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Developmental changes in cell-wall ferulate and dehydrodiferulates in sugar beet

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Abstract

Sugar beet (*Beta vulgaris* L.) seedlings were radiolabelled with ^{14}C-cinnamate applied to the leaves. Cell-wall phenolic esters were analysed by HPLC. Different phenolic distributions were found in root and shoot, with dehydrodiferulic acids relatively more prominent in the root. Radioactive dehydrodiferulic acids (but not total dehydrodiferulic acids) increased as a proportion of saponifiable phenolics with increasing time after radiolabelling. This might be due to ferulate cross-linking in the cell wall as cells matured; alternatively, incorporation of pre-formed dehydrodiferulates into the wall or ferulate turnover might be responsible. Ratios of different dehydrodiferulates changed in the root with increasing time after radiolabelling, indicating differential control of formation of different ferulate dimers. The proportion of radioactivity that was non-saponifiable increased with time, indicating increases in ether-linked phenolics.

Keywords: *Beta vulgaris*; Chenopodiaceae; Sugar beet; Biosynthesis; HPLC; Cell wall; Hydroxycinnamate; Ferulate; Dehydrodiferulate

1. Introduction

The properties of plant cell walls are greatly influenced by the nature and extent of the cross-links between cell-wall polymers (Brett & Waldron, 1996). Certain wall polysaccharides (pectins in dicots and arabinoxylans in grasses) contain ester-linked hydroxycinnamic acids, chiefly ferulic and *p*-coumaric acids, which can be oxidatively coupled to form cross-links between polysaccharides (Ishii, 1997). These cross-links may be involved in decreasing wall extensibility during cell maturation (Kamisaka, Takeda, Takahashi & Shibata, 1990; Sanchez, Pena, Revilla & Zarra, 1996). Similar correlations between increasing ferulate and dehydrodiferulate levels and decreases in wall extensibility have been observed on exposure of dark-grown *Avena* coleoptiles to light (Miyamoto et al., 1994). The dehydrodiferulate (DFA) cross-links are thought to be important in cell–cell adhesion (Ng, Harvey, Parker, Smith & Waldron, 1998), and hence have significant effects on the texture of plant-derived foods and on the maintenance of texture during cooking (Waldron, Ng, Parker & Parr, 1997). They are also thought to act as nucleation sites for lignin formation (Ralph, Grabber & Hatfield, 1995), and dehydrodiferulates and ferulate-lignin bonds limit cell-wall degradability in grasses (Grabber, Hatfield & Ralph, 1998).

At least six different dehydrodiferulic acids can be formed in vivo (Ralph, Quideau, Grabber & Hatfield, 1994). The 8–O–4 and 8–5 benzofuran (8–5B) dehydrodimers are the most abundant in most monocot (Parr, Waldron, Ng & Parker, 1996; Ralph et al., 1994; Waldron, Parr, Ng & Ralph, 1996) and dicot (Waldron et al., 1997) tissues studied, while the 8–8 aryl (8–8A) dehydrodimer is the most abundant in pine hypocotyls (Sanchez et al., 1996). Possible variations in the relative amounts of the various mono- and diferulic acids within the same plant, in different

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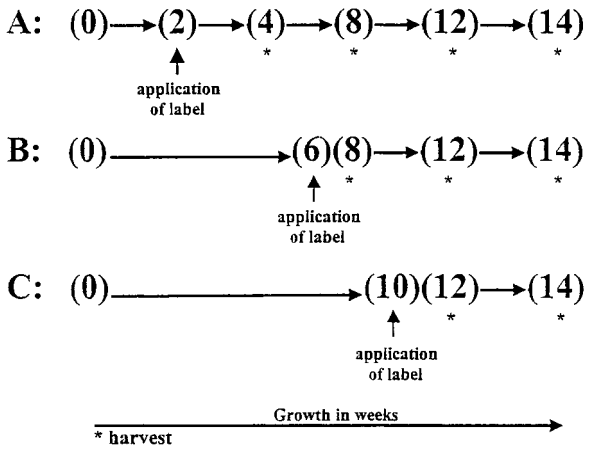

