Planta (2001) 212: 470–474

Planta
© Springer-Verlag 2001

Rapid Communication

Enzymic feruloylation of arabinoxylan-trisaccharide by feruloyl-CoA:arabinoxylan-trisaccharide *O*-hydroxycinnamoyl transferase from *Oryza sativa*

Tomoko Yoshida-Shimokawa[1], **Shigeki Yoshida**[2], **Koichi Kakegawa**[1], **Tadashi Ishii**[1]

[1]Forestry and Forest Products Research Institute, P.O. Box 16, Tsukuba Norin Kenkyuu Danchinai, Ibaraki 305-8687, Japan
[2]Institute of Applied Biochemistry, University of Tsukuba, Tsukuba-shi, Ibaraki 305-8572, Japan

Received: 14 July 2000 / Accepted: 22 August 2000

Abstract. Feruloyl-CoA:arabinoxylan-trisaccharide *O*-hydroxycinnamoyl transferase, which catalyzes the transfer of ferulic acid from Fer-CoA to arabinoxylan-trisaccharide in the formation of feruloyl arabinoxylan-trisaccharide (Fer-AXX), has been found in an ionically bound fraction and a cytosol fraction of suspension-cultured rice (*Oriza sativa* L. cv. Nipponbare) cells. Analysis of reaction products by high-performance liquid chromatography showed the formation of product A, which is one of the transfer products having the same retention time as authentic Fer-AXX. Product A was purified by reverse-phase chromatographies to characterize its structure. The isolated product A showed the same ultraviolet spectrum and molecular weight on fast atom bombardment mass spectrometric analysis as those of authentic Fer-AXX. Alakaline saponification of product A released ferulic acid and oligosaccharide. The released oligosaccharide consisted of arabinose and xylose in a molar ratio of 1:2. These results support the identity of product A as feruloylated arabinoxylan-trisaccharide and show the existence of a feruloyltransferase catalyzing the feruloylation of a hemicellulosic fragment.

Key words: Arabinoxylan – Cell wall – Ferulic acid – Feruloyl-CoA – Feruloyltransferase – *Oryza* (cell wall)

Plant cell walls contain polymer-bound hydroxycinnamic acid derivatives, such as ferulic (Fer) and *p*-coumaric acids (Hartley and Ford 1989). The wall-bound hydroxycinnamic acids are thought to cross-link the cell wall polymers through the formation of dehydrodimers by oxidative coupling (Fry 1986; Iiyama et al. 1994). Such coupling should decrease wall extensibility, and may control cell growth.

In the graminaceous cell wall, Fer is located in various tissues and organs (Harris and Hartley 1976). Feruloylation in the Gramineae mainly occurs on arabinoxylan and slightly on xyloglucan (Ishii 1997). The feruloylated arabinoxylan-oligosaccharides prepared so far from graminaceous cell walls have shown good consistency of structure. The α-L-arabinofuranoyl (Ara*f*) residues are attached to *O*-3 positions of β-1,4-linked D-xylan and are substituted at position *O*-5 with the feruloyl group (Kato et al. 1983; Wende and Fry 1997). Such a structurally specific esterification with Fer groups naturally suggests the enzyme-catalyzed formation of feruloylated arabinoxylan.

Some feruloyltransferases involved in feruloylation of peptides (Kohler and Kauss 1997), amines (Yu and Facchini 1999), quinate (Lotfy et al. 1992) and hydroxy-fatty acids (Lotfy et al. 1996) have been reported and characterized previously. As for feruloyltransferase (FTase), the enzyme presumed to catalyze the feruloylation of cell wall sugar residues, there are still many unresolved matters (Brett et al. 1999). In previous work, intraprotoplasmic feruloylation of arabinoxylans was observed in *Festuca arundinacea* cell cultures in vivo (Myton and Fry 1994), and a microsomal preparation from suspension-cultured parsley cells was reported to transfer Fer from feruloyl-coenzyme A (Fer-CoA) to an endogenous acceptor in vitro (Meyer et al. 1991). The in vitro-synthesized feruloyl polysaccharide was, however, not fully characterized. The nature of the sugar residues that were feruloylated was not determined. In addition, Myton and Fry pointed out the possibility of artifactual results in FTase assays in vitro owing to the presence of peroxidases contained in membrane preparations, because the addition of catalase or dithiothreitol suppressed the apparent FTase activities (Myton and Fry 1995; Bolwell et al. 1997).

