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SOLUBLE SINAPOYL ESTERS ARE CONVERTED TO WALL-BOUND ESTERS IN PHENYLALANINE AMMONIA-LYASE-INHIBITED RADISH SEEDLINGS

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Raphanus sativus*; Cruciferae; radish; sinapoyl esters; 2-aminoindan-2-phosphonic acid (AIP); phenylalanine ammonia lyase (PAL); phenolic transport; hypocotyl elongation; wall-bound phenolics.

Abstract—Between the third and eighth day of radish seedling growth, wall-bound esters of (E)-sinapic and (E)-*p*-coumaric acids increase about fourfold, (E)-ferulic acid about sixfold, in the cotyledons. In the hypocotyl plus roots, wall-bound ferulic and *p*-coumaric acids increase twofold but wall-bound sinapic acid levels are unchanged. When grown with 100 μM 2-aminoindan-2-phosphonic acid (AIP), a competitive inhibitor of phenylalanine ammonia-lyase (PAL), cotyledons had levels of wall-bound sinapic acid similar to controls but there was no detectable wall-bound *p*-coumaric acid and only traces of wall-bound ferulic acid. As seedlings developed, their decrease in soluble sinapoyl esters greatly exceeded levels of wall bound sinapic acid accumulated. We interpret this as evidence that *de novo* hydroxycinnamic acid synthesis was blocked and preformed soluble sinapoyl esters stored in the cotyledons were mobilized and esterified into developing cell walls. Growth with 5 μM AIP inhibited wall-bound *p*-coumaric and ferulic acid accumulation more than 90% in the hypocotyl plus roots and slightly increased hypocotyl elongation, suggesting that wall-bound phenolics may normally play a role in restricting radish hypocotyl cell wall extensibility. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Wall-bound phenolics are well known in monocots [1–4] and reported from a few dicots [5–8]. In a number of plants they are believed to restrict cell wall extensibility by forming diferulic acid linkages, interfering with enzymatic degradation of arabinoxylans or initiating lignification [9–12]. In a 1981 survey of 163 species of dicots [6], using TLC and UV fluorescence microscopy, wall-bound *p*-coumaric, ferulic and diferulic acids were reported absent from most groups and found primarily in the subclass Centrospermeae [6], including spinach where wall-bound phenolics have been well characterized [5, 9, 10]. Recent work, using more sensitive analytical techniques, reports that potato cell walls accumulate at least seven different wall-bound phenolics at levels around 10 nmol (g fresh weight)$^{-1}$ [7], and carrot cell walls yield a variety of wall-bound phenolics including ca 30 μg of ferulic acid g^{-1} [13].

Rice coleoptile elongation is closely correlated with decreased cell wall extensibility and higher levels of ferulic and diferulic acids ester-linked to arabinose residues in the cell wall [11]. Similarly, Kajita *et al.* [8] found phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity directly related to levels of wall-bound ferulic acid and coleoptile elongation in wheat seedlings, and Liu *et al.* [14] reported that UV-B increased PAL activity, increased wall-bound ferulic acid, and slowed elongation of primary leaves.

Radish (*Raphanus sativus L.*) seeds have very high levels of 6,3′-disinapoylsucrose, sinapoylcholine (sinapine), and 6-sinapoylcoraphenine [15]. During early seedling development, the latter two compounds are converted to 1-sinapoylglucose and much of that to sinapoylmalate [15]. Light inhibition of hypocotyl elongation in radish seedlings is correlated with increased accumulation of 1-sinapoylglucose which, when applied exogenously, is inhibitory to elongation of both intact and excised radish hypocotyls [16]. It is

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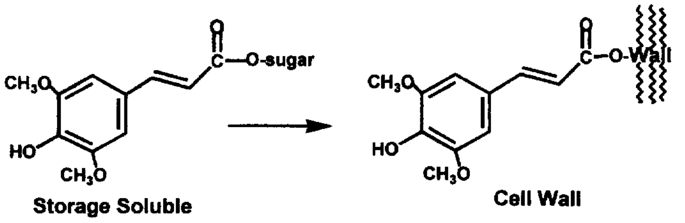

not known if this compound works directly as an inhibitor or is perhaps involved in the formation of wall-bound phenolics.

