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Ferulic acid is bound to the primary cell walls of all gymnosperm families

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Abstract

Unlignified primary cell walls containing ester-linked ferulic acid fluoresce blue in ultraviolet radiation which changes to green with increased intensity on treatment with ammonium hydroxide. Using this fluorescence behaviour, we detected ester-linked ferulic acid in the primary cell walls of all 41 species of gymnosperms we examined. These species were in 17 families representing all four extant classes of gymnosperms. In addition, we obtained cell-wall preparations containing $>95\%$ primary cell walls from nine gymnosperm species in nine families, representing all four extant classes. These preparations were analysed for ester-linked monomeric phenolic acids. We found ferulic acid (mostly *trans*) (88-1,561 μg/g cell walls) in all of the preparations and *p*-coumaric acid (mostly *trans*) (0–106 μg/g cell walls) in all except one of them. Ferulic acid ester-linked to primary cell walls has previously been found in angiosperms: in the commelinoid monocotyledons and in the dicotyledon order Caryophyllales, both monophyletic groups. From the present results, we postulate that the extant classes of gymnosperms are monophyletic and no class is sister to the angiosperms. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The hydroxycinnamic acids ferulic and *p*-coumaric occur ester-linked to the cell walls of many angiosperm species. These ester-linked phenolic acids were first found

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in cell-wall preparations from grasses and cereals (family Poaceae) containing a mixture of both unlignified primary and lignified secondary cell walls (Higuchi et al., 1967; Hartley, 1972). In these early studies, it was assumed that the ferulic acid was ester-linked only to lignin and thus occurred only in the lignified secondary cell walls. However, the presence of ester-linked ferulic acid in primary cell walls was demonstrated in two ways. First, by examining sections by ultraviolet (UV) fluorescence microscopy (Harris and Hartley, 1976). Primary cell walls in these sections fluoresced blue in water (pH 5.4), but this fluorescence changed to green and increased in intensity when the sections were treated with 0.1 M ammonium hydroxide (pH 10.3). Esters of ferulic acid are known to show this change in fluorescence colour with pH (Harris and Hartley, 1976; Fry, 1988). In contrast to the primary cell walls, the lignified secondary cell walls fluoresced blue in water and after treatment with ammonium hydroxide continued to fluoresce blue, but with increased intensity. Second, preparations containing only primary cell walls were isolated from the leaves of perennial ryegrass (*Lolium perenne*); analyses of these cell walls showed that they contained ferulic acid, together with small amounts of *p*-coumaric acid (Harris et al., 1980).

Subsequent surveys, using UV fluorescence microscopy, showed that ester-linked ferulic acid also occurred in the primary cell walls of all families of the commelinoid group of monocotyledons which comprises the following orders recognized by the Angiosperm Phylogeny Group (1998): Arecales, Commelinales, Poales, Zingiberales and some unplaced taxa (Harris and Hartley, 1980; Rudall and Caddick, 1994; Harris et al., 1997; Smith and Harris, 1999). Hartley and Harris (1981) used the same technique to show that ester-linked ferulic acid also occurs in the primary cell walls of all the families they examined of the dicotyledon order Caryophyllales as defined by Cronquist (1968, 1988). In the surveys done by Harris and Hartley (1980) and Hartley and Harris (1981), cell-wall preparations were also obtained and analysed, although these preparations contained some lignified secondary cell walls as well as primary cell walls. Ferulic acid (mostly *trans* or *E*) was found in cell-wall preparations of only those species that showed the blue to green fluorescence behaviour of their primary cell walls; these cell-wall preparations also contained *p*-coumaric acid (mostly *trans*).

Compared with the angiosperms, little is known about the occurrence of hydroxycinnamic acids in the cell walls of gymnosperms. This is in spite of there being many fewer gymnosperms; the group comprises four classes, 17 families, 86 genera, and 840 species (Mabberley, 1997). Strack et al. (1988) reported the presence of ferulic and *p*-coumaric acids in cell-wall preparations from mature leaves of 54 gymnosperm species in five families: Pinaceae, Taxodiaceae, Cupressaceae, Araucariaceae, and Taxaceae. *p*-Coumaric acid was the major hydroxycinnamic acid present in these cell-wall preparations; only small amounts of ferulic acid were found. These cell-wall preparations probably contained a high proportion of lignified secondary cell walls, and thus a high proportion of hydroxycinnamic acids probably arose from these types of cell walls rather than from primary cell walls. In the Poaceae, ester-linked *p*-coumaric acid is known to be much more abundant in lignified secondary cell walls (linked to both lignin and polysaccharides) than in primary cell walls (Mueller-Harvey et al., 1986; Ralph and Helm, 1993). Isolation of primary cell walls from gymnosperms