Fig. 1. Time-courses of radiolabelling and harvesting of sugar beet plants. Seedlings of sugar beet (*Beta vulgaris*) were grown for up to 14 weeks and ^{14}C-cinnamate applied onto the youngest leaves after 2, 6 and 10 weeks of growth. Plants were harvested as indicated.

tissues and at different stages of growth, are largely unexplored. Since any such variations would imply metabolic control of the relative levels of these components and would suggest the possibility of distinct roles for the different dehydrodiferulates, this is a potentially interesting area of investigation.

The Chenopodiaceae, of which beet (*Beta vulgaris* L.) is a member, contain higher levels of wall phenolics than most dicots (Colquhoun, Ralet, Thibault, Faulds & Williamson, 1994; Rombouts & Thibault, 1986). We have undertaken a survey of ferulate and dehydrodiferulate (dimer) content in the shoot and root of beet at various stages of seedling growth. In spinach (*Spinacea oleracea* L.), another member of the Chenopodiaceae, it has been shown that externally-supplied ^{14}C-cinnamate acts as a precursor for cell-wall phenolics (Fry, 1984). We have therefore also investigated the fate of radioactive phenolics formed from ^{14}C-cinnamate supplied to the plants at different stages of development.

2. Results

Sugar beet seedlings were grown for up to 14 weeks, and ^{14}C-cinnamate was applied to the two youngest leaves at 2, 6 or 10 weeks old (series A, B and C, respectively — see Fig. 1). Radioactively-labelled plants were then harvested at 4, 8, 12 or 14 weeks, and the alkali-labile cell-wall phenolics were analysed by HPLC for both total phenolic content (non-radioactive measurements) and for radioactively-labelled phenolics.

2.1. Alkali-labile material detected by HPLC in the alcohol-insoluble residue

Analysis by HPLC of the alkali-labile components

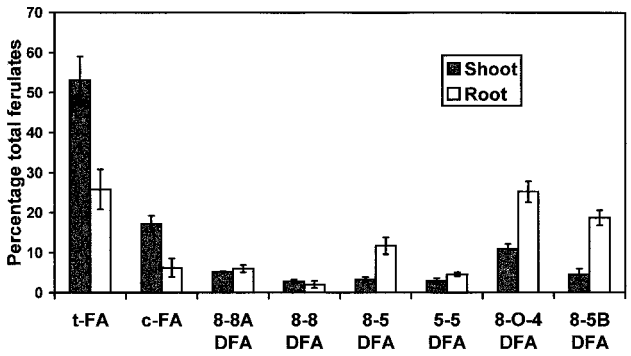

Fig. 2. Mono- and dehydrodiferulates in shoot and root as a percentage of total ferulates. Cell-walls (AIRs) from shoot and root of 12-week-old sugar beet plants were saponified with 2 M NaOH for 18 h at room temperature. Phenolics in the alkali extract were partitioned into EtOAc and analysed by HPLC (non-radioactive measurements).

of the alcohol-insoluble residue (AIR) (non-radioactive measurements) indicated that the mono- and dehydrodiferulic acids together made up at least 92% of the alkali-labile compounds detected in all the samples analysed, as judged by absorption at 280 nm, using response factors of standard compounds (Waldron et al., 1996). Small amounts of vanillin and *p*-coumaric acid were also detected. Vanillin made up 1.8–4.0% of the alkali-labile material in the shoot AIR and 1.1–5.5% in the root AIR. *p*-Coumaric acid comprised 1.6–2.7% of the alkali-labile material in the shoot AIR and 0.4–2.9% in the root AIR.