In order to apparently prove the existence of an FTase responsible for the formation of feruloylated cell wall polysaccharide fragments, an enzyme extract from

Abbreviations: α-L-Ara*f* = α-L-arabinofuranose; β-D-Xyl*p* = β-D-xylopyranose; FAB-MS = fast atom bombardment mass spectrometry; Fer = ferulic acid; Fer-CoA = feruloyl-coenzyme A; FTase = feruloyltransferase; AXX = arabinoxylan-trisaccharide; Rt = retention time

Correspondence to: T. Yoshida-Shimokawa;
E-mail: tshimo@ffpri.affrc.go.jp; Fax: +81-298-73-3795

suspension-cultured rice cells was examined for enzymic feruloylation on arabinoxylan-trisaccharide (AXX). We used a fluorometric method to detect the FTase activity. This method enabled to quantify as little as 20 pmol of the feruloylated products in HPLC analysis. Furthermore, we investigated the application of AXX as an acceptor. The structure of AXX isolated from rice straw (Yoshida et al. 1990) is the same as the sugar component of authentic feruloylated AXX (Ishii and Hiroi 1990). Non-use of radiolabelled substrates facilitated the isolation and structural analysis of the in vitro-biosynthesized feruloylated AXX by gas-liquid chromatography or fast atom bombardment mass spectrometry (FAB-MS).

Plant material

Suspension-cultured cells of rice (*Oryza sativa* L. cv. Nipponbare) were kindly provided by Dr. N. Shibuya (National Institute of Agrobiological Resources). They were maintained and subcultured every 7 d in a modified N-6 medium (Kuchitsu et al. 1993) on a reciprocal shaker at 24 °C. To exclude the big aggregates, the cells were filtered through a 20-mesh screen every subculture. Suspension-cultured cells were harvested by filtration after 12 d of subculture to prepare enzyme solutions.

Chemicals

Feruloyl-coenzyme A (Fer-CoA) was synthesized by transesterification of the *N*-hydroxysuccinimide ester of ferulic acid with CoA (Stöckigt and Zenk 1975), then purified by sequential column chromatographies on DE 52 (Whatman, Kent, UK) (Cha and Parks 1964) and DIAION HP 20 (Mitsubishi Chemical, Tokyo, Japan). The concentration of the Fer-CoA solution was determined by using its extinction coefficient; then stock solution was prepared (Stöckigt and Zenk 1975). Arabinoxylan-trisaccharide was prepared from rice-straw as described previously (Yoshida et al. 1990). Authentic feruloylated arabinoxylan-trisaccharide, Fer-AXX [3-*O*-(5-*O*-feruloyl-α-L-Ara*f*)-*β*-D-Xyl*p*-(1→4)-D-Xyl], was isolated from bamboo shoot cell walls as described previously (Ishii and Hiroi 1990).

Enzyme preparation

Harvested cells were washed with the modified N-6 medium. Washed cells (118 g) were homogenized in an extraction buffer [118 ml of 0.1 M K-phosphate buffer (pH 6.0) containing 1 mM EDTA, 1 mM DTT, and 0.4 M sucrose] with quartz sand. The homogenate was then centrifuged at 14,000 *g* for 15 min. The pellet was resuspended in extraction buffer (236 ml) containing 1 M NaCl and stirred for 30 min at 4 °C. The extract was centrifuged at 14,000 *g* for 15 min and the supernatant was brought to 60% saturation of $(NH_4)_2SO_4$ under continuous stirring. The $(NH_4)_2SO_4$-precipitated protein pellets were redissolved in 0.1 M K-phosphate buffer (pH 6.0), and then dialyzed against the same buffer. The dialyzate (32 ml, 2 mg/ml protein) was used as an ionically bound fraction. Membrane and intracellular fractions were prepared as follows. After centrifugation of the cell homogenate, the supernatant was recentrifuged at 100,000 *g* for 30 min. The resultant pellet was washed and resuspended in 0.1 M K-phosphate buffer (pH 6.0) as a membrane fraction. The supernatant obtained by centrifugation at 100,000 *g* was collected, and dialyzed against 0.1 M K-phosphate buffer (pH 6.0) as a cytosol fraction.