is not easy as they are usually woody plants. However, Sánchez et al. (1996) obtained cell-wall preparations from the hypocotyls of *Pinus pinaster* (Pinaceae) which, in contrast to the preparations from gymnosperm leaves, probably contained a high proportion of primary cell walls. Sánchez et al. (1996) identified ferulic acid as the major monomeric ester-linked hydroxycinnamic acid in these preparations; *p*-coumaric acid was present in only small amounts.

In the present study we examined the primary cell walls of 41 species of gymnosperms for the presence of ester-linked ferulic acid using UV fluorescence microscopy. These species were selected so that all 17 gymnosperm families were represented. In addition, we obtained cell-wall preparations containing $> 95\%$ primary cell walls from nine species of gymnosperms in nine families (representing all four classes). Ester-linked ferulic and *p*-coumaric acids in these preparations were quantified by capillary GC of their trimethylsilyl derivatives; ester-linked *p*-hydroxybenzoic acid, another phenolic acid, was also quantified in the same way.

2. Materials and methods

2.1. Plant material

The sources of plant material are shown in Table 1. Seeds of *Welwitschia mirabilis* and *Pinus radiata* were germinated at 25°C in the dark on moist filter paper. The *W. mirabilis* seeds were first coated with Captan™ fungicide and the filter paper moistened with Captan™ solution (2 g/l in H_2O). The *P. radiata* seeds were first surface sterilized with sodium hypochlorite solution (1% containing two drops per 100 ml of Tween 20) for 5 min and then soaked in H_2O for 24 h. Cambium (with some differentiating xylem and phloem) was collected from the trunk of a tree of *P. radiata* by peeling off and discarding the bark and gently scraping the exposed surface (tissue frozen at -20°C).

2.2. Microscopy

Bright-field and UV-fluorescence microscopy (Smith and Harris, 1995) were done on fresh transverse sections cut using a razor blade from the organs indicated in Table 1 and on cell-wall preparations obtained from these organs. Staining with the bright-field stain toluidine blue O was as described by Harris et al. (1994). Toluidine blue O stains polychromatically: lignin stains green or blue–green; polyanions such as rhamnogalacturonans stain pink or purple (O'Brien and McCully, 1981). Lignin was detected by the red colour reaction given by phloroglucinol–HCl (Harris et al., 1980). Starch was detected using I_2 in KI (Jensen, 1962). Controls were also examined in which the sections or cell walls were mounted in the solvent for the stain or colour reagent. Fluorescence of the cell walls in UV radiation was examined using sections mounted in H_2O and in 0.1 M NH_4OH (Harris and Hartley, 1976, 1980; Smith and Harris, 1995).

Table 1
Sources of gymnosperms, organs examined, and UV fluorescence of their cell walls