Are sinapoyl compounds associated with hypocotyl elongation in radish seeds produced *de novo* by pathways involving phenylalanine ammonia-lyase activity, or can soluble sinapoyl compounds carried over from the seed be 'recycled' into wall-bound forms? The latter role was suggested by Strack [17] as early as 1977 when he determined that total soluble sinapoyl esters in radish cotyledons decrease from about 200 nmol to 140 nmol, per cotyledon, during the first few days of growth and remain near that level throughout the first two weeks of growth.

To examine these alternative hypotheses, we grew radish seedlings with and without 2-aminoindan-2-phosphonic acid (AIP) which is a competitive inhibitor of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) [18]. Growth with AIP should provide plants with high levels of preformed, seed-derived, soluble sinapoyl esters but inhibited in their ability to form new hydroxycinnamic acids via PAL. By following changes in wall-bound phenolics in expanding cotyledons and elongating hypocotyls during early seedling growth, we attempted to detect conversion of preformed soluble sinapoyl esters into insoluble wall-bound sinapoyl esters. We also compared hypocotyl elongation in control and AIP-grown plants in an attempt to correlate changes in wall-bound phenolics with changes in elongation rates.

RESULTS

(E)-sinapic, (E)-ferulic, and (E)-*p*-coumaric acids were identified by HPLC and TLC as the major cell wall-bound phenolics in radish seedlings. In control plants (grown without AIP), a pair of three-day-old cotyledons contained ca 30 ng of wall-bound *p*-coumaric, 30 ng of wall-bound ferulic acid and 60 ng of wall-bound sinapic acid. By day eight, wall-bound esters in the cotyledons had increased to about 120 ng of *p*-coumaric, 150 ng of ferulic and 240 ng of sinapic acid (Fig. 1). In hypocotyls plus roots, increases in wall-bound *p*-coumaric and ferulic acids were of the

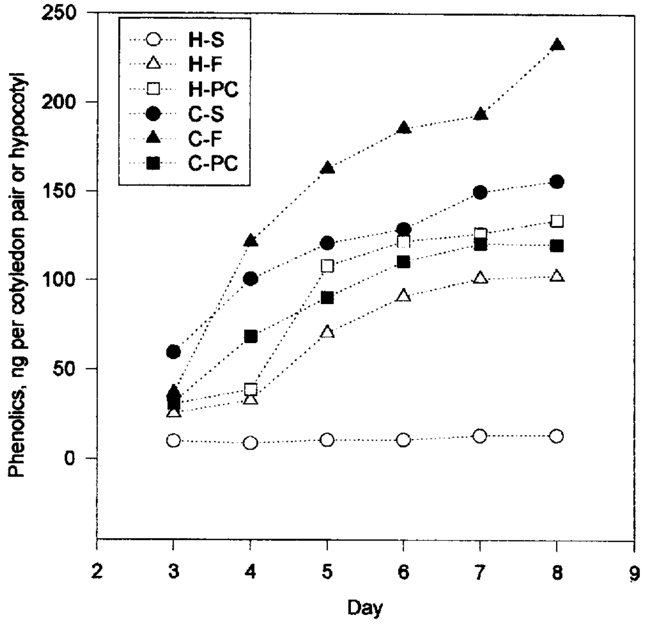

Fig. 1. Changes in wall-bound phenolics in radish seedlings grown on water-impregnated filter paper at 23°. After 2 days in the dark, plants were transferred to a plant growth chamber and given continuous 300 μM PAR from cool-white fluorescent lights. H = hypocotyl plus roots, C = cotyledon pair, S = (E)-sinapic acid, F = (E)-ferulic acid, PC = (E)-*p*-coumaric acid. Values are means of 3 experiments ($n = 3$). S.E. not shown for clarity of illustration, but similar to those in Figs 2–5.