Protein determination

Protein was determined with Coomassie brilliant blue (Bio-Rad, Hercules, Calif., USA) using bovine γ-globulins as the standard (Bradford 1976).

Detection of FTase activity

The reaction mixture consisted of 20 μl of enzyme solution, 10 μl of 0.2 M AXX and 10 μl of 2 mM Fer-CoA solution. The reaction was carried out at 30 °C for a suitable time, and then stopped by heating at 100 °C for 5 min after adding 0.1 M acetic acid (40 μl). The mixture was centrifuged at 15,000 *g* for 3 min. The supernatant was filtered through a 0.45-μm membrane (Millipore, Bedford, Mass., USA) and an aliquot (20 μl) of the filtrate was analyzed by HPLC on a Cosmosil $5C_{18}$-AR column (150 mm long, 6.0 mm i.d.; Nakalai Tesque, Kyoto, Japan) equilibrated with 30% methanol (pH was adjusted to 3.0 with dilute trifluoroacetic acid) at 40 °C with a flow rate of 1 ml/min. A linear gradient elution within 8 min from 30 to 50% methanol (pH was adjusted to 3.0) was applied 2 min after injection. Feruloyl compounds were detected and quantified by monitoring the fluorescence at 330 nm (excitation) and 435 nm (emission). The amounts of Fer and product A, which showed the same retention time as authentic Fer-AXX, were calculated based on calibration curves prepared using authentic *trans*-Fer and Fer-AXX from bamboo, respectively.

Optimum pH of FTase

The optimum pH for the FTase activity was measured using the FTase assay described above at various pHs at 30 °C for 3 h. The ionically bound fraction was further concentrated (concentrated ionically bound fraction) with a YM-3 membrane (Amicon, Beverly, Mass., USA) before use. The following buffers were used: 50 mM Na_2HPO_4-citric acid buffer (pH 3–6), 50 mM K-phosphate buffer (pH 5–7), and 50 mM Tris-HCl buffer (pH 7–9).

Isolation of transfer product (product A)

A reaction mixture (total volume, 2 ml) consisting of 50 mM AXX, 1 mM Fer-CoA, and concentrated ionically bound fraction (protein content, 10 mg) in 50 mM K-phosphate buffer (pH 6.0) was incubated at 30 °C for 12 h. The transfer product (product A) was purified by repeated column chromatographies on Cosmosil $5C_{18}$-AR as described above, then extracted with an Oasis extraction cartridge (Waters, Milford, Mass., USA) before lyophilization (yield, 200 nmol).

Characterization of product A

Isolated product A was checked for identity as Fer-AXX by four procedures:

(i) The UV spectra of product A and authentic Fer-AXX were analyzed with a UV-2400 PC spectrophotometer (Shimadzu) in 30% methanol (pH 3.0) at 24 °C.
(ii) The FAB-MS spectra were recorded with a JEOL HX110A mass spectrometer (JEOL, Tokyo, Japan) operated in the positive-ion mode with accelerating voltage of 10 kV. The sample was dissolved in water and analyzed with glycerol:thioglycerol (1:1, v/v) and xenon as matrix and bombarding gas, respectively.
(iii) Alkaline saponification was carried out to determine the hydroxycinnamic acid derivative and sugar constituent of product A. A 500-μl aliquot of 0.1 M NaOH was added to the sample (100–120 μg), followed by incubation at 4 °C for 4 h

under N_2 in the dark. The reactant was acidified with diluted HCl to pH 3–3.5, and extracted twice with equal volumes of ethyl acetate. The organic fractions were combined and evaporated to dryness, and then the residue was dissolved in 30% methanol (100 μl) for HPLC analysis as described above.