Class, family, genus, species[a]	Source[b]	Microscopy			Walls isolated
		Organs examined[c]	Parenchyma walls[d]	Epidermal walls[e]	
Class Ginkgoopsida (Ginkgoatae)					
Ginkgoaceae					
Ginkgo biloba L.	A	L	+ +	B/G	L + P
Class Pinopsida (Pinatae, incl. Taxopsida)					
Araucariaceae					
Agathis australis (D. Don) Salisb.	A	L	+ + +	Y/Y	L + P
Araucaria heterophylla (Salisb.) Franco.	A	Sts	+ + +	− /Y	NI
Cephalotaxaceae					
Cephalotaxus harringtonia (Forbes) Koch var. *drupacea*	A	Stb	+ + +	B/Y	NI
Cupressaceae					
Calocedrus decurrens (Torr.) Florin	A	Sts	+ +	c/c	NI
Chamaecyparis lawsoniana (Murray) Parl.	A	Sts	+	c/c	NI
Cupressus macrocarpa Hartw. ex Gordon	A	Sts	+ +	c/c	NI
Juniperus conferta Parl.	A	St	+ + +	c/c	NI
Juniperus squamata Buch.-Ham. Ex D. Don 'Blue Star'	A	St	+ + +	c/c	NI
Libocedrus plumosa (D. Don) Sarg.	A	Sts	+ / + +	Y/Y	NI
Microbiota decussata Komar.	A	Sts	+ +	− /Y	NI
Thuja occidentalis L. 'Rheingold'	A	Sts	+	c/c	NI
Widdringtonia schwarzii (Marloth) Mast.	F	Sts	+ / + +	c/c	NI
Phyllocladaceae					
Phyllocladus trichomanoides D. Don	A	P	+ + +	B/Y	NI
Pinaceae					
Abies concolor (Gordon & Glend.) Lindl. ex Hildebr.	F	L	+ +	B/B[f]	NI
Abies magnifica A. Murray	F	L	+ +	B/B[f]	NI
Cedrus atlantica (Endl.) Carr.	A	L	+ +	Y/Y[f]	NI
Keteleeria evelyniana Mast.	F	St	+ + +	c/c	NI
Pinus radiata D. Don (open pollinated clone 268.041)	F	C	+ + +	na	C
		FG	+ + +	na	NI
		Hy24	+ + +	B/B	NI
		Rd	+ + +	B/B	NI
		Co	+	c/G	NI
Pinus thunbergii Parl.	H	L	+	B/ − [f]	NI
Pseudotsuga menziesii (Mirb.) Franco.	F	St	+ + +	B/ −	NI
Pseudotsuga sinensis Dode.	F	St	+ + +	B/B	NI
Tsuga heterophylla (Raf.) Sarg.	F	St	+ + +	B/B	NI
Podocarpaceae					
Dacrycarpus dacrydioides (A. Rich.) Laub.	A	Stb	+ / + +	− / −	NI
Dacrydium cupressinum Sol. ex Forst.	A	Sts	+ +	− / −	NI
Lagarostrobos colensoi (Hook.) Quinn	A	Sts	+ + +	B/Y	Sts

Table 1—(*continued*)

Class, family, genus, species[a]	Source[b]	Microscopy			Walls isolated
		Organs examined[c]	Parenchyma walls[d]	Epidermal walls[e]	
Podocarpus totara G. Benn ex D. Don	A	L	+ +	Y/Y	NI
Prumnopitys ferruginea (D. Don) Laub.	A	Stb	+ +	B/G	NI
Sciadopityaceae					
Sciadopitys verticillata (Thunb.) Sieb. & Zucc.	A	L	+ +/+ + +	c/c	NI
Taxaceae					
Taxus baccata L.	A	Stb	+ + +	B/G	NI
Taxodiaceae					
Cryptomeria japonica (L.f.) D. Don 'Vilmoriniana'	A	St	+/+ +	c/c	NI
Sequoia sempervirens (D. Don) Endl.	A	St	+ + +	−/Y[f]	NI
Taiwania flousiana Gauss.	F	Sts	+ + +	−/−	NI
Taxodium distichum (L.) Rich	A	Stb	+ +	Y/Y	L + P + R
Class Cycadopsida (Cycadatae)					
Boweniaceae					
Bowenia spectabilis Hook. ex Hook. f.	AG	Pi	+	B/c	NI
Cycadaceae					
Cycas revoluta Thunb.	AG	Pi	+ +	B/c	Pi + R + P
Stangeriaceae					
Stangeria eriopus (Kunze) Nash.	AG	Pi	+/+ +	c/G	NI
Zamiaceae					
Zamia integrifolia Ait.	AG	R & Pi	+	B/B	Pi
Class Gnetopsida (Gnetatae)					
Ephedraceae					
Ephedra gerardiana Wallich	A	St	+ + +	−/−	St + L
Gnetaceae					
Gnetum gnemon L.	AG	L	+ + +	B/G	L
Welwitschiaceae					
Welwitschia mirabilis Hook. f.	S	Hy3	+ + +	B/G	NI

[a]Classification according to Mabberley (1997) and Kramer and Green (1990).

[b]Plant sources: (H) private garden at Helensville, New Zealand; (F) grounds of Forest Research, Rotorua, New Zealand; (A) grounds of The University of Auckland; (AG) glasshouses at The University of Auckland; (S) seeds from Silverhill Seeds, Silverhill Crescent, Kenilworth, 7700, RSA.