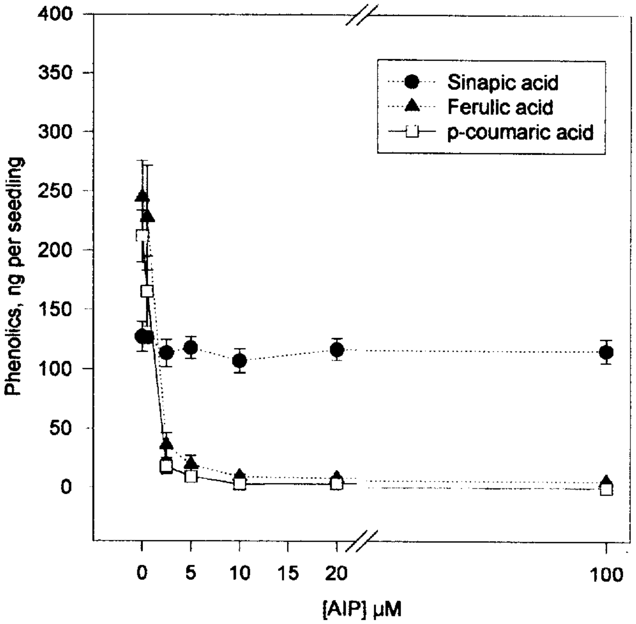

Fig. 2. Effect of varying concentrations of AIP on wall-bound phenolics in 6-day-old radish whole seedlings. Growth conditions and symbols as in Fig. 1. Values are means ±S.E., $n = 4$.

same magnitude as in cotyledons, but there was no apparent increase in levels of wall-bound sinapic acid (Fig. 1).

To establish AIP levels required to inhibit wall-bound phenolic biosynthesis, plants were grown with AIP at levels from 0.0 (controls)–100 μM and wall-bound phenolics determined in whole seedlings. On day six, growth with 5 μM AIP inhibited wall-bound ferulic and *p*-coumaric acids about 90% when compared with controls, and 10 μM or more inhibited them below our level of detection (ca 2 ng per seedling; see Experimental), but there was no inhibitory effect of AIP on accumulation of wall-bound sinapic acids (Fig. 2). Low levels of wall-bound ferulic and *p*-coumaric acids in plants grown with 10 μM, or higher, AIP were similar to levels found in seeds from which seedcoats had been removed (not shown) and we concluded that this fraction was carried over from wall-bound forms in the seed. From these experiments, we concluded that AIP is highly effective in blocking *de novo* hydroxycinnamic acid biosynthesis in radish seedlings.

To determine the effects of AIP on wall-bound phenolics as seedlings developed, plants were grown with 100 μM AIP in an attempt to completely inhibit PAL activity. Wall-bound phenolics were determined in cotyledons and in hypocotyls plus roots from day three to day eight (3–8). Under these conditions, the cotyledons of AIP-grown plants had almost as much wall-bond sinapic acid at any age as did control cotyledons, but only a trace of wall-bound ferulic acid and no detectable wall-bound *p*-coumaric acid (Fig. 3). Hypocotyls plus roots from these plants had no detectable wall-bound *p*-coumaric or ferulic acid and only a trace of wall-bound sinapic acid (Fig. 3).

To determine if radish seeds and seedlings had levels of preformed soluble sinapoyl compounds sufficient to account for increased wall-bound sinapic acid in AIP-grown seedlings (Fig. 3), we used TLC and HPLC procedures of Strack who had earlier characterized the metabolism of these compounds in radish seedlings [15, 17, 19]. We found, as had Strack, that seeds contain primarily sinapoylcholine (sinapine), 6-sinapoylglucoraphanine and 6-3′-disinapoylsucrose. Between days 3–8, cotyledons (AIP-treated or control) contained primarily 1-sinapoylglucose, sinapoyl malate, 6-3′-disinapoylsucrose and 1,2-disanopylglucose. Hypocotyls plus roots of AIP-treated plants showed a pattern similar to cotyledons, but control hypocotyls plus roots showed additional, unidentified, compounds.