(iv) The aqueous fraction obtained above was deionized with Dowex 50W-X8 before lyophilization and the glycosyl residue compositions in the aqueous fraction were determined as the alditol acetate derivatives by gas-liquid chromatography on an SP-2330 capillary column (30 m long, 0.25 mm i.d.) as described by York et al. (1985).

During the search for FTase activity from the suspension-cultured rice cells, we tried to detect formation of Fer-AXX using AXX and Fer-CoA as the feruloyl acceptor and donor, respectively (Fig. 1). We succeeded in sensitively detecting Fer-AXX in HPLC analysis by using its fluorescence intensity. Authentic Fer-AXX showed two peaks due to the α and β anomers of the reducing xylosyl residue in Fer-AXX on the HPLC analysis (Ishii and Hiroi 1990). However, a good correlation was obtained between the amount of Fer-AXX and the calculated peak areas [retention time (Rt) = 8.87–9.67 min] of Fer-AXX, at least from 20 to 1,500 pmol on the HPLC analysis.

When the ionically bound fraction and cytosolic fraction from 12-d-cultured cells were incubated with Fer-CoA and AXX in K-phosphate buffer at pH 6, the formation of product A, a transfer product showing the same Rt as authentic Fer-AXX, was detected. The formation of product A was found to be maximal at pH 6. No product A was detected during the incubation period with the boiled enzyme solution, indicating that an enzyme was involved the formation of product A.

The total amount FTase activity contained in the cytosolic fraction from 118 g of fresh cells was almost the same as that of ionically bound FTase, but the cytosolic fraction simultaneously contained much more peroxidase activity than did the ionically bound fraction. In previous efforts to reveal FTase activity, the presence of peroxidase activity in the membrane fraction hindered the detection of putative FTase activity since peroxidase catalyzed the oxidative polymerization of [^{14}C]feruloyl-CoA (Myton and Fry 1995; Bolwell et al. 1997). To minimize the unwanted effect of peroxidase on the detection of FTase activity, the ionically bound fraction was used for further study.

As shown in Fig. 2, the amount of product A in the reaction mixture increased with reaction time. An approximately linear relationship existed between the amount of product A and the reaction time (Fig. 2B). A considerable amount of Fer, which may be produced by the hydrolysis of both Fer-CoA and the newly produced feruloylated compound, was also detectable. Other transfer product(s) eluting at Rt = 16.2 min remain unknown.

Product A was detected only in the presence of AXX, Fer-CoA and enzyme solution in the reaction mixture (Fig. 3). Product A could not be detected when Fer-CoA was eliminated from the reaction mixture (Fig. 3, trace a). This result showed that product A was formed by enzymic feruloylation on AXX, not from the hydrolytic cleavage of a glycosidic bond in the feruloylated arabinoxylan, which might be contained in the enzyme fraction. The extent of production of Fer by non-enzymic hydrolysis of Fer-CoA was very small under the experimental conditions (Fig. 3, trace b), though the Fer-CoA was an unstable compound. In the reaction mixture without AXX (Fig. 3, trace c), Fer and an unknown product (Rt = 16.2) were detected after the incubation period, but not product A. Consequently, product A was formed by transesterification on AXX using Fer-CoA.

The protein content of the ionically bound fraction in the reaction mixture was also proportional to the amount of product A formed (Fig. 4). When reaction mixture containing 200 μg protein was incubated for 12 h at 30 °C, 6.5% of the Fer-CoA was converted into product A. In contrast, no measurable FTase activity was detected in the membrane fraction, though the protein content was over 800 μg in the reaction mixture.

Product A was purified by HPLC and sequential solid-phase extractions. The purified product A showed the same Rt in co-HPLC analysis and the same UV spectrum (λmax = 326 nm, λmin = 264 nm) as authentic Fer-AXX. On FAB-MS analysis, product A gave $(M + H)^+$ and $(M + Na)^+$ ions at $m/z = 591$ and 613, respectively, consistent with the values predicted for the Fer-AXX (MW = 590).