[c]Organs and tissues examined: (C) cambium with some differentiating xylem and phloem; (Hy3) hypocotyl from a 3 day old seedling; (Hy24) hypocotyl from a 24 day old seedling; (FG) female gametophyte; (L) leaf; (P) petiole; (Pi) pinnule/pinna; (R) rachis; (Rd) radicle from a 24 day old seedling; (St) young stem; (Sts) young stem plus scale-like leaves; (Stb) young stem plus bases of leaves; (Co) cotyledon from a 24 day old seedling; (NI) cell walls not isolated.

[d]Intensity of the green fluorescence of the primary walls of parenchyma cells after treatment with ammonium hydroxide: + + + = intense fluorescence; + + = moderate fluorescence; + = weak fluorescence.

[e]Fluorescence colour of walls of epidermal cells, excluding stomatal guard cells, before and after treatment with ammonium hydroxide: (B) blue; (G) green; (Y) yellow; (c) fluorescence of cell walls could not be determined because of intense fluorescence of cell contents; — little or no fluorescence.

[f]Epidermal walls lignified.

2.3. Isolation of cell walls

Cell-wall preparations containing $< 5\%$ lignified cell walls (estimated microscopically after treatment with phloroglucinol–HCl) were obtained from young plant material, collected in September or October, that contained only small proportions of lignified cell walls (Table 1). All procedures were carried out at 4°C. Cell-wall isolation and all manipulations of the cell-wall preparations, the phenolic acids and their TMSi derivatives were done in illumination from a halogen reflector lamp with a UV filter to avoid UV radiation which causes *cis–trans* (or *Z–E*) isomerization (Kahnt, 1967; Hartley and Jones, 1975).

Plant material (1.5–2 g fresh weight) was cut into pieces (3 mm × 3 mm) and homogenized in 35 ml of Mops-KOH buffer (20 mM, pH 6.8) containing 20 mM sodium metabisulphite using a Polytron blender (4 min). The homogenates were centrifuged (750 g, 5 min) and the pellets divided into three equal batches each of which was further homogenized in fresh buffer (5 ml) using a Tenbroeck ground-glass homogenizer. The homogenates were centrifuged (as above), the pellets washed with buffer three times by centrifugation, resuspended in buffer, sonicated (for 1 min at full power), and centrifuged. The pellets were washed with H_2O (35 ml) five times by centrifugation, and filtered onto nylon mesh (pore size 11 μm). The residue on the mesh was washed with H_2O (750 ml) until the filtrate was clear, dried using solvent exchange by successively washing with EtOH, MeOH, and *n*-pentane (50 ml of each), and stored under vacuum over Si gel.

2.4. Removal of contaminating starch

Tris-maleate buffer (1 ml, 5 mM, pH 6.9) was added to cell-wall preparations (40 mg) contaminated with starch and the starch gelatinized by heating for 5 min at 85°C. After cooling to 37°C, an equal volume was added of tris-maleate buffer (15 mM, pH 6.9) containing 2 mM $CaCl_2$ and porcine pancreatic α-amylase (300 units, Type 1-A, Sigma, St. Louis, MO, USA) and incubated for 1 h at 37°C. The suspension was washed with H_2O on to nylon mesh, and further washed with H_2O until the filtrate was clear. The cell-wall preparations were dried and stored as described above. No starch was detected in these preparations.

2.5. Treatment of cell walls with NaOH

The method was based on those used by Ford and Hartley (1988) and Turner et al. (1993). Cell walls (15 mg) were shaken with 1 M NaOH (2 ml) containing 3,4-dimethoxycinnamic acid (10 μg/ml) as the internal standard for 20 h at 20°C under Ar. The suspension was filtered (glass microfibre filter, type GF/C, Whatman, Maidstone, UK) and the residue washed with H_2O (4 × 1 ml); the extracts and washings were combined. The filtrate was adjusted to pH 1.5 with 6 M HCl (370 μl), saturated with NaCl, and the phenolic acids extracted by shaking with Et_2O (4 × 6 ml). The combined Et_2O extracts were evaporated in a stream of N_2, and dried under vacuum over P_2O_5. Reference phenolic acids (20 and 60 μg each of *p*-hydroxybenzoic,

trans-p-coumaric, and *trans*-ferulic acids) were also carried through the NaOH treatment and extraction procedure as above. The internal standard 3,4-dimethoxycinnamic acid (20 μg) was added to the reference phenolic acids before the NaOH treatment.

