Hydroxycinnamic Acids and Their Dimers Involved in the Cessation of Cell Elongation in Mentha Suspension Culture

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The contents of wall-bound hydroxycinnamic acids and their dimers were compared between elongated and non-elongated cells of suspension-cultured Mentha. Wall-bound peroxidase activity was also investigated. The main hydroxycinnamic acids esterified to these two kinds of cell walls were ferulic and caffeic acids. Eleven dehydrodicafeic acid isomers and six dehydrodiferulic acid isomers formed through C-C and C-O-C coupling processes, were detected by GC-MS from the extract released from the walls of non-elongated cells. On the other hand, only four dehydrodicafeic acid isomers and three dehydrodiferulic acid isomers were found in the walls of elongated cells. Amounts of monomers of ferulic and caffeic acids and their 5,5'-dehydrodimers in non-elongated cell walls were about ten and twenty times higher, respectively, than those in the elongated cell walls. There was a close correlation between the amount of 5,5'-dehydrodimers and activity of wall-bound peroxidase in non-elongated and elongated cells. The level of 5,5'-dehydrodimers accumulated at a higher rate than monomers in non-elongated cell walls. These results suggest that the dimerization of ester-linked ferulic and caffeic acids by peroxidase and the increase in amounts of their 5,5'-dehydrodimers are important factors in the cessation of cell elongation in Mentha suspension culture.

Key words: cell elongation; cell wall; dehydrodicafeic acid; dehydrodiferulic acid; wall-bound peroxidase

The presence of wall-bound hydroxycinnamic acid derivatives such as ferulic acid (FA) and p-coumaric acid (PCA) is well known in monocotyledons1-5 and reported from a few dicotyledons.6-9 In addition to the FA and PCA, other hydroxycinnamic acids such as sinapic10 and caffeic acids (CA)10-11 have been shown to be ester-linked to the plant cell wall, and other phenolic acids such as 4-hydroxybenzoic acid and vanillic acid, as well as their aldehydes (4-hydrobenzaldehyde and vanillin), have also been demonstrated to be ester-linked to the primary cell wall.12

The concentrations of hydroxycinnamic acids range from 0.5 – 1.5% of the dry walls in monocotyledons and 0.3% of the dry walls in dicotyledons (Chenopodiaceae).13 Hydroxycinnamic acids in growing plant cell wall such as PCA and FA have been identified as being ester linked to arabinoxylans in monocotyledons,14 and to pectic arabinans and galactans in dicotyledons.15 Ester-linked PCA and FA are able to undergo dimerizations through photodimerisation to produce stereocyclodimers,16 or through peroxidase-mediated oxidative coupling16-17 to produce dehydrodimers which cross-link matrix polysaccharides.18-20 The formation of these stereocyclodimers and dehydrodimers in growing plant cell walls is believed to restrict cell-wall extensibility, and to be involved in the cessation of cell elongation.19,21 Indeed, there have been few detailed investigations on wall-bound phenolics in dicotyledons. In monocotyledons, it has been reported that the increase in the amounts of wall-bound FA and 5,5'-dehydrodiferulic acid (5,5'-DFA) in growing cell walls of oat and rice coleoptiles is closely correlated with a decrease in cell-wall extensibility.22-26 However, most of these published works on the cessation of cell elongation were done using the plant tissues such as coleoptiles that contain both un lignified and lignified walls.

Plant cell suspension cultures have been favorite subjects for studies of cell growth and wall biochemistry, in part because their walls are more homogeneous than those of complex tissues.27-28 We have reported that two distinct types of cells with different growth pattern of suspension culture of Mentha were formed, when the cells maintained in the medium containing 1000 μg L-1 2,4-D were trans-
ferred into medium containing different 2,4-D concentrations.\textsuperscript{29} When cells were cultured in a medium containing high concentrations of 2,4-D (500 – 2000 μg l\(^{-1}\)), cell elongation occurred after cell division (elongated cells); when cells cultured in a medium containing low concentrations of 2,4-D (1 – 300 μg l\(^{-1}\)), cell elongation stopped, and the short non-elongated cells longitudinally connected with each other (non-elongated cell). The Mentha cells release a large amount of extracellular polysaccharides, which have been identified as typical primary wall-polysaccharides.\textsuperscript{30-32} These cells are useful for studying the mechanisms of both cell elongation and cessation of cell elongation.