2.2. Differences in dehydrodiferulate (dimer) content between root and shoot (non-radioactive measurements)

The amounts of *trans*- and *cis*-ferulate and the major dimers, expressed as a percentage of the total ferulates, were significantly different between shoot and root in 12-week-old plants. The overall percentage of dimers was more than twice as great in root (68%) as in shoot (30%), and this was accounted for by large differences in ferulic acid (*cis* and *trans*) and in each of the three major dimers (8–O–4, 8–5B and 8–5) (Fig. 2). There was much less difference in the amounts of the three minor dimers (5–5, 8–8 and 8–8A). In the shoots, *trans*-ferulic acid was by far the largest component, while in the root *trans*-ferulic acid and the 8–O–4 dimer were present in similar amounts. Similar results for root and shoot were obtained for 8- and 14-week-old plants (results not shown).

2.3. Changes in dehydrodiferulate content during growth (non-radioactive measurements)

The shoot showed no major changes in the total ferulate content (monomers plus dimers) of cell walls (1.8–2.3 μg/mg) over the growth period, and the pro-

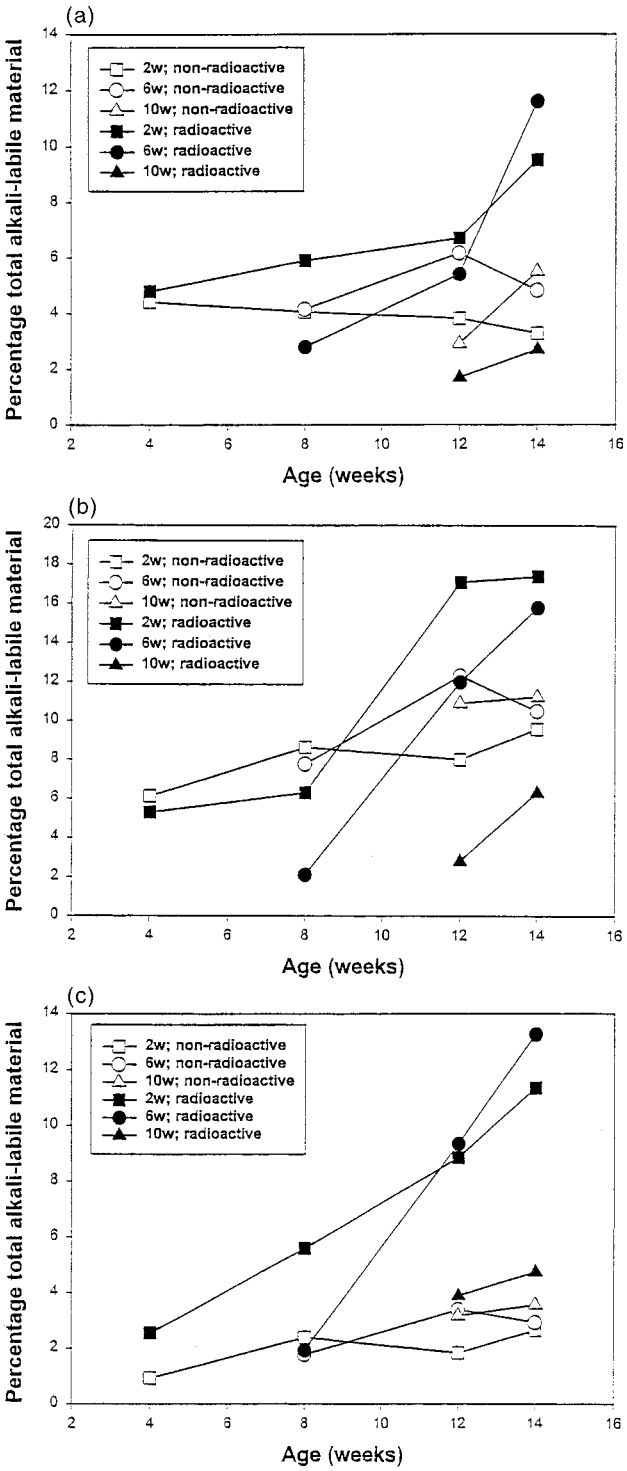

Fig. 3. Changes in radioactive and non-radioactive dehydrodiferulates as a percentage of total alkali-labile material in sugar beet shoots during growth. Cell-walls (AIRs) from shoots of sugar beet plants were saponified with 2 M NaOH for 18 h at room temperature. Phenolics in the alkali extract were partitioned into EtOAc and analysed by HPLC. (a) 8–5B dehydrodiferulate; (b) 8–O–4 dehydrodiferulate; (c) 5–5 dehydrodiferulate. Series A, B and C (radiolabelled at 2, 6 and 10 weeks) represented by squares, circles and triangles, respectively. Closed symbols: radioactive measurements; open symbols: non-radioactive measurements.

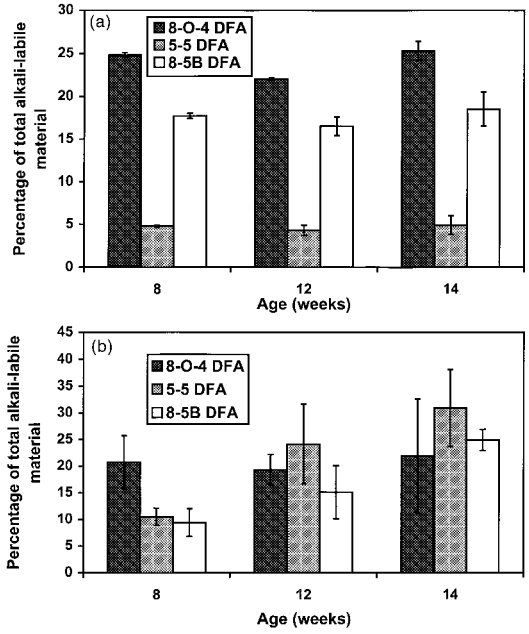

