK8 varied in the amount of IDT they contained, the ratio of IDT to Tyr ranging from ~0.4 to 1; YL8 preparations consistently showed a lower molar IDT to Tyr ratio of ~0.4. FK9 lacked IDT consistent with the absence of Tyr. Di-IDT was present only in the cross-linked products of YK20, YK8, YL8, and FK9 (see “Experimental Procedures” for IDT and di-IDT isolation). We estimated the that highly cross-linked YK20 (cross-linked overnight) had a di-IDT/IDT/Tyr molar ratio of 1:2:4.

Partial N-terminal Sequences of FK9-EGFP and Deglycosylated YK20-EGFP, YK8-EGFP, and YL8-EGFP—Partial N-term-
minal sequences of deglycosylated YK20-EGFP (dYK20-EGFP), deglycosylated YK8-EGFP (dYK8-EGFP), deglycosylated YL8-EGFP (dYL8-EGFP), and of glycosylated FK9-EGFP confirmed the sequences predicted from their respective synthetic genes: RRPSOOOOYYYKSOOOOSOS for YK20 (O denotes Hyp), RR-OSOOOOYYYKSOOOOSOS for YK8, RRPSOOOOYYYLSOO-OOSOS for YL8, and SOOOOSOSOOOOFFFKSOOOOSOSO-OOFFFKS for FK9.

Neutral Sugar Composition Analyses—Neutral sugar analyses (Table II) indicated that all of the fusion glycoproteins contained arabinose and galactose. The small amount of glucose present in some of the samples was probably a contaminant. Likewise, the rhamnose and xylose in YK8-EGFP and YL8-EGFP were also probably contaminants, given the Hyp-glycoside profiles of the molecules.

Hyp-glycoside Profiles—Hyp-glycoside profiles for the fusion glycoproteins demonstrated that most of the Hyp residues were tetra- and tri-O-arabinosylated (Table III). A small amount of large molecular weight Hyp-glycan was detected only in YL8-EGFP.

In Vitro Cross-linking Assays Using the pl 4.6 Extensin Peroxidase from Tomato—Anion exchange chromatography of the tomato culture medium yielded a fraction containing the anionic extensin peroxidase characterized earlier (20). Cross-linking was assayed by the decrease in glycoprotein monomer and corresponding increase in multimers via gel permeation chromatography on Superose-6 (Fig. 5).

The absolute cross-linking rates (mg cross-linked min⁻¹ mg enzyme⁻¹) were estimated from the decrease in monomer peak area after 1 min of cross-linking compared with a time 0 control. YK20, YK8, YL8, and tomato P1 extensin were all cross-linked in vitro by the peroxidase fraction; however, the absolute cross-linking rates after a 1-min reaction varied from one enzyme preparation to another due to variations in the purity of the enzyme preparations. For instance, the P1 cross-linking rates for two separate enzyme preparations were 8,784 and 17,055 mg of P1 cross-linked min⁻¹ mg enzyme⁻¹. However, the relative rates of cross-linking compared with the cross-linking of tomato extensin P1 were consistent across individual enzyme preparations (Fig. 6). The relative cross-linking rate for YK20 was greater than the rates for native P1 extensin and much greater than the smaller tyrosine-containing glycomodules YK8 and YL8. Most significantly, FK9 was not cross-linked in vitro by the enzyme. Controls showed that all cross-linking reactions required hydrogen peroxide and the pl 4.6 peroxidase (20, 23). The basic peroxidase eluted from the tomato cell surface showed no significant cross-linking activity at the enzyme concentrations used in the assays.