In this study, we investigated the qualitative differences in hydroxycinnamic acids and their dimers, and quantitative differences in monomers and 5,5\textquotesingle-dehydrodimers of FA and CA ester-linked to the walls between elongated and non-elongated cells of suspension-cultured Mentha. We also examined the activity of wall-bound peroxidase, which has been thought to be a key enzyme in stiffening the cell wall, probably through the formation of biphenyl bridges between wall polymers.\textsuperscript{39}

**Materials and Methods**

**Cell suspension culture.** Suspension cells of a Mentha hybrid (F1 of M. arvensis cv. sanbi × M. spicata car. Crispa) were cultured as previously described.\textsuperscript{30} Elongated and non-elongated cells were cultured and harvested as reported previously.\textsuperscript{29}

**Preparation and purification of cell-wall materials from non-elongated and elongated cells.** Cell-wall materials of elongated and non-elongated cells were prepared and purified by the method reported by Selvendran and Ryden.\textsuperscript{39} Elongated and non-elongated cells harvested on different days were washed several times with deionized water, a small amount of 5 mM Na\(_2\)SO\(_4\) was added to prevent polyphenol oxidase activity, and cells were then frozen overnight. The cells, which had been lyophilized, were homogenized in aqueous 1.5\% sodium dodecyl sulfate (SDS) containing 5 mM Na\(_2\)SO\(_4\) for 2 h followed by centrifugation. The precipitate was again homogenized in aqueous 0.5\% SDS containing 3 mM Na\(_2\)SO\(_4\) for 1 h, followed by centrifugation to remove the protein. To remove the starch, 90\% dimethyl sulfoxide (DMSO) was added to the washed precipitate, followed by sonication for 10 min, stirring for 16 h, and then centrifugation. The 90\% DMSO was again added to the precipitate, followed by sonication for 30 min, and stirring for 1 h. The homogenates were then centrifuged and washed six more times until no more polysaccharides were detected. The washed precipitate was suspended in deionized water and dialyzed with water for 24 h. The dialyzed precipitate was finally lyophilized to obtain the purified cell walls.

**Extraction and analysis of phenolic acids in cell walls.** Before the extraction of wall-bound hydroxycinnamic acids, the free phenolics were removed by ethanol from the purified cell walls.\textsuperscript{32} Hydroxycinnamic acids ester-linked to polysaccharides were then released from the cell walls as described by Ralph et al. as follows.\textsuperscript{75} The purified cell-walls (100 – 500 mg) washed with ethanol were suspended and shaken under N\(_2\) in solution of 2 n NaOH (100 ml) at room temperature for 24 h. The suspension was acidified to pH 1–2 with 6 N HCl, then centrifuged, and finally, the residue was washed with deionized water. The supernatant and washings were combined and then extracted with ethyl acetate (3 × 50 ml). The extracts were divided into two parts, one for trimethylsilylation (TMS) and the another one for methylation, and dried by evaporation under vacuum respectively. Then a part of the divided extracts was trimethylsilylated with N\(_2\)-O-bis(trimethylsilyl)-trifluoroacetamide (30 μl) in pyridine (15 μl) for 30 min at 60°C. For methylation, another part of the divided extracts was resolved in a small amount of methanol; then, etheral diazomethane (10 – 20 ml) was added and kept for 10 – 20 min. After the ether was removed by evaporation under vacuum, it was resolved in a small amount of ethyl acetate for GC-EI-MS.\textsuperscript{35} Trimethylsilylated and methylated derivatives were each separated by a DB-1 column (30 m × 3.0 mm) on a gas chromatograph (HP 5890) using He as carrier gas at a flow rate 4.0 ml/min and detected with a mass spectrometer (Jeol SX102, ion source temp. 250°C, ionization voltage of 70 eV). The column was held at 150°C for 1 min, programmed at 6°C min\(^{-1}\) to 300°C, and held for 30 min.

For qualitative estimation of hydroxycinnamic acids, the retention times and mass data of authentic substances were used. The dehydrodimers of ferulic acid were identified by their relative retention times to 5,5\textquotesingle-diferulic acid and mass data reported for their TMS derivatives.\textsuperscript{13,36}

For quantitative determinations of FA, CA, 5,5\textquotesingle-DFA, and 5,5\textquotesingle-dehydrodicaffeic acid (5,5\textquotesingle-DCA), [9-\textsuperscript{13}C\(_3\)]-FA and 5,5\textquotesingle-[9,9\textquotesingle-\textsuperscript{13}C\(_2\)]-DFA were used as internal standards added before saponification of samples.\textsuperscript{37} The amounts of FA and 5,5-DFA were estimated with trimethylsilylated derivatives by GC-EI-MS. Since CA (or 5,5-DCA) gives the same product as FA (or 5,5\textquotesingle-DFA) after the methylation, the amount of FA (or 5,5\textquotesingle-DFA) estimated with methylated derivatives is the sum of FA and CA (or 5,5-DFA and 5,5-DCA) when the plant extracts contain either FA and 5,5\textquotesingle-DFA or CA and 5,5-DCA. The amount of CA (or 5,5\textquotesingle-DCA) from the cell-walls of Mentha was estimated with trimethylsilylated and methylated derivatives by GC-
EI-MS, respectively and expressed as a quantitative difference between the amount of methylated FA (or 5,5'-DFA) and the amount of trimethylsilylated FA (or 5,5'-DFA).