2.6. Silylation

The residues were silylated by incubating with a mixture of pyridine (100 μl) and BSTFA [*N*,*O*-*bis*(trimethylsilyl) trifluoroacetamide] containing 1% TMCS (trimethylchlorosilane) (100 μl) for 60 min at 20°C under Ar with occasional shaking. Dichloromethane (0.5 ml for the cell wall samples, 3 ml for the reference phenolic acids) was added before GC. Reference standards (1 mg plus 1 mg of 3,4-dimethoxycinnamic acid) of each of the following were also silyated: *trans*-caffeic acid, *p*-hydroxybenzaldehyde, *trans*-sinapic acid, syringaldehyde, syringic acid, vanillic acid, and vanillin. Mixtures of *cis*- and *trans*-isomers of caffeic acid, ferulic, *p*-coumaric, and sinapic acids were obtained by exposing solutions of the *trans*-forms (1 mg in 3 ml of MeOH) to UV radiation (maximum λ 365 nm) for 8 h. These solutions were evaporated to dryness and silylated.

2.7. GC

The TMSi derivatives of the phenolic acids were separated and quantified on a 30 m × 0.32 mm id, 0.25 μm film thickness DB-1 column (J&W Scientific, Folsom, CA, USA) with a deactivated fused silica retention gap (2 m) in a gas chromatograph fitted with a FID and a dedicated cool on-column capillary inlet. The carrier gas used was He at a column head pressure of 40 kPa; 1 μl aliquots were injected. The following oven temperature programme was used: 38°C for 30 s after injection; to 150°C at 50°C/min; 150°C for 15 min; to 260°C at 5°C/min; and 260°C for 5 min. The detector temperature was at 290°C. The phenolic acids in cell-wall extracts were quantified using the internal standard. The *cis*-isomers of ferulic and *p*-coumaric acids were quantified using the response factor of their *trans*-isomer; this has previously been shown to be valid (Turner et al., 1993).

3. Results

3.1. UV fluorescence microscopy

The walls of all cell types in all gymnosperm species examined fluoresced (Table 1). The only exceptions were epidermal cell walls that showed little or no fluorescence in some species (Table 1); however, the walls of all other cell types in these species fluoresced. As the fluorescence behaviour of epidermal cell walls varied among species, it is described separately below and in Table 1. The walls of all the other cell types fluoresced blue when the sections were mounted in water. After treatment with

ammonium hydroxide, the fluorescence of lignified cell walls (identified histochemically using phloroglucinol–HCl and toluidine blue O, including the cell walls of xylem tracheary elements, and sclerenchyma fibres) remained blue, but the fluorescence of unlignified primary cell walls (including those of phloem and most parenchyma cells) changed to green indicating the presence of ester-linked ferulic acid (Harris and Hartley, 1976). Following treatment with ammonium hydroxide, the intensity of the fluorescence also increased. The intensity of the fluorescence of the primary cell walls varied among species, from intense to only just detectable. An estimate of the intensity of the fluorescence of parenchyma cell walls after treatment with ammonium hydroxide is shown in Table 1. Isolated cell walls showed the same fluorescence behaviour as in sections, although the cell types from which the cell walls were derived usually could not be determined. The two histochemical methods we used to identify lignified cell walls gave results that agreed closely. Cell walls that gave a positive colour reaction with phloroglucinol–HCl stained blue/green with toluidine blue O; cell walls that gave no colour reaction with phloroglucinol–HCl stained purple.

In the epidermis, the walls of the stomatal guard cells were always lignified and fluoresced blue before and after treatment with ammonium hydroxide. The walls of the other epidermal cells were lignified in five of the species examined: *Abies concolor*, *A. magnifica*, *Cedrus atlantica*, *Pinus thunbergii*, and *Sequoia sempervirens*. The fluorescence of these walls is described in Table 1. The unlignified epidermal cell walls of some species, e.g. *Ginkgo biloba* (Table 1) showed the same blue to green fluorescence behaviour as the walls of other cell types. However, the unlignified epidermal walls of the other species showed a variety of fluorescence behaviours, including a yellow fluorescence, either after treatment with ammonium hydroxide, or in some, both before and after treatment, e.g. *Libocedrus plumosa*. These species with epidermal walls that showed a yellow fluorescence were all in the class Pinopsida.