Total soluble sinapoyl levels in radish seeds and seedlings were estimated by HPLC using sinapic acid as a standard. As shown in Fig. 4, radish seeds contain approximately 45 μg of soluble sinapoyl compounds, decreasing over the first 8 days of growth to about 20 μg (control) of 15 μg (AIP-grown) in the cotyledons and about 5 μg in hypocotyls plus roots in plants grown with or without AIP. This decrease of approximately 20 μg of soluble sinapic acids during the transition from seed to 8-day-old seedling is more than adequate to account for 100 ng of wall-bound sinapic acids which accumulates in cotyledons of AIP-grown plants between days 3 and 8 (Fig. 3).

We believed that these results provide strong evidence that wall-bound sinapic acid in cotyledons of

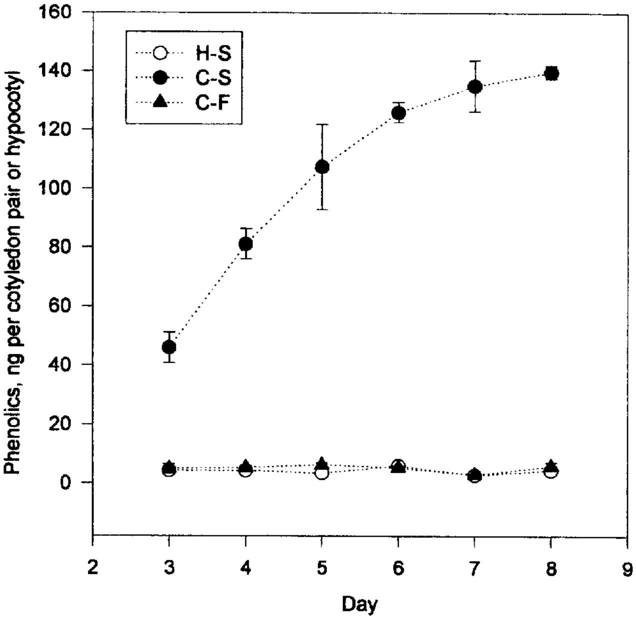

Fig. 3. Effect of growth with 100 μM AIP on developmental changes in wall-bound phenolics in cotyledon pairs and in hypocotyls plus roots. Growth conditions and symbols as in Fig. 1. Values are means ±S.E., $n = 4$.

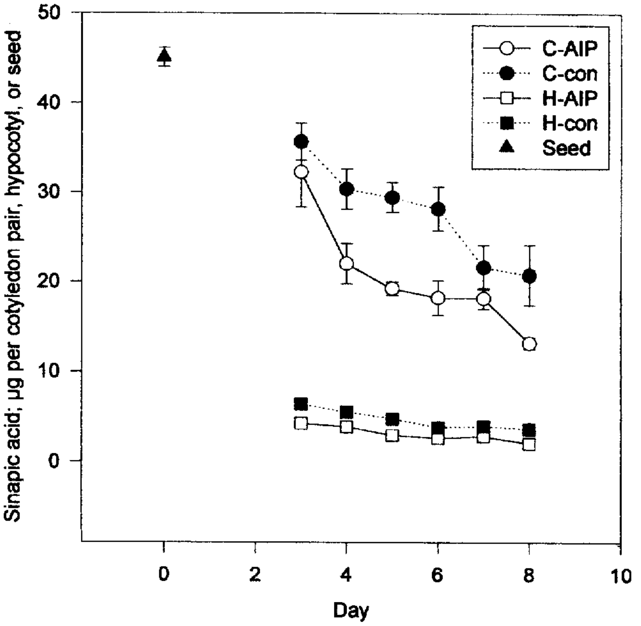

Fig. 4. Developmental changes in total soluble sinapoyl esters in cotyledon pairs and in hypocotyls plus roots of radish seedlings grown with 0 (control) or 100 μM AIP. Growth conditions as in Fig. 1. C = cotyledon pair, H = hypocotyl plus roots. Values are means ±S.E., $n = 3$.

AIP-grown seedlings is derived from soluble sinapoyl esters present in the seed. They also suggest that wall-bound sinapic acid in cotyledons of control plants originates largely from soluble sinapic acid esters, rather than via pathways involving PAL, since levels are similar in plants grown with or without AIP.