The HPLC analysis of the organic phase obtained by ethyl acetate extraction of saponified product A showed the presence of *trans*-Fer. No other hydroxycinnamic acid derivatives, such as *p*-coumaric acid (Rt = 10.6 min) were detected. The glycosyl residue composition of

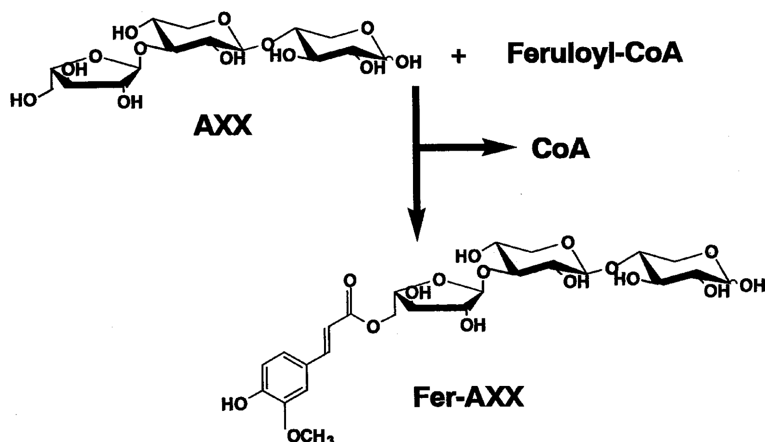

Fig. 1. Presumed reaction scheme for the formation of Fer-AXX catalyzed by FTase

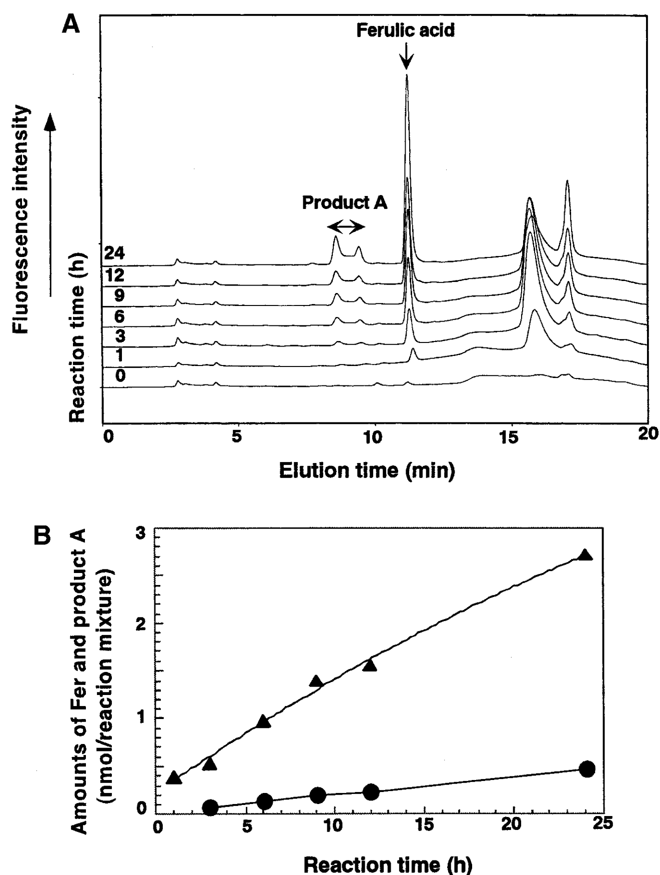

Fig. 2A,B. Time courses of product A formation and Fer liberation during the rice FTase reaction. The reaction mixture contained 50 mM AXX, 500 μM Fer-CoA, and ionically bound fraction. **A** HPLC chromatograms of reaction products. Product A and *trans*-Fer were detected at Rt = 8.8–9.7 and 11.2 min, respectively. For clarity of presentation, the blank, which was incubated with boiled enzyme solution for each of the reaction times, is shown in only one of the chromatograms (3 h) as reaction time 0 h. **B** Quantitative analysis of product A formation (●) and Fer liberation (▲). Background corrections were made on all data points using each blank in a single measurement

