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Changes in Cell Wall-Bound Phenolic Acids in the Internodes of Submerged Floating Rice

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Abstract : Submergence induces rapid elongation of internodes in floating rice (*Oryza sativa* L.). We examined the distributions of *p*-coumaric, ferulic and 5-5-coupled diferulic acids ester-linked to cell walls along the axis of highest internodes of submerged and air-grown floating rice stem segments. The amounts of ferulic and 5-5-diferulic acids per cell wall weight were lowest around the intercalary meristem, and increased as the distance from the meristematic zone increased toward the upper part of the internode in both air-grown and submerged stem segments. The ratio of 5-5-diferulic acid to ferulic acid also increased toward the upper, old parts of internodes in both air-grown and submerged stem segments. These observations suggest that the feruloylation of cell wall polysaccharides and the formation of diferulic acids contribute to the cessation of internodal cell elongation and that the formation of diferulic acids in cell walls is controlled by the coupling reaction in addition to the feruloylation. The amounts of *p*-coumaric acid per unit length and per cell-wall weight were markedly low in the newly elongated region of submerged internodes, and closely correlated with cell-wall dry mass in both air-grown and submerged internodes, suggesting that the deposition of *p*-coumaric acid in cell walls is related to the formation of secondary cell walls in floating rice internodes.

Key words : Cell elongation, *p*-Coumaric acid, Deepwater rice, Diferulic acid, Ferulic acid, Floating rice, Internode, Submergence.

Floating or deepwater rice plants respond to submergence by a large increase in the rates of internodal elongation (Catling, 1992). The enhanced growth of internodes is known to be mediated by hypoxia, ethylene, gibberellin and abscisic acid (Kende et al., 1998; Azuma et al., 2003b). The hypoxic conditions caused by submergence enhance the production of ethylene, and the ethylene increases the responsiveness of cells to endogenous gibberellins, at least in part, because of a reduction in the content of endogenous abscisic acid (Raskin and Kende, 1984b; Cohen and Kende, 1987; Hoffmann-Benning and Kende, 1992; Azuma et al., 1995). We have previously shown that hypoxia also can promote internodal elongation independently of enhanced ethylene production (Azuma et al., 2001).

The cell-wall extensibility of rapidly elongating submerged internodes is much higher than that of air-grown internodes (Kutschera and Kende, 1988). The major constituents of growing plant cell walls are cellulosic and noncellulosic matrix polysaccharides such as pectins and hemicelluloses. The cell walls in the elongating zone of floating rice internodes have been reported to contain considerably less cellulosic and noncellulosic polysaccharides and to show a relatively low ratio of cellulosic to noncellulosic polysaccharides (Azuma et al., 1996). These properties of cell-wall polymers may be involved in the high extensibility of the cell walls of submerged internodes. Cell walls of *Poaceae* also contain significant amounts of phenolic acids such as ferulic acid and *p*-coumaric acid, esterified to matrix polysaccharides (Harris and Hartley, 1976; Shibuya, 1984). Ferulic acids bound to the cell walls can form diferulic bridges through a coupling reaction by peroxidase, and such bridges cross-linked among cell wall polysaccharides would lead to a decrease in cell wall extensibility (Fry, 1986).

In the present study, we investigated differences between the spatial distributions of phenolic acids ester-linked to cell walls in rapidly elongating submerged internodes and those of air-grown internodes of floating rice. Our results show that the deposition of ferulic and diferulic acids into cell walls is one of the processes of suppressing rapid elongation of floating rice internodes.

Materials and Methods

1. Plant materials

Caryopses of floating rice (*Oryza sativa* L. cv. Habiganj Aman II) were germinated, and the seedlings were grown as described previously (Azuma et al., 1990). Stem segments, 20 cm long and containing the youngest elongating internodes, were prepared from two- to three-month-old plants by the method of Raskin and Kende (1984a). Segments with the youngest internodes ranging from 6 to 7 cm were used.

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Abbreviations : HPLC, high performance liquid chromatography.