Since hypocotyls of AIP-grown plants contained only traces of wall-bound sinapic acid, this suggests that there was no appreciable transport of soluble sinapoyl esters from the cotyledons into the hypocotyl.

Hypocotyls of plants grown with 5 μM AIP were slightly, but consistently, longer than those of control plants from day five–seven (Fig. 5) and they had only about 10% as much wall-bound *p*-coumaric and ferulic acid as controls (Fig. 6). These results are consistent

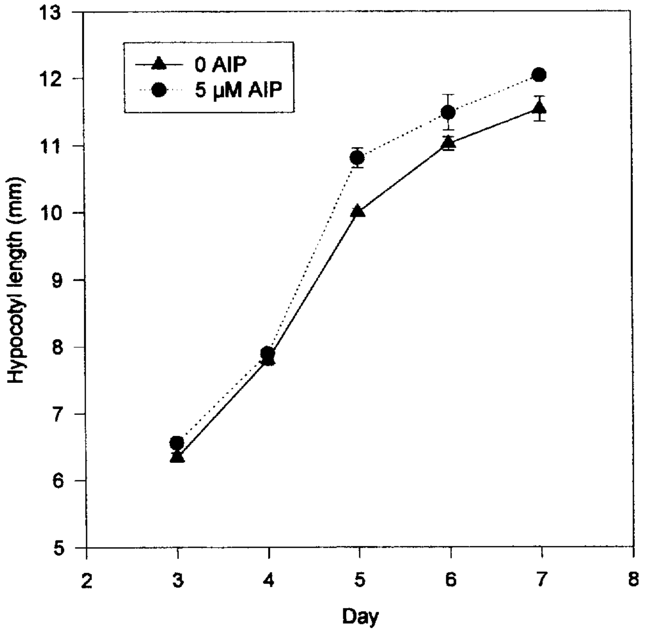

Fig. 5. Effect of 5 μM AIP on radish seedling hypocotyl elongation. Growth conditions as in Fig. 1. 0 AIP = control. Values are means of ca 48 seedlings ±S.E., $n = 4$.

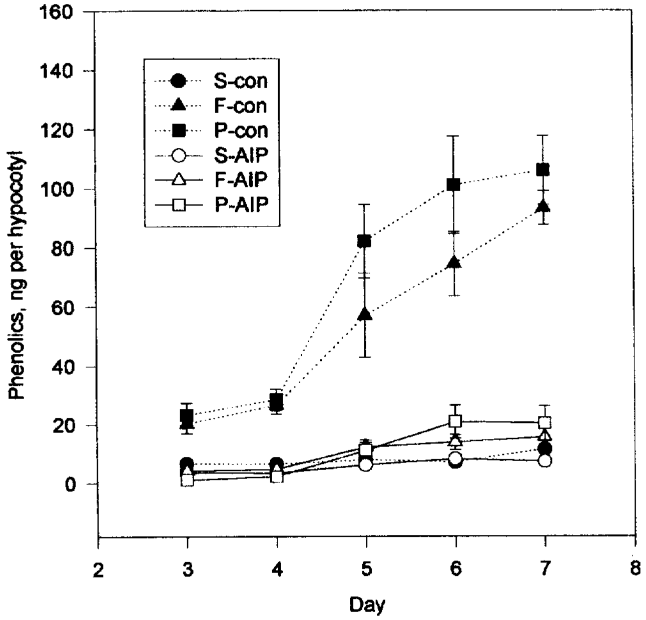

Fig. 6. Effect of 5 μM AIP on wall-bound phenolics in radish hypocotyls whose length is shown in Fig. 4. Growth conditions and symbols as in Fig. 1. con = control, AIP = 5 μM AIP. Values are means ±S.E., $n = 4$.

with the view that wall-bound phenolics can regulate hypocotyl elongation in radish seedlings. However, hypocotyls of plants grown with 10 μM AIP were not significantly different in length from controls (Fig. 7) and the plants had no detectable wall-bound *p*-coumaric or ferulic acid (Fig. 2). Visual examination of the seedlings showed no apparent toxic effect of AIP at levels used in this research.