Fig. 4. Changes in radioactive and non-radioactive dehydrodiferulates as a percentage of total alkali-labile material in sugar beet roots during growth. Cell-walls (AIRs) from roots of sugar beet plants were saponified with 2 M NaOH for 18 h at room temperature. Phenolics in the alkali extract were partitioned into EtOAc and analysed by HPLC. (a) non-radioactive measurements; (b) radioactive measurements (Series A plants, radiolabelled at 2 weeks (see Fig. 1)).

portion of dimers as a percentage of total ferulates also remained quite stable (22–30%), as did the relative amounts of the different dimers (Fig. 3a–c, open symbols). In the root, the total ferulate content declined from 3.4 to 1.4 μg/mg between 8–14 weeks, during which time the total amount of wall material in the root increased 5-fold or more as the storage root expanded. The proportion of dimers as a percentage of total ferulates remained approximately constant (65–69%) in the root during this period, and the relative proportions of the different dimers also remained approximately constant (Fig. 4a).

2.4. Changes in total radioactivity in the AIR with time on a whole organ basis

When ^{14}C-cinnamate was applied to young leaves, radioactivity was incorporated into AIRs in both shoot and root. Changes in the total radioactivity in shoot and root AIRs (i.e. alkali-labile plus alkali-stable components) with time are shown in Table 1. Shoots of plants to which ^{14}C-cinnamate was applied at 2 weeks (Series A) showed a marked drop in total radioactivity in the AIR between 8–14 weeks. In plants to which ^{14}C-cinnamate was applied after 6 weeks (Series B), an initial drop in total radioactivity was seen after 8 weeks, followed by an increase after 12 weeks, and a similar increase after 12 weeks was seen in Series C

Table 1
Analysis of radioactively-labelled phenolics in beet cell walls. Cell-walls (AIRs) from shoot and root of sugar-beet plants were saponified with 2 M NaOH for 18 h at room temperature[a]