The cuticles of all the gymnosperms examined fluoresced, and the intensity was increased by treatment with ammonium hydroxide. In some species, e.g. *Bowenia spectabilis*, *Dacrydium cupressinum*, *G. biloba*, *Gnetum gnemon*, they fluoresced blue, both before and after treatment with ammonium hydroxide; in others, e.g. *Agathis australis*, *Dacrycarpus dacrydioides*, *L. plumosa*, they fluoresced yellow or orange. In others, e.g. *Ephedra gerardiana*, *Sciadopitys verticillata*, *Widdringtonia schwarzii*, they fluoresced blue when mounted in water which changed to yellow after treatment with ammonium hydroxide. The only exception to the above was the cuticle of *Welwitschia mirabilis* which when mounted in water fluoresced yellow and changed to blue after treatment with ammonium hydroxide. Unlike the yellow fluorescence of the epidermal cell walls, the yellow fluorescence of the cuticles was not confined to the class Pinopsida.

3.2. Phenolic acids released from the cell walls

Ferulic, *p*-coumaric, and *p*-hydroxybenzoic acids were released from the cell-wall preparations following treatment with sodium hydroxide. Caffeic, sinapic, syringic or vanillic acids were not detected, nor were *p*-hydroxybenzaldehyde, syringaldehyde, or vanillin. The amounts of ferulic acid found ranged from 88 μg/g for *Zamia integrifolia*

Table 2
Amounts (μg/g) of ferulic, *p*-coumaric, and *p*-hydroxybenzoic acids released from the cell walls by treatment with sodium hydroxide

Class, family, genus, species[a]	Ferulic acid		*p*-Coumaric acid		*p*-Hydroxy benzoic acid
	trans-	*cis*-	*trans*-	*cis*-	
Class Ginkgoopsida (Ginkgoatae)					
Ginkgoaceae					
Ginkgo biloba	79[b]	11	22	14	—
Class Pinopsida (Pinatae, incl. Taxopsida)					
Araucariaceae					
Agathis australis	1007	144	34	11	17
Pinaceae					
Pinus radiata	465	13	—	—	—
Podocarpaceae					
Lagarostrobos colensoi	1459	102	86	20	45
Taxodiaceae					
Taxodium distichum	74	19	13	9	—
Class Cycadopsida (Cycadatae)					
Cycadaceae					
Cycas revoluta	106	7	11	—	13
Zamiaceae					
Zamia integrifolia	69	19	60	30	31
Class Gnetopsida (Gnetatae)					
Ephedraceae					
Ephedra gerardiana	128	27	32	21	—
Gnetaceae					
Gnetum gnemon	264	124	34	21	—

[a] Classification according to Mabberley (1997) and Kramer and Green (1990).
[b] Averages of determinations on two samples.

to 1561 μg/g for *Lagarostrobos colensoi* (Table 2). Smaller amounts of *p*-coumaric acid were found. The amounts of *p*-coumaric acid found ranged from none detected in the walls of *P. radiata* to 106 μg/g for *L. colensoi*. In *Z. integrifolia* there was slightly more *p*-coumaric acid released from the cell walls than ferulic acid. For all species examined, the *trans*-forms of both ferulic and *p*-coumaric acids were more abundant than the *cis*-forms. *p*-Hydroxybenzoic acid was found in extracts of the cell walls of only four species: *Agathis australis*, *Cycas revoluta*, *L. colensoi*, and *Z. integrifolia*.

4. Discussion

4.1. Implications of cell-wall bound ferulic acid for systematics

The primary cell walls in all species of gymnosperms examined showed the same blue to green pH-dependent change in UV fluorescence as in the commelinoid

monocotyledons (Harris and Hartley, 1980; Rudall and Caddick, 1994; Harris et al., 1997; Smith and Harris, 1999) and the dicotyledon order Caryophyllales (Hartley and Harris, 1981), indicating the presence of ester-linked ferulic acid (Harris and Hartley, 1976); the UV fluorescence of epidermal walls was anomalous in some species and is discussed below. In the angiosperms, both the commelinoid monocotyledons and the Caryophyllales have been shown to be monophyletic using nucleotide sequences of the *rbc*L gene (e.g. Chase et al., 1993).