A possible explanation of such a dose-dependent response is that a 90% reduction in wall-bound phenolics by 5 μM AIP might be enough reduction to interfere with cross-linking and allow more cell expansion [8, 14], but still leave enough phenolics in the wall to serve as screening pigments for a blue-UV photoreceptor involved in hypocotyl photoinhibition [11]; and from this, it would follow that inhibition of

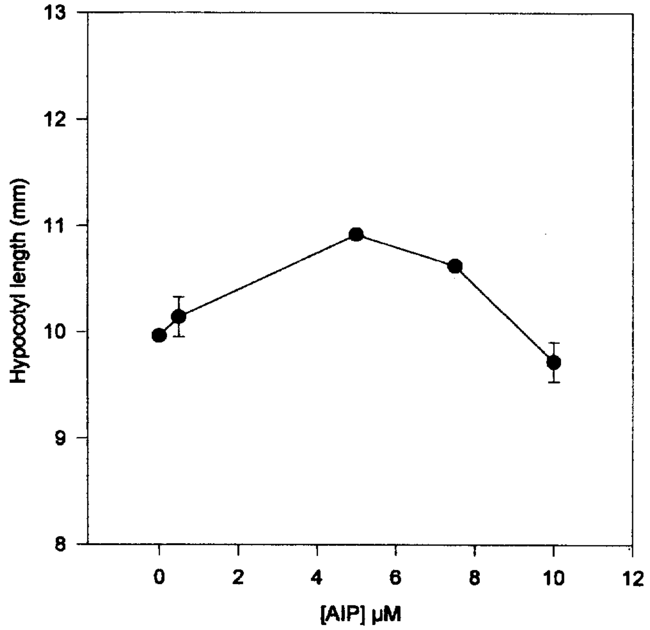

Fig. 7. Effect of varying concentrations of AIP on radish hypocotyl elongation at day 5. Growth conditions as in Fig. 1. Values are means of ca 48 seedlings ±S.E., $n = 3$.

phenolics below our level of detection with 10 µM AIP would provide essentially no such screening and allow more photoinhibition of hypocotyl elongation [11].

Alternately, the more effective inhibition of phenolic biosynthesis by 10 µM AIP might inhibit lignification to such an extent that the vascular system collapses and hypocotyl elongation is inhibited. With this in mind, we are currently extending our work on AIP inhibition of PAL in radish seedlings to a study of changes in lignification.

DISCUSSION

Cell wall preparations from 6 day-old-radish seedlings yield about 1 mg wall-bound phenolics per g cell wall in contrast to 2–5 mg in preparations from cultured spinach cells [5], and 10–50 mg in young seedlings of barley [4, 14]. Recent reports of wall-bound phenolics from a number of dicots [5–9] suggest that these compounds may be a more common feature of this group than was earlier determined [6].

Our results correlating reduced levels of wall-bound ferulic and *p*-coumaric acid (Fig. 6) with increased hypocotyl elongation (Fig. 5) are consistent with elongation responses found in young organs of other species [11–14, 20] and further extends the range of plants where cell expansion and tissue elongation appears to be influenced by wall-bound phenolics.

If soluble sinapoyl esters stored in the seed are converted into insoluble wall-bound sinapoyl esters in the cell wall of PAL-inhibited plants, this implies that they are transported out of the protoplasm into the apoplast. If so, are they esterified to sugar precursors of cell walls within the protoplasm and the sinapoyl-sugar ester incorporated into the growing cell wall, or are they transported out into the apoplast and there esterified to cell wall polysaccharides?