Isolation and Characterization di-IDT from Cross-linked YK20—Extended cross-linking of YK20 (18–20 h) for isolation of the unknown cross-link amino acid yielded a flocculent precipitate that remained insoluble after removal of the saccharide adducts by HF deglycosylation. We isolated the A₂₈₀ nm absorbing peak, designated unknown in Fig. 4, from the hydrolysates of cross-linked YK20. The unknown and an authentic di-IDT standard co-eluted on the polyhydroxyethyl A column and co-migrated when analyzed by paper chromatography; the unknown also exhibited intense blue fluorescence when visualized under UV light after exposure to ammonia vapor (not shown). MALDI-TOF MS of the unknown and the di-IDT standard yielded molecular ions of m/z 719. The one-dimensional ¹H NMR spectrum of the unknown showed four groups of resonances in the aromatic proton region (Fig. 7). The resonances at 7.203 (dd, J = 8.4 Hz, 2.2 Hz) and 6.965 ppm (dd, 8.4 Hz, 2.2 Hz) (Fig. 7, peaks B and D, respectively) indicated that the unknown contained 1,4-substituted benzene rings. Two mutually coupled single-proton doublet resonances (J = 2.2 Hz) at 6.900 and 6.970 ppm (Fig. 7, peaks C and E) suggested the existence of 1,2,3,5-substituted benzene rings. These couplings...
were confirmed by a two-dimensional HH-COSY spectrum (not shown), which showed signal correlations between 7.203 and 6.965 ppm and between 6.970 and 6.900 ppm. The N-linked protons gave rise to resonances at 8.330 ppm (Fig. 7, peak A).

Three groups of aliphatic protons with signals at 3.91, 3.21, and 3.02 ppm in the one-dimensional spectrum corresponded to $H_{9251}$, $H_{9252}$, and $H_{9252}/H_{11032}$, respectively, of tyrosine moieties. The two-dimensional HH-COSY spectrum showed correlations of $H_{9251}$ to $H_{9252}$, $H_{9251}$ to $H_{9252}/H_{11032}$, and $H_{9252}$ to $H_{9252}/H_{11032}$, which confirmed the aliphatic proton systems of di-IDT. However, because of resonance overlapping, we could not assign these signals to individual tyrosine moieties of di-IDT.

The integrated area ratios of signals A/B/C/D/E in the one-dimensional $1H$ NMR spectrum (Fig. 7) was 11.3:4.2:2.1:3.9:2.0, which was consistent with the expected ratio of 10.8:4:2:4:2 for a di-IDT spectrum run in 10% $D_2O$.

**DISCUSSION**

Synthetic genes enabled the elucidation of O-Hyp glycosylation codes and validated the Hyp contiguity hypothesis as a useful predictive tool (25–27). Here we extended this approach not only to apply the Hyp contiguity hypothesis to known extensin motifs but also to test putative codes for other post-translational modifications that lead to the formation of specific cross-links. Such cross-links help define the extensin networks that contribute to wall self-assembly and architecture ranging from embryogenesis (4) to stress responses (6, 7). We designed a series of P3-type extensin analogs for expression and characterization because P3 extensins are difficult to isolate, yet play a crucial role in the cell wall judging from their ubiquity and their highly conserved repetitive sequences that include palindromic motifs (boldface) and numerous short intramolecular IDT motifs (underlined): Ser-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Tyr-Tyr-Tyr-Lys.

Such palindromic supersymmetry (1) may enhance self-as-

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**TABLE I**

Amino acid compositions of YK20, YK8, YL8, FK9, and YK20XL compared with compositions predicted from their respective gene sequences

| Amino acid | YK20 Protein | YK20 Protein Gene | YK8 Protein | YK8 Gene | YL8 Protein | YL8 Gene | FK9 Protein | FK9 Gene |
|------------|--------------|-------------------|------------|---------|------------|---------|------------|---------|
| Hyp        | 60           | NA                | 57 NA      | NA      | 60 NA      | 56 NA   | 53 NA      | NA      |
| Pro        | 0            | 55.4              | 2 NA       | NA      | 0 54.1     | 0 54.1  | 0 55.3     | NA      |
| Ser        | 18           | 19.0              | 18 NA      | NA      | 18 19.3    | 20 19.3 | 18 19.5    | NA      |
| Tyr        | 9            | 18.3              | 11 NA      | NA      | 9 17.8     | 13 NA   | 0 0.0      | NA      |
| 1⁄2IDT     | 9            | NA                | 3 NA       | NA      | 8 NA       | 5 NA    | 0 NA       | NA      |
| 1⁄4di-IDT  | 0            | NA                | 4 NA       | NA      | 0 NA       | 0 NA    | 0 NA       | NA      |
| Phe        | 0            | 0.0               | 0 NA       | NA      | 0 0.0      | 0 0.0   | 21 17.5    | NA      |
| Lys        | 4            | 6.4               | 6 NA       | NA      | 5 6.7      | 0 0.7   | 7 6.5      | NA      |
| Leu        | 0            | 0.0               | 0 NA       | NA      | 0 0.0      | 6 0.0   | 0 0.0      | NA      |
| Thr        | 0            | 0.3               | 2 NA       | NA      | 0 0.7      | 0 0.7   | 0 0.0      | NA      |
| Met        | 0            | 0.2               | 0 NA       | NA      | 0 0.7      | 0 0.7   | 1 0.6      | NA      |
| Val        | 0            | 0.2               | 0 NA       | NA      | 0 0.7      | 0 0.7   | 0 0.6      | NA      |