**Assays for peroxidase activity.** The isolation and assays of peroxidase activity were done by the method of Jaeger et al. The washed cells (1 g) were suspended in 5 ml ice-cold buffer (0.1 M sodium phosphate buffer, pH 6.3) and homogenized in a chilled glass homogenizer for 1 min followed by centrifugation at 12,000 × g for 15 min at 4°C to remove the soluble peroxidase. The pellet was washed twice with the same buffer to isolate the cell walls by centrifugation. The cell walls were resuspended in 5 ml of the same buffer containing 1 M NaCl followed by centrifugation to remove the CaCl2-extractable peroxidase (ion bound). After the pellet was washed twice with the same buffer, it was resuspended for one night in 8 ml of 0.1 M acetate buffer (pH 4.0) containing cellulase (EC 3.2.1.4, 60 mg 1−1) and pectinase (EC 3.2.1.15, 60 mg 1−1). The mixture was centrifuged at 15,000 × g for 30 min, and the supernatant was dialyzed against the same buffer for 5 h. The dialyzed supernatant was used as the wall-bound peroxidase fraction.

The activity of peroxidase was assayed as follows. The extract (1 ml) was added to a reaction mixture containing 0.5% pyrogallol (1 ml), 0.5% H2O2 (0.1 ml), and H2O (8 ml) and incubated at 30°C for 3 min; the reaction was then stopped by addition of 1 ml 10% H2SO4. The purpurogallin produced was measured using a standard curve of purpurogallin based on the absorbance at 430 nm. The protein was measured by the method of Lowry et al. using bovine serum albumin as a standard.

**Results and Discussion**

*Identification of monomers and dimers of hydroxycinnamic acids derivatives in the cell walls of non-elongated and elongated cells*

**Table 1.** Main Hydroxycinnamic Acids and Dehydrodiferulic Acids Released from Walls of Non-elongated Cells of Suspension-Cultured Mentha by Alkali

<table>
<thead>
<tr>
<th>Hydroxycinnamic acids</th>
<th>RRTa</th>
<th>RRTb</th>
<th>Major ions (relative abundance, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-FAa</td>
<td>1.00</td>
<td>0.36</td>
<td>338 (M, 100), 323 (51), 308 (46), 293 (21), 279 (9), 249 (33), 219 (13), 147 (8), 73 (44)</td>
</tr>
<tr>
<td>trans-CAa</td>
<td>1.10</td>
<td>0.39</td>
<td>396 (M, 100), 381 (24), 307 (10), 219 (61), 191 (10), 147 (3), 73 (50)</td>
</tr>
<tr>
<td>8-8′-DFA</td>
<td>2.54</td>
<td>0.79</td>
<td>674 (M, 12), 659 (12), 556 (100), 467 (37), 435 (12), 273 (12), 73 (25)</td>
</tr>
<tr>
<td>8-8′-DFA*</td>
<td>2.69</td>
<td>0.82</td>
<td>674 (M, 7), 659 (16), 556 (100), 467 (78), 410 (17), 380 (10), 273 (10), 147 (10), 73 (98)</td>
</tr>
<tr>
<td>8-5′-DFA</td>
<td>2.92</td>
<td>0.89</td>
<td>674 (M, 100), 659 (43), 556 (13), 526 (17), 147 (37), 75 (25), 73 (95)</td>
</tr>
<tr>
<td>5-5′-DFA</td>
<td>3.27</td>
<td>1.00</td>
<td>674 (M, 100), 659 (19), 586 (11), 497 (9), 407 (10), 281 (11), 207 (57), 147 (6), 73 (52)</td>
</tr>
<tr>
<td>8-O4′-DFA*</td>
<td>3.14</td>
<td>0.96</td>
<td>602 (M, 62), 587 (22), 484 (100), 457 (21), 281 (19), 147 (3), 73 (100)</td>
</tr>
<tr>
<td>5-O4′-DFA</td>
<td>3.33</td>
<td>1.02</td>
<td>602 (M, 100), 598 (87), 587 (42), 583 (14), 572 (22), 513 (11), 499 (10), 357 (38), 209 (26), 73 (76)</td>
</tr>
</tbody>
</table>

a, relative retention time to [9-13C]-ferulic acid; b, relative retention time to 5,5′-[9-13C]-diferulic acid. *; also detected in elongated cell walls.
On the other hand, only three isomers of dehydrodiferulic acid (Table 1) and four isomers of dehydrodicticaffeic acid (retention time at 22.67, 23.83, 26.67, and 28.54 min, respectively) were detected in the walls of elongated cells.