In pulse-chase studies of feruloylation of [^{3}H] arabinose and its incorporation into cell walls of spinach cultures, Fry *et al.* [5] concluded that feruloylation takes place intracellularly and feruloyl-arabinosides are transported from the cytoplasm to the developing cell wall. In contrast, Yamamoto and Towers [3] found that wall-bound sinapic and ferulic acids continued to increase in barley seedlings after there was no measurable increase in cell wall dry weight and suggested that wall-bound phenolics are added to preformed polysaccharides in the cell walls. Subsequently, Yamamoto *et al.* [21] found that [2-^{14}C] feruloyl CoA was incorporated into barley cell wall preparations at a linear rate, which they interpreted as further support of apoplastic feruloylation in this species, but they also indicated that whether the binding was specific or not was not clearly established. In barley, feruloyl glucose was poorly incorporated into wall preparations [21] but Strack *et al.* [22], in pulse-labeled experiments with *Chenopodium* suspension cultures, suggested that 1-feruloyl glucose was transported into the cell wall region and esterified to the wall by a simple transacylation reaction. In this regard, 1-sinapoylglucose is a major soluble phenolic constituent of radish seedlings [15] and could perhaps serve as a transacylation substrate for apoplastic esterification of sinapic acid onto polysaccharides in radish seedling cell walls.

In a recent review, Carpita [23] concludes that primary cell walls of grasses differ from those of all other flowering plant species, varying in types of aromatic substances and structural cross-linking of the walls. When the phenolic chemistry and biochemistry of grass cell walls is compared with these aspects of well-studied dicots such as spinach (10), one cannot exclude the possibility that esterification of ferulic acid, and presumably other hydroxycinnamic acids, to sugars in the cell wall, may take place intracellularly in some plants but *in muro* in others.

EXPERIMENTAL

Plant materials, growth conditions and sampling

Radish seeds, *Raphanus sativus L.* cv Cherry Bell, were purchased from W. A. Burpee Co. (Warminster, PA 18974, U.S.A.). For each experimental replication, 16 seeds were selected for uniform size and sown on two layers of filter paper moistened with 10 ml distilled water (control) or aqueous AIP in a 10 cm diameter plastic petri dish. The petri dishes were maintained at a high relative humidity at 23°. Seedlings were grown in the dark for two days to facilitate germination and stimulate hypocotyl elongation before transfer to continuous 300 μM PAR from cool white fluorescent lamps. Depending on the experiment (see results), whole seedings were analyzed, cotyledons were separated from the hypocotyl plus root by cutting at the node, or cotyledons and roots cut from the hypocotyl and hypocotyl length determined. Hypocotyl length was determined to the nearest 0.1 mm using a dial calliper.

Synthesis of 2-aminoindan-2-phosphonic acid (AIP)

AIP was synthesized and purified by procedures described in Zon and Amrhein [18]. Identity was confirmed by comparison of IR spectra and m.pt with published values [18] and with an authentic sample of AIP generously provided by Professor Amrhein.

Extraction of wall-bound phenolics

Plant material was ground to a slurry with a mortar and pestle, transferred to a 15 ml centrifuge tube, and soluble phenolics removed by sequentially stirring, plus centrifugation, with water (2X), 50% aqueous MeOH + 0.1% v/v HOAc (4X), and Me_2CO (5X). The resultant cell wall fraction was dried under N_2. To solubilize wall-bound phenolics, cell walls were saponified with 2N NaOH under N_2 for 2 h at room temp. At the end of saponification, the pH was adjusted to 1.0 with HCl and samples were extracted with Et_2O (3X), the Et_2O fraction taken to dryness under N_2, and the residue dissolved in 1 ml of MeOH for HPLC.

Analysis of wall-bound phenolics

Phenolics were assayed by HPLC as previously described [14]. Briefly, from 5–15 μl of sample was autoinjected and separated on an Absorbosphere HS (Altech) 250 × 4.6 mm column of 7 μm C-18 particles using an isocratic solvent of 1% (v/v) aqueous H_3PO_4–MeOH–tetrahydrofuran (67:21:12) at a flow rate of 0.7 ml/min with absorbance detection at 320 nm. Limits of detection were ca 50 ng of authentic *p*-coumaric, ferulic or sinapic acid per ml^{-1} when 10 μl samples were injected. By our procedures, this corresponds to a lower limit of detection of about 2 ng per pair of cotyledons or a hypocotyl plus roots.