*a* Both IDT and di-IDT were assayed by gel permeation chromatography rather than standard amino acid analysis techniques. The composition of YK20XL (shown here) was determined on material cross-linked for only 15 min, and di-IDT was present but the levels were low compared with the preparations, such as highly cross-linked YK20, that were used for di-IDT characterization.

*b* NA, not applicable.

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**TABLE II**

Neutral sugar compositions of YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP

| Glycosyl residue | YK20-EGFP* | YK8-EGFP | YL8-EGFP | FK9-EGFP |
|-----------------|------------|----------|----------|----------|
| Arabinose       | 90         | 91       | 84       | 91       |
| Galactose       | 8          | 7        | 12       | 9        |
| Rhamnose        | 1          | 1        | 2        | 0        |
| Xylose          | 0          | 0        | 1        | 0        |
| Glucose         | 1          | 1        | 1        | 0        |

*a* EGFP is not glycosylated (25).

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**TABLE III**

Hyp-glycoside profiles of YK20-EGFP, YK8-EGFP, YL8-EGFP, and FK9-EGFP

| Hyp-glycoside | YK20-EGFP | YK8-EGFP | YL8-EGFP | FK9-EGFP |
|---------------|-----------|----------|----------|----------|
| % total Hyp   |           |          |          |          |
| Hyp-PS        | 0         | 3        | 0        | 0        |
| Hyp-Ara$_4$   | 56        | 56       | 55       | 42       |
| Hyp-Ara$_3$   | 32        | 31       | 31       | 40       |
| Hyp-Ara$_2$   | 4         | 4        | 6        | 5        |
| Hyp-Ara$_1$   | 5         | 5        | 5        | 5        |
| NG-Hyp        | 3         | 4        | 5        | 8        |

*a* The abbreviations used are as follows: Hyp-PS, Hyp polysaccharide; Hyp-Ara$_n$, Hyp-oligoarabinoside$_{1–4}$; NG-Hyp, non-glycosylated Hyp.

protons gave rise to resonances at 8.330 ppm (Fig. 7, peak A). Three groups of aliphatic protons with signals at 3.91, 3.21, and 3.02 ppm in the one-dimensional spectrum corresponded to $H_a$, $H_b$, and $H_b’$, respectively, of tyrosine moieties. The two-dimensional HH-COSY spectrum showed correlations of $H_a$ to $H_b$, $H_a$ to $H_b’$, and $H_b$ to $H_b’$, which confirmed the aliphatic proton systems of di-IDT. However, because of resonance overlapping, we could not assign these signals to individual tyrosine moieties of di-IDT.

The integrated area ratios of signals A/B/C/D/E in the one-dimensional $1H$ NMR spectrum (Fig. 7) was 11.3:4.2:2.1:3.9:2.0, which was consistent with the expected ratio of 10.8:4:2:4:2 for a di-IDT spectrum run in 10% $D_2O$.
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FIG. 5. Superose-6 gel permeation assay of the YK20 15-min in vitro cross-linking reaction. A, the time 0 control yielded a major peak corresponding to monomeric YK20 and reagent peaks that eluted after 25 min. B, after 15 min of cross-linking, most of the YK20 monomeric peak shifted to the void volume of the column. Similar profiles (not shown) were observed for YK8 and YL8, except the monomers, which are smaller than YK20 and eluted later from the column.