Series and harvest date (weeks)	8–5B (Bq)	8–O–4 (Bq)	5–5 (Bq)	Saponifiable phenolics (Bq)	Non-saponifiable phenolics (Bq)	Total phenolics (Bq)
Shoot A						
4	61.5	67.3	32.8	1280	676	1956
8	87.1	93.2	83.1	1485	502	1987
12	16.4	41.4	21.5	403	476	879
14	10.9	18.7	12.9	114	258	372
Shoot B						
8	55.0	41.8	38.1	1979	227	2206
12	54.9	122.0	94.8	1015	519	1534
14	148.9	201.8	169.6	1280	1561	2841
Shoot C						
12	16.9	27.6	38.2	980	214	1194
14	77.5	180.6	135.3	2847	506	3353
Root A						
8	0.4	1.4	0.5	6.4	11.8	18.2
12	0.7	0.6	1.2	4.9	10.1	15.0
14	4.0	2.0	6.7	21.8	39.3	61.1
Root B						
8	0.3	0.2	0.4	2.3	3.8	6.1
12	0	0.4	0.1	2.1	2.0	4.1
14	0	0.6	1.0	9.0	13.3	22.3

[a] Phenolics in the alkali extract were partitioned into EtOAc and analysed by HPLC (radioactive measurements). Radioactivity in the three resolved dehydrodiferulates is shown, together with total radioactivity recovered from the HPLC column ('Saponifiable phenolics'), radioactivity remaining in the AIR after saponification ('Non-saponifiable phenolics') and the sum of these two fractions ('Total phenolics').

(^{14}C-cinnamate applied after 10 weeks). In the root, incorporation of radioactivity into AIRs was very much lower than in the shoot. Plants fed with ^{14}C-cinnamate at 2 or 6 weeks (Series A and B) both showed a slight fall in total radioactivity between 8–12 weeks, followed by a 4- to 5-fold increase between 12–14 weeks (Table 1).

2.5. Changes in radioactively-labelled dehydrodiferulates in the shoot during growth

Analysis of the ^{14}C-labelled dimers formed following the application of ^{14}C-cinnamate to young leaves gave results that were significantly different from those of non-radioactive dimers (Fig. 3 and Table 1; see Fig. 1 for timings of labelling and harvest). The three dimers whose radioactivity was resolved on HPLC (8–O–4, 8–5B and 5–5 DFAs) all steadily increased as a proportion of total radioactive alkali-labile material in the AIR in the 12 weeks after ^{14}C-cinnamate was applied to 2-week-old plants (Series A, Fig. 1), even though little change was seen in the corresponding non-radioactive dimers (Fig. 3). Even greater increases were observed in the plants to which ^{14}C-cinnamate was applied at 6 weeks and the ^{14}C-alkali-labile material measured over the following 8 weeks (Series B, Fig. 1), and increases were also seen in plants to which ^{14}C-cinnamate was applied at 10 weeks and changes monitored between 12–14 weeks (Series C, Fig. 1). By 14 weeks old, the sum of these three ^{14}C-dimers (8–O–4, 8–5B and 5–5) reached 38.2% of the total ^{14}C-alkali-labile material in the case of the plants to which ^{14}C-cinnamate was applied at 2 weeks old (Series A) and 40.2% in the case of plants to which ^{14}C-cinnamate was applied at 6 weeks old (Series B). In contrast, the sum of the three dimers as a percentage of total non-radioactive alkali-labile material was only 18.0% at 14 weeks old.

2.6. Changes in radioactively-labelled dehydrodiferulates in the root during growth

As in the shoot,^{14}C-labelled dimers gave results that were significantly different from those of non-radioactive dimers. Only the plants to which ^{14}C-cinnamate was applied at 2 weeks and changes monitored between 8–14 weeks (Series A) gave sufficient radioactivity in the roots for accurate analysis. No data were obtained for the 4 week time-point because the root was too small for extraction of cell-wall material. Data for the three dimers whose radioactivity was resolved on HPLC are shown in Fig. 4. Non-radioactive dehydrodiferulates changed relatively little during growth as a proportion of total alkali-labile material (Fig. 4a), with the 8–O–4 dimer present in greatest quantity and the 5–5 dimer present at around 20% of the 8–O–4 dimer. The ^{14}C-8–O–4 dimer also remained relatively constant as a proportion of total ^{14}C-alkali-labile ma-