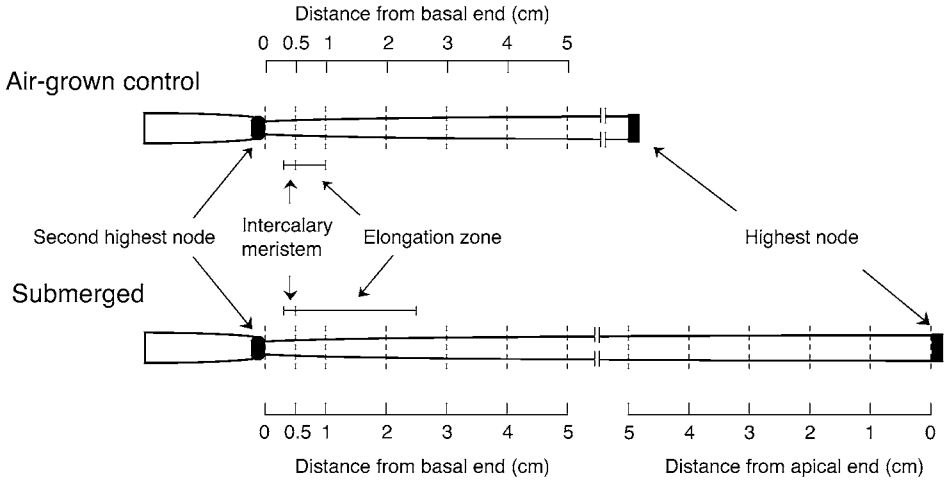

Fig. 1. Diagram of the internodal regions used for analysis of cell wall-bound phenolic acids. Twenty-cm-long stem segments with the highest internodes of 6 to 7 cm in length were submerged or grown in air for two days. From both submerged and air-grown stem segments, two 0.5-cm long sections from the basal end of the internode and four succeeding 1-cm sections were cut for the analysis of phenolic acids. Furthermore, five succeeding 1-cm sections from the apical end of submerged internodes were cut.

2. Submergence of segments

Stem segments were placed upright in a 100-mL beaker and were fixed with grass beads to prevent them from floating upwards. The beaker containing the segments was lowered to the bottom of a one-liter, 50-cm-deep cylinder that was filled with distilled water. Control stem segments were placed upright in a 100-mL beaker that contained 30 mL of distilled water, and the beaker was placed in a plastic cylinder (height, 60 cm; inner diameter, 20 cm) through which ethylene-free air was passed (Azuma et al., 2003a). The stem segments were incubated at 27˚C under fluorescent lights at 100 μmol m^{-2} sec^{-1} for two days.

3. Preparation of cell walls

After the incubation, stem segments in which internodes had elongated by more than 5 cm as a consequence of submergence were used for the analysis of cell wall-bound phenolic acids in internodes. From both submerged and air-grown stem segments, two 0.5-cm long sections from the basal end of the internode and four succeeding 1-cm sections were cut for the analysis of phenolic acids (Fig. 1). Furthermore, five succeeding 1-cm sections from the apical end of submerged internodes were cut. These sections were killed by boiling in methanol and then homogenized in water in a mortar with a pestle. Starch in the homogenate was removed by extraction with dimethyl sulfoxide and by treatment with pancreatic α-amylase (Azuma et al., 1996). Then, cell-wall material was washed with water, acetone, a methanol:chloroform mixture (1:1, v/v), methanol and water. The pellet obtained was frozen, lyophilized and weighed.

4. Extraction of wall-bound phenolic acid

The cell wall material was extracted with 20 mM ammonium oxalate (pH 4.0) at 70˚C for 2 hr to remove pectic substances. The residue was suspended in 1 M NaOH solution containing 0.05 mg mL^{-1} $NaBH_4$ in a vial sealed under N_2. Sinapic acid was added to the vial as an internal standard. The mixture was agitated at 37˚C with a magnetic stir bar for 24 hr. After hydrolysis, the mixture was centrifuged and the supernatant was acidified to pH 2.0 with HCl and then extracted with diethyl ether. The ethereal extract was dried over anhydrous sodium sulfate, evaporated under vacuum and then stored at –30˚C.