FIG. 6. The relative cross-linking rates of the P3 analogs YK20, YK8, YL8, and FK9 compared with tomato P1 extensin. The absolute cross-linking rates averaged from reactions carried out using two different enzyme preparations were used to determine the relative rates presented here. The error bars represent the estimated standard deviation. FK9 showed no cross-linking whatsoever.

assembly of tightly packed networks (cf. Ref. 40) knitted together by intra- and intermolecular covalent cross-link sites involving IDT. Furthermore, the related extensin RSH, required for cell plate orientation during cytokinesis (4), also contains numerous putative IDT motifs and a repetitive symmetry that, although not palindromic, should also favor similar close packing. It was therefore of considerable interest to express simple P3 analogs with Tyr → Phe and Lys → Leu mutations in the putative cross-link site.

Post-translational Hydroxylation and Glycosylation—Because endogenous P3 extensins have not been isolated in their precursor forms, P3 glycosylation profiles can only be inferred from their corresponding genes (39) and from Hyp-glycoside profiles of P3 glycopeptides enzymically released from the walls (18, 41). The Hyp contiguity hypothesis (1, 25–27, 42) predicts that P3 extensins, with their abundant contiguous Hyp and nonclustered single Hyp residues, should be extensively arabinosylated mainly with tetra- and triarabino-oligosaccharides but lack arabinogalactan polysaccharides. The monosaccharide compositions and Hyp-glycoside profiles of YK20-EGFP, YKS-EGFP, YL8-EGFP, and FK9-EGFP were consistent with earlier P3 glycopeptide profiles (18) and with the predictions for Hyp-glycosylation, the exception being a small amount of Hyp-polysaccharide in YL8-EGFP (Tables II and III). These large molecular weight Hyp-glycans were likely arabinogalactan polysaccharides given the monosaccharide composition of YL8-EGFP which contained somewhat more galactose and rhamnose than the other glycoproteins (Table II). Comparing with earlier Hyp-glycosylation profiles of HRGP analogs having only tandem repeats of the pentapeptide (Ser-Hyp-Hyp-Hyp-Hyp), or the dipeptide (Ser-Hyp), (25, 26), we infer that an arabinogalactan adduct probably occurred on two of the eight lone Hyp residues occupying the center (underlined) of the palindromic repeats: Ser-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Tyr-Tyr-Tyr-Leu.

From their amino acid compositions (Table I), we determined that YK20 contained about 180 Hyp residues, YK8 and YL8 contained about 72, and FK9 about 81. Of the total Hyp residues in each glycoprotein, only 3–8% (i.e., 3–7 Hyp residues/glycoprotein) were not glycosylated, which indicated that generally all Hyp residues in the 16-residue repeats were arabinosylated, including arabinosylation of the noncontiguous Hyp residues. This contrasts with polysaccharide addition to AGP noncontiguous Hyp residues that are generally, but not always, clustered, unlike the sporadic lone Hyp residues of extensins. This is consistent with earlier work demonstrating that in extensins contiguous Hyp residues are the preferred arabinosylation sites, whereas noncontiguous Hyp residues can be arabinosylated or remain nonglycosylated (42). The occurrence of arabinosylated, noncontiguous Hyp residues in the P3-type extensins, but addition of arabinogalactan polysaccharide to noncontiguous Hyp that is not highly clustered in the AGPs (43), raises questions about what determines the glycosylation of nonclustered, noncontiguous Hyp residues. Perhaps conformational differences between the extensin polypeptide, which is rich in polyhydroxyproline that determines a polyproline-II helix, and the AGP polypeptide, which possesses a more flexible “random coil” conformation (26), control the specificities on the glycosyltransferases involved in Hyp O-glycosylation. On the other hand, judging by the proline hydroxylation patterns and Hyp-glycosylation specifics of a series of synthetic genes expressed earlier in BY2 cells, the amino acid residues flanking Pro and Hyp residues may also play a role in both proline hydroxylation and Hyp O-glycosylation (27).