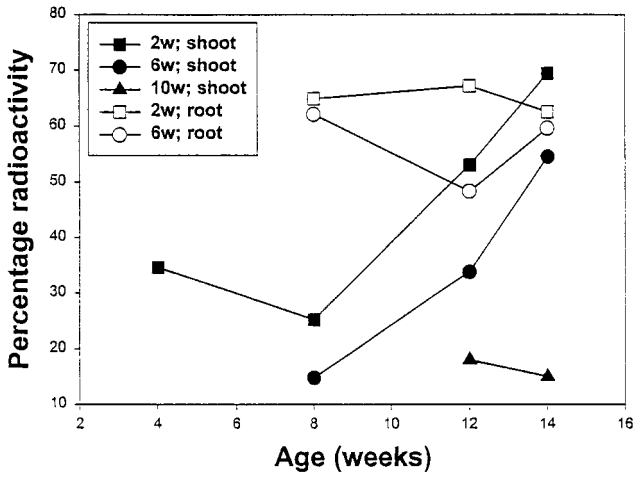

Fig. 5. Alkali-stable radioactivity as a percentage of total radioactivity recovered. Radioactivity in non-saponified cell-wall material as a percentage of saponified radioactivity plus non-saponifiable radioactivity (see Table 1).

terial during growth, while both ^{14}C-8–5B and ^{14}C-5–5 diferulates increased considerably (Fig. 4b). As a result, while the 8–O–4 dimer was the major radioactive dimer at 8 weeks, the 5–5 and 8–5B radioactive dimers were present in equal or greater amounts compared to the 8–O–4 dimer at 12 and 14 weeks.

2.7. Changes in relative amounts of alkali-labile and alkali-stable radioactivity in the AIR with time

Alkali-stable radioactivity in the AIR is shown in Table 1. It increased markedly in the shoot over the time period as a percentage of total (alkali-labile plus alkali-stable) radioactivity recovered from the AIR (Fig. 5). In the root, the proportion of alkali-stable radioactivity remained at a relatively high level throughout the time period.

2.8. Distribution of radioactivity in root and shoot

An indication of the distribution of radioactivity in the shoot and root was obtained by autoradiographic imaging of the plant organs. Much radioactivity remained in the leaf to which it had been applied. Other leaves showed a uniform distribution of radioactivity within each leaf, the next oldest leaves (i.e. those forming at the time of application) being most heavily labelled (Fig. 6). In the root, radioactivity was found in the epidermis and central vascular tissue (stele) of the storage root and also in the absorptive root (Fig. 7). At least 80% of the radioactivity remained in the leaves to which it had been applied, and of this radioactivity at least 90% could be extracted with 80% ethanol. In the other leaves (such as those shown in Fig. 6) and in the root, i.e. those parts of the plant to which radioactivity had been transported, about half of the radioactivity could be removed with 80% ethanol, and the relative abundance of radioactivity in the different parts of the leaves and roots was not significantly altered after ethanol extraction.

3. Discussion

Analysis of total (non-radioactive) alkali-labile ferulates in beet shoot and root highlighted major differences between the two organs. Total ferulate in the shoot remained approximately constant during the growth period on a ng/mg cell wall basis, as did the proportions of each of the dimers. In the root, total ferulate content was initially higher than in the shoot, but decreased by more than 50% during the growth period, and by 14 weeks it had dropped below that of the shoot. This indicates that root tissues laid down during the latter part of the 14-week growth period were much poorer in ester-linked ferulates than those deposited early in root growth. Since ferulate content has been reported to be inversely correlated with growth rate in some tissues (Kamisaka et al., 1990; Sanchez et al., 1996), this change may be related to the major expansion of the storage root during the latter part of the growth period. Throughout the growth period, the percentage of total diferulates in the root was much higher than in the shoot, due to 2- to 4-fold higher percentages of the 8–O–4, 8–5 and 8–5B dimers. The low dimer levels in the shoot may also be related to high rates of cell expansion, since expanding leaf parenchyma is the major tissue type in the shoot.