Identification of wall-bound phenolics

The wall-bound phenolics from 7-day-old seedlings (150 specimens) were applied to a cellulose microcrystalline TLC plate and developed in 4% (v/v) aqueous HCOOH. R_f and colors under UV of the major three compounds were identical with authentic samples of (E)-sinapic acid, (E)-ferulic acid, and (E)-*p*-coumaric acid. HPLC as above, or using a linear gradient of A: 1.5% (v/v) aqueous H_3PO_4 and B: 80% (v/v) aqueous MeCN from 35%–50% B over 20 min at a flow rate of 1 ml/min, further confirmed this identification.

Estimation of total soluble sinapoyl esters

Samples of 10 seeds from which seedcoats had been removed, or 15 pairs of cotyledons or hypocotyls plus roots, were placed in 10 ml of 50% aqueous MeOH (v/v) containing 1% (v/v) HoAC and extracted for a week in the dark at room temperature. The extract was filtered for HPLC and analyzed using the linear gradient described above for wall-bound phenolics. Total peak area of the major soluble sinapoyl esters (see Results) were determined and total soluble sinapoyl esters estimated using sinapic acid as a standard.

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REFERENCES

1. Hartley, R. D. and Jones, E. C., *Phytochemistry*, 1976, **15**, 1157.
2. Harris, P. J. and Hartley, R. D., *Biochemical Systematics and Ecology*, 1980, **8**, 153.
3. Yamamoto, E. and Towers, G. H. N., *Journal of Plant Physiology*, 1985, **117**, 441.
4. Ohashi, H., Yamamoto, E., Lewis, N. G. and Towers, G. H. N., *Phytochemistry*, 1987, **26**, 1915.
5. Fry, S. C., *Planta*, 1987, **171**, 205.
6. Hartley, R. D. and Harris, P. J., *Biochemical Systematics and Ecology*, 1981, **9**, 189.
7. Keller, H., Hohlfeld, H., Wray, V., Hahlbrock, K., Scheel, D. and Strack, D., *Phytochemistry*, 1996, **42**, 389.
8. Kajita, S., Hishiyama, S., Tomimura, Y., Katay-

ama, Y. and Omori, S., *Plant Physiology*, 1997, **114**, 871.
9. Fry, S. C., *Phytochemistry*, 1984, **23**, 59.
10. Fry, S. C., *Annual Review of Plant Physiology*, 1986, **37**, 165.
11. Tan, K.-S., Hoson, T., Masuda, Y. and Kamisaka, S., *Plant and Cell Physiology*, 1992, **33**, 103.
12. Wakabayashi, K., Hoson, T. and Kamisaka, S., *Plant Physiology*, 1997, **113**, 967.
13. Parr, A. J., Ng, A. and Waldron, K. W., *Journal of Agricultural and Food Chemistry*, 1997, **45**, 2468.
14. Liu, L., Gitz, D. C. III and McClure, J. W., *Physiologia Plantarum*, 1995, **93**, 72.
15. Linscheid, M., Wendisch, D. and Strack, D., *Zeitschrift für Naturforschung*, 1980, **35c**, 907.
16. Hasegawa, K. and Hase, T., *Plant and Cell Physiology*, 1981, **22**, 303.
17. Strack, D., *Zeitschrift für Pflanzenphysiologie*, 1977, **84**, 139.
18. Zon, J. and Amrhein, N., *Liebigs Annalen der Chemie*, 1992, 625.
19. Strack, D., Dahlbender, B., Grotjahn, L. and Wrath, V., *Phytochemistry*, 1984, **23**, 657.
20. Wakabayashi, K., Hoson, T. and Kamisaka, S., *Plant Physiology*, 1997, **113**, 67.
21. Yamamoto, E., Bokelman, G. H. and Lewis, N. G., In *Plant Cell Wall Polymers, Biogenesis and Biodegradation*, ed. N. G. Lewis and M. G. Paice, 89, American Chemical Society, Washington DC, pp. 68–88.
22. Strack, D., Bokern, M., Berlin, J. and Sieg, S., *Zeitschrift für Naturforschung*, 1984, **39c**, 902.
23. Carpita, N. C., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1996, **47**, 445.