Whether or not extant gymnosperms are monophyletic has been the subject of much recent discussion. In particular, the relationships of the Gnetales have been controversial. Morphological data have indicated that the Gnetales are a sister group to the angiosperms and the gymnosperms are not monophyletic (Doyle, 1998). Molecular data have indicated a variety of relationships for the Gnetales, but support for these has often been weak (Doyle, 1998; Frohlich, 1999). However, a recent study of sequence data from the chloroplast *rpo*C1 gene showed strong support for gymnosperms being monophyletic and none of the extant classes being a sister group to the angiosperms (Samigullin et al., 1999). Another recent study, involving sequence data from MADS-box genes, also showed strong support for the Gnetales not being a sister group to the angiosperms (Frohlich, 1999; Winter et al., 1999). Using our data on the occurrence of cell-wall bound ferulic acid as a character, the most parsimonious hypothesis would be to postulate that the gymnosperms are monophyletic and none of the extant classes are sister to the angiosperms. Although, as indicated above, some angiosperm families have this character, these families are away from the root of the angiosperms and appear to have acquired this character independently.

4.2. Amounts of hydroxycinnamic acids in isolated cell walls

Our analyses of isolated gymnosperm primary cell walls confirmed the presence of ferulic and *p*-coumaric acids. As in angiosperm primary cell walls, both these acids occurred mostly in their *trans*-forms and, with one exception, *Zamia integrifolia*, there was more ferulic than *p*-coumaric acid. The amount of ferulic acid (200–460 μg/g) found in the cell walls of *Pinus pinaster* hypocotyls by Sánchez et al. (1996) was similar to the amount we found in the cell walls of *Pinus radiata* (478 μg/g); although they found small amounts of *p*-coumaric acid, we were unable to detect any. However, the amounts of ester-linked ferulic acid we found in gymnosperm primary cell walls (88–1561 μg/g) were less than in primary cell walls of angiosperms, although there have been few quantitative studies. Examples of amounts of ferulic acid found in primary cell-wall preparations from angiosperms are as follows: 5700–6300 μg/g in mesophyll cell walls of *Lolium perenne* (Poaceae) (Harris et al., 1980); 14,600 μg/g in walls of suspension culture cells of *Zea mays* (Poaceae) (Grabber et al., 1995); 7217 μg/g in parenchyma cell walls of *Eleocharis dulcis*, a commelinoid monocotyledon, (Cyperaceae) (Parr et al. 1996); and 4592 and 6939 μg/g in parenchyma cell walls of sugarbeet and beetroot, respectively (*Beta vulgaris* subsp. *vulgaris*) (Chenopodiaceae) (Waldron et al., 1997). In all these examples, only comparatively small amounts of *p*-coumaric acid were found in the cell walls.

Even the primary cell walls of gymnosperm species with the smallest amounts of ferulic acid showed the blue to green fluorescence behaviour. Thus, this fluorescence was shown even with ferulic acid amounts of 88 μg/g (*Zamia integrifolia*), 90 μg/g (*Ginkgo biloba*) and 93 μg/g (*Taxodium distichum*). Smaller amounts of ester-linked ferulic acid (29.1 μg/g) have been reported to occur in the primary cell walls of the dicotyledon carrot (*Daucus carota*) (Apiaceae) (Parr et al., 1997). However, the cell walls of this species did not show this characteristic fluorescence (Hartley and Harris, 1981). Thus $\sim$ 90 μg/g of ferulic acid may be near the lower limit for detection using fluorescence microscopy. Furthermore, it is interesting that the primary cell walls of *Zamia integrifolia* still showed the blue to green fluorescence colour change despite containing slightly more *p*-coumaric than ferulic acid. Esters of *p*-coumaric acid do not fluoresce at neutral or acidic pHs but fluoresce blue at alkaline pHs (Fry, 1988). This indicates that the green fluorescence of ferulic esters dominates the wall fluorescence and masks the fluorescence of the *p*-coumarate esters.