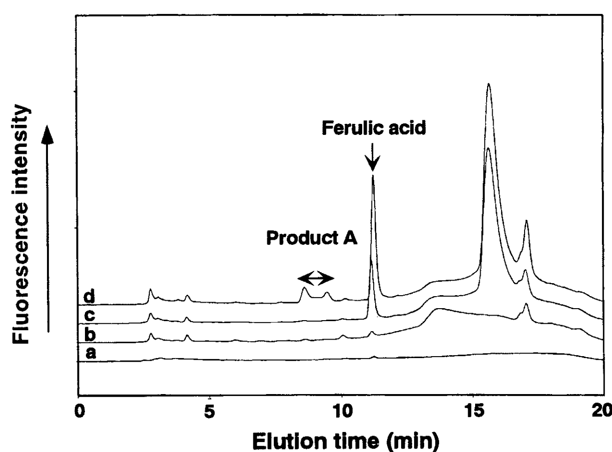

Fig. 3. The conditions required for product A formation. The reaction was performed at 30 °C for 6 h. *Trace a* Reaction product from AXX and ionically bound fraction. *Trace b* Reaction product from AXX and Fer-CoA. *Trace c* Reaction product from Fer-CoA and ionically bound fraction. *Trace d* Reaction product from AXX, Fer-CoA, and ionically bound fraction

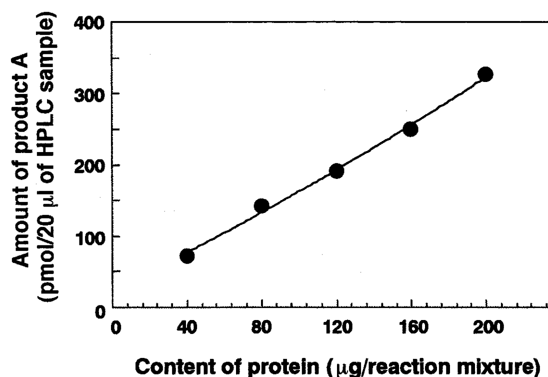

Fig. 4. Effects of protein content in the reaction mixture on product-A formation. Concentrated ionically bound fraction was diluted in series by 0.1 M K-phosphate buffer (pH 6.0). The enzyme reaction was performed at 30 °C for 12 h

product A was Ara and Xyl, and the ratio of Ara:Xyl was about 1:2. Thus, since the oligosaccharide moiety of product A was composed of AXX, the product A was identified as feruloylated AXX.

The detection of in vitro-synthesized feruloylated AXX (product A) in this study directly proves the existence of FTase, feruloyl-CoA:arabinoxylan-trisaccharide *O*-hydroxycinnamoyl transferase. But many questions still remain to be answered about feruloylation by FTase. The application of AXX as an acceptor for the enzyme reaction enabled us to detect FTase, but it was not clear whether AXX was a suitable acceptor for FTase or not. As for the feruloyl donor, the possibility of an intermediary feruloyl donor generated via Fer-CoA could not be excluded in this study. The cellular location of feruloylation of primary cell wall polysaccharide is thought to occur intracellularly (Fry 1987; Myton and Fry 1994). However, we detected FTase activity not only in the cytosolic fraction but also in the ionically bound fraction, which is considered to be derived from the cell wall. Yamamoto and Towers (1985) have suggested that feruloylation might occur in the cell wall. The extensive characterization of this enzyme should yield some reasonable answers to the many questions about cell wall feruloylation, such as what the natural acceptor and feruloyl donor for the FTase is, and where feruloylation occurs.

This work was supported by a Domestic Research Fellowship from the Japan Science and Technology Corporation (to T.Y.-S.). Part of this study is funded by a research grant (Biodesign Project BDP-00-II-1-2) from the Ministry of Agriculture, Forestry and Fisheries.