Analysis of radioactive phenolics following ^{14}C-cinnamate application gave results that showed marked differences compared to the non-radioactive measurements. Total radioactivity (alkali-labile plus alkali-stable) in the cell-wall (AIR) decreased by 30–50% in both roots and shoots between 8–12 weeks. This may indicate some loss of radioactivity from cell walls, perhaps due to metabolic turnover. Between 12–14 weeks, radioactivity in the AIR in both roots and (in most cases) shoots showed a marked (2- to 5-fold) rise, indicating a large net increase in radioactive wall-bound phenolics during this period of rapid growth. This implies that a significant pool of radioactive phenolic precursors must have remained present and available for further wall synthesis at 12 weeks, even though the radioactive precursor had been supplied to the plants up to 10 weeks earlier. The observation that substantial amounts of radioactivity could be extracted from the leaves and roots with 80% ethanol is consistent with this, and Fry (1984) found that a large pool of methanol-soluble radioactive metabolites were formed from ^{14}C-cinnamate fed to spinach cells. The exception to the pattern of increased total radioactivity in the

Fig. 6. Autoradiographic image of radioactivity in leaves of plant radiolabelled at 2 weeks (Series A) and harvested at 8 weeks. The two-heavily labelled leaves visible are the first pair formed after the seed leaves. The seed leaves, which were more heavily labelled, were senescent at this time and are not shown. The second pair of leaves formed after the seed leaves were more lightly labelled (visible on the left of the image). Two further, younger pairs of leaves were also present in this preparation, but were very lightly labelled (on the upper part and on the right of the image, but too lightly labelled to be clearly visible). The leaves were cut from the plant and placed directly into the imager, without drying or solvent extraction. (×0.25).

AIR after 12 weeks was the shoots radiolabelled at 2 weeks (Series A): here radioactivity continued to decline after 12 weeks. The most likely explanation for this is that the heavily-labelled leaves (the seed leaves, to which ^{14}C-cinnamate had been applied at this early labelling point) had been shed from the plant by 12 weeks and their radioactivity thus not recorded in the total. Alternatively, the cell walls of these heavily-labelled leaves may have undergone breakdown due to senescence, in which case some of the radioactivity may have been exported to the root and have contributed to the rise in radioactivity in the root after 12 weeks. This latter possibility is currently under investigation.

In the shoot, radioactivity in the 8–O–4, 5–5 and 8–5B dimers increased as a percentage of total alkali-

Fig. 7. Autoradiographic image of radioactivity in root of plant radiolabelled at 2 weeks and harvested at 8 weeks. The storage root was cut into 1 mm transverse sections which are seen in sequence, with the uppermost at the top left of the image. The absorptive root is on the right, in longditudinal view. All parts of the root were included in the preparation, without drying or solvent extraction. (×0.5).

labile radioactivity in the wall over the period subsequent to the application of the radioactivity. Autoradiographic imaging showed that the radioactivity was present chiefly in the leaves to which the ^{14}C-cinnamate had been applied and in the next oldest pair of leaves, and that younger leaves contained very little radioactivity. Thus cell-wall ferulates in these leaves became increasingly cross-linked as these leaves aged. Since the shoot increased in size throughout the growth period due to the continual formation of fresh leaves, the average degree of cross-linking, as measured by the analysis of non-radioactive ferulates, remained relatively low. It is notable, however, that the percentage of the major dimer, the 8–O–4, was initially lower in the radioactive measurements than on a non-radioactive basis in the samples to which radioactivity was applied at 6 and 10 weeks (Series B and C). Hence newly-formed leaves probably had a lower degree of ferulate cross-linking than average at these age points.