5. Determination of phenolic acids

The samples were analyzed by reversed-phase HPLC using a Shimadzu model SCL-6B system equipped with a Mightysil RP-18 GP column (250 × 4.6 mm, Kanto Chemical Co., Tokyo, Japan) and a UV detector (Shimadzu, model SPD-6A), according to the method of González et al. (1999) with following modifications. The flow rate was 1 mL min^{-1} and the elution was performed with an acetonitrile convex gradient against 3% acetic acid at 50˚C. After sample injection, the acetonitrile was kept at 7.5% for 5 min, increased to 12.5% in 30 min and then to 50% in an additional 15 min. The proportion of acetonitrile was held at 50% for 5 min and brought back to 7.5% for an additional 5 min. Phenolic acids were monitored by absorbance at 320 nm and identified by retention time using *p*-coumaric, ferulic and 5-5-coupled-diferulic acids as standards; 5-5-coupled diferulic acid was a kind gift from Prof. Kamisaka (Toyama University, Japan). The quantities of phenolic acids were calculated according

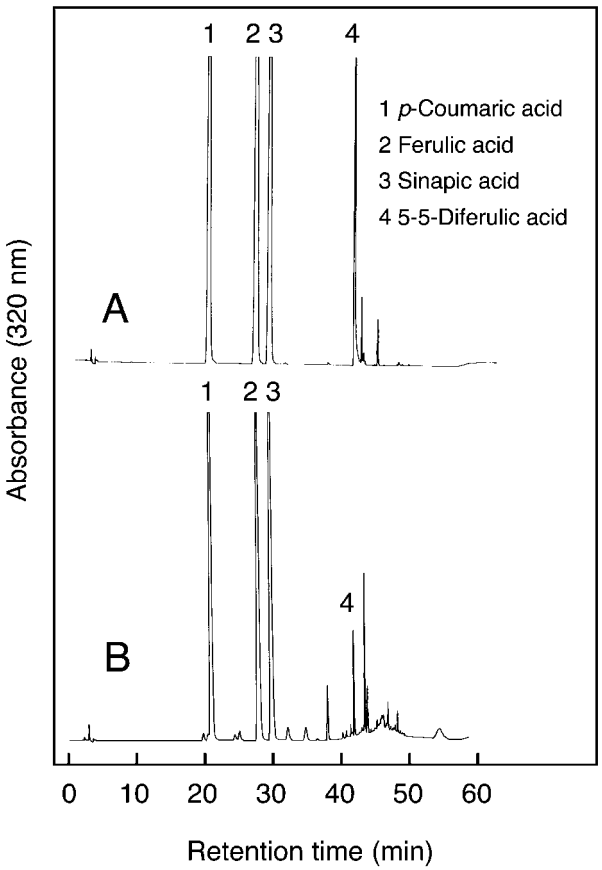

Fig. 2. HPLC profile of phenolic acids extracted from cell walls of floating rice internodes. Sinapic acid was used as an internal standard. A Standards. B An alkali extract of the cell walls of mature tissue.

to the yield of the internal standard.

Results and Discussion

Fig. 2B shows the HPLC elution profile of phenolic acids extracted with 1 M NaOH from the cell walls of mature region of floating rice internodes. *p*-Coumaric acid and ferulic acid were identified as the major phenolic components in the internodal cell walls, 5-5-coupled diferulic acid as a minor compound. These phenolic compounds have already been reported to exist in the cell walls of rice endosperm (Shibuya, 1984) and rice coleoptiles (Tan et al., 1992b). In Fig. 2B, several unknown peaks were observed around the peak of 5-5-coupled diferulic acid. Diferulic acid had been considered to be produced by 5-5 dimerization of ferulic acid ester-linked to the cell wall. However, Ralph et al. (1994) identified new diferulic acids produced by 8-5, 8-8, 8-*O*-4 and 4-*O*-5 dimerization, which were formed by peroxidases in grass walls. Furthermore, Fry et al. (2000) reported that trimers and larger products of ferulate appear to make the major contribution to cross-linking of wall polysaccharides in cultured maize cells. Therefore, the unidentified peaks around the peak of the 5-5-coupled diferulic acid in Fig. 2B might