4.3. Fluorescence of epidermal cell walls

Unlike angiosperms examined in previous surveys (Harris and Hartley, 1980; Hartley and Harris, 1981; Rudall and Caddick, 1994; Harris et al., 1997; Smith and Harris, 1999), the unlignified primary walls of epidermal cells in certain gymnosperm species did not show the blue to green change in UV fluorescence following treatment with ammonium hydroxide. In some species, all in the class Pinopsida, these epidermal cell walls fluoresced yellow, either after, or both before and after, treatment with ammonium hydroxide. This fluorescence may be due, at least in part, to the presence of flavonol glycosides which have been detected, using the histochemical reagent Naturstoffreagenz A, in the epidermal cell walls of the leaves of *Pinus sylvestris* (Schnitzler et al., 1996) and *Picea abies* (Hutzler et al., 1998). Strack et al. (1988) also found that cell-wall preparations from leaves of the Pinaceae contained flavonoid glycosides, most commonly astragalin (kaempferol 3-O-glucoside). Although in some gymnosperm species we found that the epidermal cell walls showed little or no fluorescence in UV radiation, the cuticle fluoresced in all the species we examined. Harris and Hartley (1980) and Hartley and Harris (1981) also found that the cuticle fluoresced in all the angiosperm species they examined. This fluorescence is probably due to phenolic components, including hydroxycinnamic acids and flavonoids, known to occur in cuticles (Bacic et al., 1988; Laguna et al., 1999).

4.4. Ester-linkage of hydroxycinnamic acids to polysaccharides

Which polysaccharides the ferulic and *p*-coumaric acids are ester-linked to in the primary cell walls of gymnosperms are unknown. In the angiosperms where this has been determined, they are attached to the predominant non-cellulosic polysaccharides in the cell walls. In the Poaceae, where these are the glucuronoarabinoxylans, the acids are esterified via their carboxyl groups to the C(O)5 hydroxyl of single α-L-arabinofuranosyl residues located on the C(O)3 of xylopyranosyl residues in the polysaccharide backbone (Kato and Nevins, 1986). However, in spinach (*Spinacia

oleracea) and sugarbeet (Chenopodiaceae, Caryophyllales) the predominant non-cellulosic polysaccharides in the primary cell walls are pectic polysaccharides. The ferulic acid is esterified via its carboxyl groups to the C(O)6 hydroxyl of galactopyranosyl residues in pectic galactans and to the C(O)2 hydroxyl of arabinofuranosyl residues in pectic arabinans (Ishii and Tobita, 1993; Colquhoun et al., 1994). In the gymnosperm primary cell walls the ferulic and *p*-coumaric acids may also be linked to the most abundant non-cellulosic polysaccharides which in the few species that have been examined (mostly conifers) are pectic polysaccharides (Edashige et al., 1995; Edashige and Ishii, 1996).

4.5. Ferulic acid dimers

Dimerization of ferulic acid residues linked to different polysaccharides chains results in the cross-linking of these chains. In the present study, we did not analyse for ferulic acid dimers ester-linked to the primary cell walls. However, such dimers have been reported from hypocotyl cell walls in *Pinus pinaster* (Sánchez et al., 1996). Three dehydrodimers of ferulic acid were found: two 8-8′ dimers and a 8-5′dimer. These, together with other ferulic acid dehydrodimers, have also recently been found in primary cell walls of the Poaceae (Grabber et al., 1995; Ralph et al., 1994), *Eleocharis dulcis* (Cyperaceae) (Parr et al., 1996), and sugarbeet and beetroot (Chenopodiaceae) (Waldron et al., 1997).

For many years, only one ferulic acid dehydrodimer, the 5-5′ dimer, was known, and was first found in cell walls of the Poaceae (Hartley and Jones, 1976; Markwalder and Neukom, 1976). This dimer was found by Harris and Hartley (1980) and Hartley and Harris (1981) in their surveys of angiosperm cell walls; they found it in almost all the monocotyledon cell-wall preparations that contained ferulic acid, and in some of the Caryophyllales cell-wall preparations that contained ferulic acid. Thus, ferulic acid dehydrodimers often accompany ferulic acid when it occurs in primary cell walls. It is interesting that Sánchez et al. (1996) were unable to detect the 5-5′ dimer in the hypocotyl cell walls of *P. pinaster*. Definitive evidence for the crosslinking of primary cell-wall polysaccharides by ferulic acid dehydrodimers has been obtained for the cell walls of the Poaceae. Here the 5-5′ dimer was shown to link glucuronoarabinoxylan molecules by the isolation and characterization of a diferuloyl arabinoxylan hexasaccharide (Ishii, 1991). This cross-linking of cell-wall polysaccharides by diferulate has been shown in the Poaceae to make the primary cell walls mechanically more rigid (Tan et al., 1991) and impede their degradation by a mixture of fungal carbohydrases (Grabber et al., 1998). Similar effects of polysaccharide cross-linking may also occur in the primary cell walls of the gymnosperms. This polysaccharide cross-linking may thus protect the cell walls from degradation by enzymes produced by pathogenic microorganisms.

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