The cell walls of suspension-cultured Mentha contained either dehydrodiferulates or dehydrodicticaffeates. In particular, non-elongated cell walls contain a much wider variety of isomeric dehydrodimers of FA and CA than elongated cell-walls. These results suggest that the formation of both dehydrodiferulates from feruloyl and dehydrodicticaffeates from caffeoyl residues contribute to wall cross-links which cause a decrease in cell-wall ability for elongation as proposed previously.\textsuperscript{18,41}

Quantitative estimation of FA, CA, and their 5,5'-dehydrodimers in the cell walls of non-elongated and elongated cells

Changes in amounts of CA, FA, and their 5,5'-coupled dimers associated with the cell walls of suspension-cultured Mentha were investigated.

Figure 1 shows the changes in the amounts of FA and CA released from the walls of elongated and non-elongated cells of Mentha during the culture period. The amounts of FA ester-linked to the wall of non-elongated cells increased significantly from day 7 to day 14, while the amounts of CA increased little during the culture period (Fig. 1A). In contrast with non-elongated cells, the amounts of FA in the walls of elongated cells were small. The amount of CA increased during the culture period, but it was lower than that in the walls of non-elongated cells (Fig. 1B). A total amount of FA and CA in the walls of non-elongated cells were nearly ten times higher than those in the walls of elongated cells.

Figure 2 shows the changes in the amounts of 5,5'-DFA and 5,5'-DCA esterified to the cell walls of elongated and non-elongated cells of Mentha during the culture period. The amounts of 5,5'-DFA and 5,5'-DCA in the walls of non-elongated cells increased significantly from day 7 to 14 during the culture period (Fig. 2A). In contrast, only 5,5'-DCA was detected from the walls of elongated cells; its level was lower, and changed scarcely during the culture period (Fig. 2B). The total amounts of 5,5'-DFA and DCA released from non-elongated cell walls were approximately twenty times greater than those from elongated cell walls.

These results suggest that the increase in amount of dehydrodimers of FA and CA are involved in the cessation of cell elongation by increasing wall cross-links. The increase in wall-bound FA and CA may also decreases cell-wall extensibility, possibly by changing the physical and chemical properties of matrix polysaccharides.\textsuperscript{42} In agreement with our results, it has been reported that the cessation of elongation growth of coleoptiles in rice and oat is closely correlated with the higher levels of FA and 5,5'-DFA ester-linked to cell-wall polysaccharides.\textsuperscript{22-25} In a cell suspension culture of rice, it is reported that the increases in levels of esterified FA and 5,5'-DFA are involved in the cell aggregation.\textsuperscript{43} Furthermore, it was reported that in vitro feruloylated arabinoxylans oligosaccharides inhibit auxin-stimulated cell elongation in rice, suggesting ferulate fragments are involved in the regulation of plant growth.\textsuperscript{44-45}

Interestingly, the amount of 5,5'-DCA in non-elongated cell walls was higher than that of 5,5'-DFA (Fig. 2A), while the amount of CA was lower than that of FA (Fig. 1A). The ratio of 5,5'-DCA/CA was higher during the culture period and increased after 10 days (Fig. 3), while the ratio of 5,5'-DFA/FA was lower and did not increase after 10 days. These results showed the consumption of esterified CA was at higher rate than that of esterified FA. Recently, it was reported that cysteinyl CA and CA have similar effects on laccase-catalyzed gelation of wheat water-extractable arabinoxylans, in which the CA and cys-

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Fig. 1. Changes in the Amounts of Monomeric Hydrocinnamic Acids Released by Alkaline Treatment from Elongated and Non-elongated Cell-Walls.

A, non-elongated cell-walls. B, elongated cell-walls. ■, FA. ◇, CA. The mean values shown are an average of three different experiments. Variation was less 20% of the mean values.
teinyl CA provoked a delay in gelation and in the consumption of the esterified FA as well as in the dehydrodimers production. These effects are explained by the oxidation of CA and cysteinyl CA with concomitant reduction of the FA semiquinones into the original FA immediately as they are formed. Our present results suggest that the esterified CA would compete with esterified FA for the active site of peroxidase to form dehydrodimers.