In addition to arabinosides, extensins generally contain monogalactosylated Ser (18, 42, 44, 45), and this seems likely for YK20, YK8, FK9, and probably for YL8, although at least some of the Gal in YL8 probably occurs in the few larger molecular weight Hyp-glycans. The monosaccharide compositions of YK20, YK8, and FK9 (Table II) showed they contained about 1 mol of Gal for every 10–11 mol of Ara, indicating monogalactose occurred on each Ser residue in the P3-type module. This also is in agreement with native P3 glycopeptides isolated and characterized earlier from tomato cell walls (46) but not with the glycosylation profiles of (Ser-Hyp-Hyp-Hyp), and (Ser-Hyp-Hyp), (26) which contained abundant Ara but no Gal. Thus, the presence of Lys in the galactosylated glycopeptide motifs and the lack of Lys in the nongalactosylated (Ser-Hyp-Hyp-Hyp),
and (Ser-Hyp-Hyp), motifs suggests an amino acid sequence code may also direct monogalactosylation of Ser residues in the extensins.

Cross-linking of Expression Products by a pI 4.6 Extensin Peroxidase—A pI 4.6 peroxidase from tomato covalently cross-links monomeric P1 extensin, a natural substrate that has multiple Val-Tyr-Lys putative cross-link motifs (1, 20). Although the pI 4.6 isozyme has a specific activity 2–3 orders of magnitude greater than a basic tomato peroxidase that cross-links P1 extensin in vitro (23), its substrate range remains undefined. Conceivably, the cross-linking of multiple extensins might involve multiple extensin peroxidases, as several exten- sin peroxidases have been identified in a range of species (20, 23, 24, 47). We therefore tested a P3 analog, YK20, as a sub- strate. The pI 4.6 isozyme, but not the basic peroxidase isozyme, cross-linked YK20 at rates somewhat greater than the P1 substrate (Fig. 6), and the cross-linking was intermolecular rather than intramolecular as the products remained insoluble after deglycosylation, unlike HF-deglycosylated monomeric YK20. The cross-linking of YK20 was somewhat surprising because the Tyr-Tyr-Tyr motif in YK20 is a precursor of inte- molar cross-link in P3 extensin rather than intramolecular as the products remained insoluble after deglycosylation, unlike HF-deglycosylated monomeric YK20. The cross-linking of YK20 was somewhat surprising because the Tyr-Tyr-Tyr motif in YK20 is a precursor of in- termolecular cross-link in P3 extensin rather than intramolecular as the products remained insoluble after deglycosylation, unlike HF-deglycosylated monomeric YK20. The cross-linking of YK20 was somewhat surprising because the Tyr-Tyr-Tyr motif in YK20 is a precursor of in- termolecular cross-link in P3 extensin rather than intramolecular as the products remained insoluble after deglycosylation, unlike HF-deglycosylated monomeric YK20. The cross-linking of YK20 was somewhat surprising because the Tyr-Tyr-Tyr motif in YK20 is a precursor of intermolecular cross-link in P3 extensin rather than intramolecular as the products remained insoluble after deglycosylation, unlike HF-deglycosylated monomeric YK20.

Parameters other than the presence of Tyr influenced the cross-linking rates of the P3 analogs, primarily the molecular length of the substrate, as YK20 cross-linked more efficiently than the smaller YK8. Residues flanking the IDT motif may also contribute in subtle ways to influence intermolecular cross-linking as the Lys → Leu mutation in YL8 significantly decreased the cross-linking rate compared with YK8. This suggests a role for Lys in enzymic recognition of the cross-linking site. However, the Tyr → Phe mutation in FK9 completely abolished cross-linking, confirming direct tyrosine involvement.

The unknown amino acid isolated from hydrolysates of cross-linked YK20 was identified as di-IDT through its characteristic blue fluorescence, absorbance at 280 nm, chromatographic behavior, molecular mass, and NMR spectra (Fig. 7). Most interestingly, we found no pulcherosine in our cross-linked products, although Fry and c-workers (22) also isolated this tri-tyrosine derivative from cell wall hydrolysates and suggested a role in extensin cross-linking. Possibly pulcherosine in muro mediates cross-linking between P-3 type IDT motifs and the lone Tyr residues occurring in the Val-Tyr-Lys motifs common in P1- type extensin, which lack IDT. Alternatively, pulcherosine may have been a cross-linking intermediate that was rapidly incor- porated into di-IDT during our cross-linking reactions.