References

Bolwell GP, Gerrish C, Salaun JP (1997) Changes in enzymes involved in suberisation in elicitor-treated french bean cells. Phytochemistry 45: 1351–1357

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brett CT, Wende G, Smith AC, Waldron KW (1999) Biosynthesis of cell-wall ferulate and diferulates. J Sci Food Agric 79: 421–424

Cha S, Parks RE (1964) Succinic thiokinase. J Biol Chem 239: 1961–1967

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol 37: 165–186

Fry SC (1987) Intracellular feruloylation of pectic polysaccharides. Planta 171: 205–211

Harris PJ, Hartley RD (1976) Detection of bound ferulic acid in cell walls of the Gramineae by ultraviolet fluorescence microscopy. Nature 259: 508–510

Hartley RD, Ford CW (1989) Phenolic constituents of plant cell walls and wall biodegradability. In: Lewis NG, Paice MG (eds) Plant cell wall polymers, biogenesis and biodegradation. ACS Symp Ser 399. American Chemical Society, pp 137–149

Iiyama K, Lam TBT, Stone BA (1994) Covalent cross-links in the cell wall. Plant Physiol 104: 315–320

Ishii T (1997) Structure and functions of feruloylated polysaccharides. Plant Sci 127: 111–127

Ishii T, Hiroi T (1990) Isolation and characterization of feruloylated arabinoxylan oligosaccharides from bamboo shoot cell-walls. Carbohydr Res 196: 175–183

Kato A, Azuma J, Koshijima T (1983) A new feruloylated trisaccharide from bagasse. Chem Lett 137–140

Kohler A, Kauss H (1997) Transfer of hydroxycinnamoyl residues to microsomal proteins from parsley. Phytochemistry 44: 225–228

Kuchitsu K, Kikuyama M, Shibuya N (1993) N-Acetylchitooligosaccharides, biotic elicitor for phytoalexin production, induce transient membrane depolarization in suspension-cultured rice cells. Protoplasma 174: 79–81

Lotfy S, Fleuriet A, Macheix JJ (1992) Partial purification and characterization of hydroxycinnamoyl CoA: transferases from apple and date fruits. Phytochemistry 31: 767–772

Lotfy S, Javelle F, Negrel J (1996) Purification and characterization of hydroxycinnamoyl-Coenzyme A: ω-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase from tabacco (*Nicotiana tabacum* L.) cell-suspension cultures. Planta 199: 475–480

Meyer K, Kohler A, Kauss H (1991) Biosynthesis of ferulic acid esters of plant cell wall polysaccharides in endomembranes from parsley cells. FEBS Letters 290: 209–212

Myton KE, Fry SC (1994) Intraprotoplasmic feruloylation of arabinoxylans in *Festuca arundinacea* cell cultures. Planta 193: 326–330

Myton KE, Fry SC (1995) Dithiothreitol and cobalt effects on membrane-associated peroxidases oxidizing feruloyl-CoA. Phytochemistry 38: 573–577

Stöckigt J, Zenk MH (1975) Chemical syntheses and properties of hydroxycinnamoyl-coenzyme A derivatives. Z Naturforsch Teil C 30: 352–358

Wende G, Fry SC (1997) 2-*O*-β-D-Xylopyranosyl-(5-*O*-feruloyl)-L-arabinose, a widespread component of grass cell walls. Phytochemistry 44: 1019–1030

Yamamoto E, Towers GHN (1985) Cell wall bound ferulic acid in barley seedlings during development and its photoisomerization. J Plant Physiol 117: 441–449

York WS, Darvill AG, McNeil M, Stevenson TT, Albersheim P (1985) Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol 118: 3–40l

Yoshida S, Kusakabe I, Matsuo N, Shimizu K, Yasui T, Murakami K (1990) Structure of rice-straw arabinoxylan and specificity of *Streptomyces* xylanase toward the xylan. Agric Biol Chem 54: 449–457

Yu M, Facchini PJ (1999) Purification, characterization, and immunolocalization of hydroxycinnamoyl-CoA: tyramine *N*-(hydroxycinnamoyl)transferase from opium poppy. Planta 209: 33–44