Activity of peroxidase bound to cell wall of elongated and non-elongated cells of suspension-cultured Mentha

The biochemical interest in hydroxycinnamates is due to their apparent ability to undergo oxidative coupling by peroxidase in vivo to yield cross-linked polysaccharides.

The activity of peroxidase bound to the Mentha cell walls as a possible agent for the oxidative coupling of hydroxycinnamates has also been studied. Figure 4 shows the changes in the activity of peroxidase bound to the walls of elongated and non-elongated cells during the culture period. The wall-bound peroxidase activity of non-elongated cells increased with the culture time, while that of elongated cells did not increase. The increase in peroxidase activity (Fig. 4) was accompanied by an increase in the amounts of 5,5′-dehydrodimers of FA and CA in non-elongated cell-walls (Fig. 2). On the other hand, the increases in peroxidase activity and the amounts of 5,5′-dehydrodimers of FA and CA in the walls of elongated cells were not observed. The changes in

![Graph](image)

Fig. 2. Change in Amounts of Dimeric Hydroxycinnamic Acids Released by Alkali from Elongated and Non-elongated Cell-Walls. A, non-elongated cell walls. B, elongated cell walls. ■, 5,5′-DFA. □, 5,5′-DCA. The mean values shown are an average of three different experiments. Variation was less 30% of the mean values.

![Graph](image)

Fig. 3. Ratio of 5,5′-DCA to CA and 5,5′-DFA to FA in Non-elongated Cell-Walls during the Culture Period. – – –, 5,5′-DCA to CA. – – – –, 5,5′-DFA to FA. The ratio of 5,5′-DCA to CA and 5,5′-DFA to FA were calculated using the data from Fig. 1A and 2A.

![Graph](image)

Fig. 4. Changes in Wall-Bound Peroxidase Activity of Elongated and Non-elongated Mentha Cells During the Culture Period. – – – –, elongated cells. – – – , non-elongated cells. Wall-bound peroxidase activity was expressed as the formation of purpurigallin from pyrogallol by isolated peroxidase. The mean values shown are an average of three different experiments. Error bars represent SE with n = 3.
peroxidase activity showed a good correlation to the changes in amounts of 5,5'-dehydrodimers of FA and CA in both elongated and non-elongated cell walls. The level of 5,5'-dehydrodimers of FA and CA in non-elongated cell walls accumulated at a higher rate than monomers as shown by the decrease of the monomers/dimers ratio, while the level of dehydrodimers in elongated walls accumulated at a lower rate than monomers as shown by the increase of the monomers/dimers ratio (Fig. 5). These results suggest that the syntheses of CA and FA dehydrodimers in non-elongated cell walls are preferentially associated to the activity of wall-bound peroxidase. Therefore, it seems possible that the formation of dehydrodimers of FA and CA is a peroxidase-mediated coupling process in which peroxidase generates the active (free radical) intermediates of esterified FA\(^7\) and CA\(^9\) in Mentha suspension culture. In agreement with our results, an inverse relationship has been found between peroxidase activity and growth capacity in tissues of pine hypocotyl.\(^{34}\) On the other hand, Grabber et al.\(^{18}\) have demonstrated that in vitro the proportion of dehydrodimers to total ferulates increases from about 20 to 45% when diluted hydrogen peroxide is added to maize suspension-cultured cell walls which contain bound peroxidase.

We have previously reported that there is no difference in the amount of cell wall components and in the neutral sugar composition of wall matrix polysaccharides between elongated and non-elongated cells.\(^{29}\) A further experiment showed that pectin-depleted cell walls of non-elongated cells have a cellulase-resistant property during hydrolysis (unpublished data) similar to the Driselase-resistant property of spinach cell wall due to oxidatively coupled phenolics.\(^{43}\) In this study, we have presented evidence that the dimerization of FA and CA in primary cell walls of suspension-cultured cells of Mentha is a peroxidase-mediated coupling process, as has been proposed in other plant tissues. In conclusion, the increase in peroxidase activity accompanying with an increase in the level of 5,5'-dehydrodimers of FA and CA strongly suggest that the dimerization of ester-linked FA and CA by peroxidase is an important factor in the cessation of cell elongation and the wall regidifying. Further investigations of other isomeric dimers of dehydroferrulic and dehydrocaffeic acids make up the study in progress.

**Acknowledgments**

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