The Role of Cross-linking—EGFP fluorescence appeared both in the growth medium and the walls of plasmolysed cells expressing YK20-EGFP. This confirmed the association of YK20-EGFP with the wall and is consistent with our recent isolation of a P3 peptide from a native extensin in the tobacco HF-insoluble cell wall, although the salt-elutable, soluble P3 monomers were not detected.2 Our ability to readily isolate constitutively expressed YK20-EGFP from the growth medium suggests that the resistance to isolation of native P3 extensins is not due to their peculiar molecular characteristics but may simply be due to a very small P3 precursor pool in muro, unlike the much larger P1 pool that is also readily isolated (44). Such differences may imply functionally distinct roles for IDT-rich and IDT-poor extensins, suggested earlier (15) and exemplified by the recent isolation of the lethal RSH mutant (4) correspond- ing to the loss of a putatively IDT-rich extensin with an essen- tial role in cross-wall formation. Thus self-assembly of these extensin networks in muro may indeed involve self-recognition mediated by the inherent hydrophobicity of the putative cross-linking sites (21, 48) aided by concurrent alignment of the highly regular and hydrophilic Ser-(Hyp)₄ glycomodules (1, 21).

General Conclusions—With the identification of a di-IDT intermolecular extensin cross-link formed here in vitro, this work extends the use of synthetic genes from identifying post-translational codes for glycosylation (26–27) to codes for cross-linking and aspects of wall self-assembly. The ramifications include not only the self-assembly of extensin networks in muro but the module-by-module design of new glycoprotein- 

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2 M. Kieliszewski, and K. Terneus, unpublished results.
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Based biopolymers that combine the special properties conferred by glycosylation and specific peroxidatic cross-linking. Although the wall is frequently described as “cellulose microfibrils embedded in an amorphous matrix,” the evidence presented here for the rapid alignment and specific cross-linking of P3-type extensins indicates the wall is more accurately viewed as an exquisitely designed self-assembling (49, 50) polysaccharide and glycoprotein nanofiber networks crucially dependent on peroxidatic cross-linking.