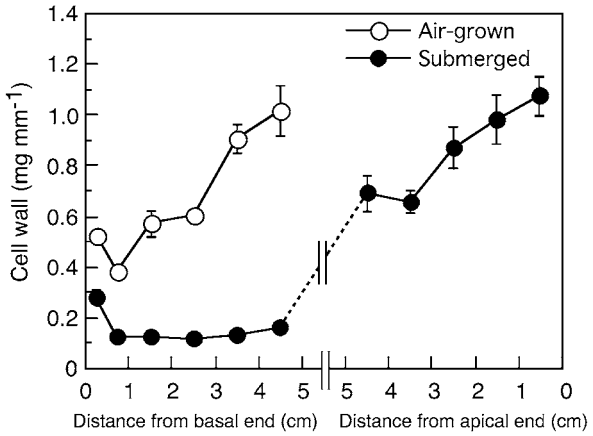

Fig. 3. Dry weight of cell walls in each section of the highest internodes of floating rice stem segments. Stem segments were submerged or grown in air for two days. Each value is the mean ± SE of results from four experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

be those of diferulic acids excluding the 5-5-coupled isomer or those of trimers and larger products of ferulic acid.

We incubated floating rice stem segments in water or in a stream of air for 2 days and examined the distributions of phenolic acids ester-linked to cell walls along the axis of the highest internodes. Fig. 3 shows the dry weight of the cell wall in each section of internode after the incubation. Dry weight of cell wall per millimeter internode was considerably low between 0.5 and 1 cm from the basal end in air-grown internodes and between 0.5 and 5 cm from the basal end in submerged internodes, and increased toward the apical end in both air-grown and submerged internodes. This indicates that deposition of secondary cell walls occurs in the region 1 cm above the lower node in air-grown internodes and in the region at least 5 cm above the lower node in submerged internodes. Bleecker et al. (1986) compared the cell sizes from the basal towards the apical end of internodes in air-grown and submerged deepwater rice and showed that the cell elongation zone of internode is between 0.5 and 1 cm above the basal end in air-grown plants and 0.5 and 2.5 cm in submerged plants (Fig. 1). Therefore, it is obvious that the cell walls in the elongation zone of both air-grown and submerged internodes are extremely thin, and that neither cell elongation nor secondary cell wall deposition occurs in the region 2.5-5 cm above the lower node in submerged internodes (Figs. 1 and 3).

Figs. 4, 5 and 6 show the respective contents of *p*-coumaric, ferulic and 5-5-coupled diferulic acids bound to the cell walls in the highest internode of air-grown and submerged stem segments. The amounts (per unit length) of all these phenolic compounds increased as the distance from the basal end increased

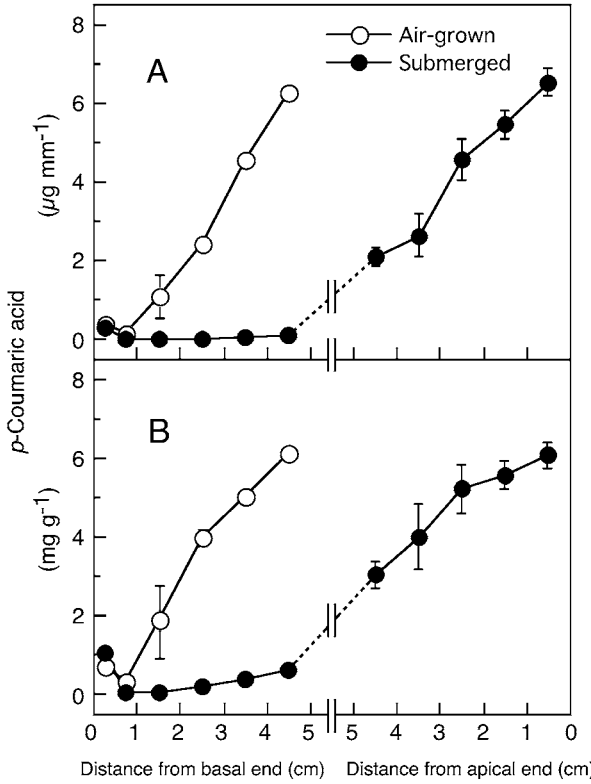

Fig. 4. The amount of *p*-coumaric acid per millimeter length (A) and per gram of cell wall (B) in cell walls in each section of the highest internodes of floating rice stem segments. Each value is the mean ± SE of results from four experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