The mechanism by which the degree of cross-linking increased in the radioactively-labelled leaves is not clear. Cross-linking in muro is likely to have occurred, but an alternative possibility is that pre-formed dimers were incorporated into the wall as the leaves aged, especially since the amount of radioactivity in the leaves of series B and C plants increased markedly in the last two weeks of the time-course. This dimerisation might have occurred by cross-linking of ferulate linked to nascent polysaccharides in the Golgi apparatus (Brett, Wende, Smith & Waldron, 1999). It is also possible that some breakdown of wall-bound ferulate may have occurred, contributing to the increase in the proportion of dimers if monomeric ferulate turned over more rapidly than the dimers. Formation of additional, ether cross-links would have the same effect if monomeric ferulate was preferentially ether-linked.

In the roots, radioactive analyses indicated that the 8–O–4 dimer was initially the main dimer cross-linking cell-wall polysaccharides, but that cross-links formed later included 8–O–4, 5–5 and 8–5B dimers in similar amounts. This may indicate the development of different cell types with different patterns of dimer formation. Future work will be directed to determining whether this is indeed the case.

In the shoot, there was an increase in the proportion of alkali-stable (ether-linked) radioactivity as a percen-

tage of total radioactivity recovered over the period of the experiment. This might have been due to an increase in lignin synthesis in the maturing plants, or to some loss of alkali-labile radioactivity, as discussed above, or both. These two processes may be linked, since alkali-labile wall phenolics may have become incorporated into lignin by further cross-linking in the wall, and hence become alkali-stable.

In conclusion, the results reported here indicate that significant differences in wall-bound phenolics occur between different parts of the plant and in the same cells and tissues at different stages of development. These observations indicate that the dynamic nature of the plant cell wall includes a changing phenolic content, and imply that the nature and extent of phenolic cross-linking is under close metabolic control.

4. Experimental

4.1. Plant material

Seeds of sugar beet (*Beta vulgaris* L., var. Saxon) were soaked in vigorously aerated water at 22°C overnight. Seeds were then germinated in vermiculite and the seedlings transferred into 5× strength Hoaglands solution plus 0.5% ferrous sulfate and 0.4% tartaric acid. The plants were grown in the presence of white fluorescent light and in the dark (16 and 8 h, respectively) at 22°C.

4.2. Uptake of radiolabel into plant tissues

[Side-chain-3-^{14}C]*trans*-cinnamic acid (185 kBq; 529 Mbq mmol^{-1}) in 10 μl 50% EtOH was applied to the two youngest leaves of the seedlings (3 per time-point) after 2, 6 and 10 weeks of growth (5 μl per leaf, placed as a single spot at the centre of the leaf). The plants were harvested up to a total growth of 14 weeks (Fig. 1). Alcohol insoluble residue (AIR) was prepared separately from the shoot and the root of each of two plants. Plant tissue was homogenised in liquid nitrogen, suspended in 80% EtOH, centrifuged (1500 *g*, 5 min), and the supernatant discarded. The pellet was washed twice in 80% EtOH, resuspending and centrifuging each time, twice in 100% EtOH and finally twice in Me_2CO, and dried overnight at 50°C. The third plant was monitored for uptake of radioactivity using an autoradiographic image analyser (Packard Instant Imager).

4.3. Analysis

Cold-alkali-labile cell-wall phenolics were extracted separately from the shoot and root AIRs with 2 M NaOH at room temperature for 18 h, and the phenolic material partitioned into EtOAc (three extractions, recovering at least 98% of the phenolic material) and analysed by HPLC (Waldron et al., 1996). This technique resolved *cis*- and *trans*-ferulic acid and the six dehydrodiferulic acids, together with other phenolics (Fig. 1 of reference (Waldron et al., 1996)). However, for radioactive work it was necessary to collect fractions from the effluent from the HPLC column. This resulted in a loss of resolution, so that only the 8–O–4, 5–5 and the 8–5B dehydrodimers could clearly be resolved. Hence results of the radioactivity feeding experiments are reported as the radioactivity in each of these dimers, which are the major ones in beet, and as the proportion of each dimer relative to total alkali-labile radioactivity recovered from the column.

Acknowledgements

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