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REFERENCES

1. Kieliszewski, M. J., and Lamport, D. T. A. (1994) Plant J. 5, 157–172
2. Roberts, K. (1974) Philos. Trans. R. Soc. Lond. B Biol. Sci. 268, 129–146
3. Goodenough, U. W., Gebhart, B., Mercham, R. P., and Heuser, J. E. (1986) J. Cell Biol. 103, 405–417
4. Hall, Q., and Cannon, M. C. (2002) Plant Cell 14, 1161–1172
5. Shirsat, A. H., Bell, A., Spence, J., and Harris, J. N. (1996) Planta 199, 618–624
6. Showalter, A. M., Zhou, J., Rumeau, D., Worst, S. G., and Varner, J. E. (1991) Plant Physiol. 95, 157–172
7. Cassab, G. I. (1986) Planta 168, 441–446
8. Van Dam, H., Govers, F., Louwerse, J., van K den, A., and Bisseling, T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4495–4499
9. Frueauf, J. B., Dolata, M., Leykam, J. F., Lloyd, E. A., Gonzalez, M., Vanden-Bosch, K., and Kieliszewski, M. J. (2000) Phytochemistry 55, 429–438
10. Lamport, D. T. A. (1983) Plant Physiol. 73, 1438–1440
11. Lamport, D. T. A. (1986) Nature 321, 1232–1234
12. Kieliszewski, M. J. (2001) Phytochemistry 67, 319–323
13. Smith, J. J., Muldoon, E. P., Willard, J. J., and Lamport, D. T. A. (1986) Phytochemistry 25, 1021–1030
14. Lamport, D. T. A. (1984) Phytochemistry 23, 1241–1246
15. Lamport, D. T. A. (1989) Biochemistry 8, 1155–1163
16. Lamport, D. T. A., and Epstein, L. (1983) in Proceedings of the Annals of Plant Biochemistry and Physiological Society Symposium (Randall, D. D., Blevins, D. G., Larson, R. L., Rapp, B. J., Pallardy, S. G., Nelson, C. J., Pelaez, J. C., and George, M. F., eds) Vol. 2, pp. 73–83, University of Missouri, Columbia, MO
17. Lamport, D. T. A. (1996) Plant J. 9, 477–489
18. Lamport, D. T. A., and Sadler, I. H., and Fry, S. C. (1996) Biochem. J. 315, 323–327
19. Brady, J. D. (1996) Phytochemistry 47, 349–353
20. Everdeen, D. S., Kiefer, S., Willard, J. J., Muldoon, E. P., Dey, P. M., Li, X.-B., and Lamport, D. T. A. (1988) Plant Physiol. 87, 616–621
21. Brownleander, M. D., Ahmed, N., Trevan, M. F., and Dey, P. M. (1995) Plant Physiol. 109, 1115–1123
22. Shpak, E., Leykam, J., and Kieliszewski, M. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14786–14791
23. Shpak, E., Barbar, E., Leykam, J. F., and Kieliszewski, M. J. (2001) J. Biol. Chem. 276, 11272–11278
24. Tan, L., Leykam, J., and Kieliszewski, M. J. (2003) Plant Physiol. 129, 3362–3369
25. McGrath, K. P., Tirrell, D. A., Kawai, M., Mason, T. L., and Fournier, M. J. (1990) Biotechnol. Prog. 6, 186–192
26. Van Dam, H., Govers, F., Tire, C., Gien, J., Villarol, R., Genetello, C., Van Montagu, M., Depicker, A., and Inze, D. (1991) Gene (Amst.) 99, 95–100
27. Bevan, M. W. (1984) Nucleic Acids Res. 12, 8711–8721
28. Lewis, R. V., Hinman, M., Kohvakka, S., and Fournier, M. (1996) Protein Expression Purif. 7, 400–406
29. An, G., Ebert, P. R., Mitra, A., and Ha, S. B. (1988) Plant Molecular Biology Manual, pp. 1–19, Martinus Nijhoff, Dordrecht, Netherlands
30. McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R. (1986) Plant Cell Rep. 5, 81–84
31. Albersheim, P., Nevins, D. J., English, P. D., and Karr, A. (1967) Carbohydr. Res. 5, 340–345
32. Lamport, D. T. A., and Miller, D. H. (1971) Plant Physiol. 48, 454–456
33. Sanger, M. P., and Lamport, D. T. A. (1983) Anal. Biochem. 132, 66–70
34. Bergman, T., Carliquist, M., and Jornvall, H. (1986) in Advanced Methods in Protein Microsequence Analysis (Wittman-Liebold, B., et al., eds) pp. 45–55, Springer-Verlag, Berlin
35. Nakajima, R., and Yamasaki, I. (1979) J. Biol. Chem. 254, 872–878
36. Zhou, J., Rumeau, D., and Showalter, A. M. (1992) Plant Mol. Biol. 20, 5–17
37. Knapp, C., and Squire, J. M. (2003) The Scientific World 3, 558–577
38. Lamport, D. T. A. (1977) in Recent Advances in Phytochemistry (Loewus, F. A., and Runcell, V. C., eds) Vol. 11, pp. 79–115, Plenum Publishing Co., New York
39. Kieliszewski, M. J., O’Neill, M., Leykam, J., and Orlando, R. (1995) J. Biol. Chem. 270, 2541–2549
40. Zhao, Z. D., Tan, L., Showalter, A. M., Lamport, D. T. A., Kieliszewski, M. J. (2002) Plant J. 31, 431–444
41. Lamport, D. T. A., Katona, L., and Roerig, S. (1973) Biochem. J. 133, 125–131
42. Smith, J. J., Muldoon, E. P., and Lamport, D. T. A. (1984) Phytochemistry 23, 1233–1239
43. Lamport, D. T. A. (1973) First International Protoxplast Colloquium, Versailles, pp. 27–31, Institut de la Recherche Agronomique, Versailles, France
44. Price, N. J., Pinheiro, C., Soares, C. M., Ashford, D. A., Ricardo, C. P., and Jackson, P. A. (2003) J. Biol. Chem. 278, 41389–41399
45. Whitesides, G. M., Mathias, J. P., and Soto, C. T. (1991) Science 254, 1312–1319
46. Kieliszewski, M. J., and Lamport, D. T. A. (1988) in Self-Assembling Architecture. 4th Symposium for the Society for Developmental Biology (Varner, J., ed) Vol. 46, pp. 61–76, Alan R. Liss, Inc., New York
47. Zhang, S. (2002) Biotechnol. Adv. 20, 321–339