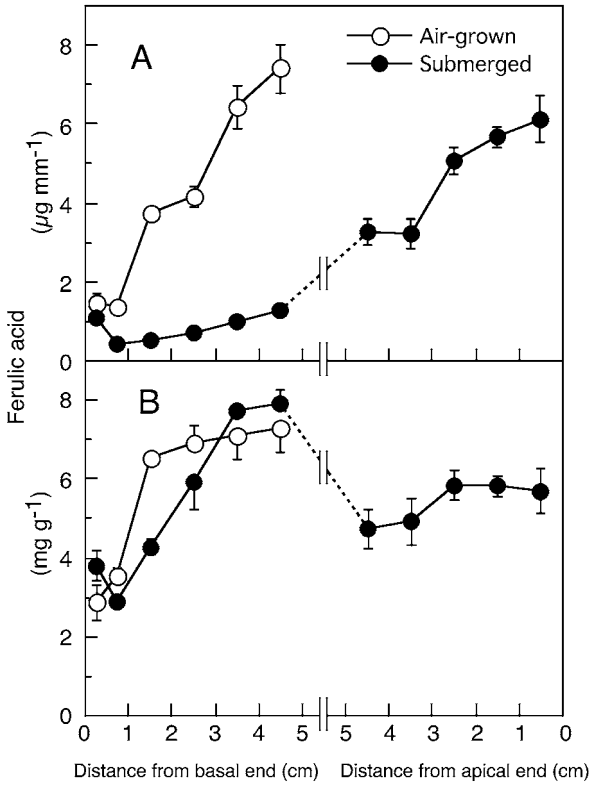

Fig. 5. The amount of ferulic acid per millimeter length (A) and per gram of cell wall (B) in cell walls in each section of the highest internodes of floating rice stem segments. Each value is the mean ± SE of results from four experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

(as the tissue aged) in both air-grown and submerged stem segments (Figs. 4A, 5A and 6A). The rate of increase was prominent in air-grown internodes and in the apical region of submerged internodes, but was low in the basal region of submerged internodes (Figs. 4A, 5A and 6A). The patterns of changes in the phenolic acids were similar to those of cell wall mass (Fig. 3).

The amounts of ferulic and 5-5-coupled diferulic acids per unit cell-wall mass increased markedly with the increasing distance from the base both in air-grown and submerged internodes (Figs. 5B and 6B). In the submerged internodes, the amounts of these two phenolic acids (especially ferulic acid) decreased toward the apical region of internodes, and they were almost constant in the apical 0-5 cm region of the internodes. On the other hand, the amount of *p*-coumaric acid also increased rapidly toward the upper parts in air-grown internodes and in the apical region of submerged internodes, although in the basal region of submerged internodes such a rapid increase was not seen (Fig. 4B). This pattern of change in *p*-coumaric acid content was similar to that of the cell-wall dry mass (Fig. 3), suggesting that the deposition of *p*-coumaric acid into cell walls is related to the formation of secondary cell walls. In the rapidly growing submerged internodes, the basal 0-0.5 cm region contained larger amounts of all the phenolic acids than the next region (0.5-1 cm). This is probably because the most basal region (0-0.5 cm) contains nonelogation zone below the intercalary meristem, which is located 0.3 to 0.5 cm from the basal end (Bleecker et al., 1986).

It should be noted that the levels of ferulic and 5-5-coupled diferulic acids per cell wall weight were lowest around the intercalary meristem and increased significantly with increasing distance in the zones of cell elongation and differentiation above it in internodes of both air-grown and submerged stem segments (Figs. 5B and 6B). These results suggest that the cell wall deposition of both ferulic and diferulic acids is not a consequence but a cause of the cessation of cell elongation in floating rice internodes. In submerged stem segments, the amounts of ferulic and 5-5-coupled-diferulic acids per unit cell wall mass were highest in the newly elongated but non-growing region of internodes (3-5 cm from the basal end; Figs. 5B and 6B). Because neither cell elongation nor secondary cell wall deposition occurs in the newly elongated but

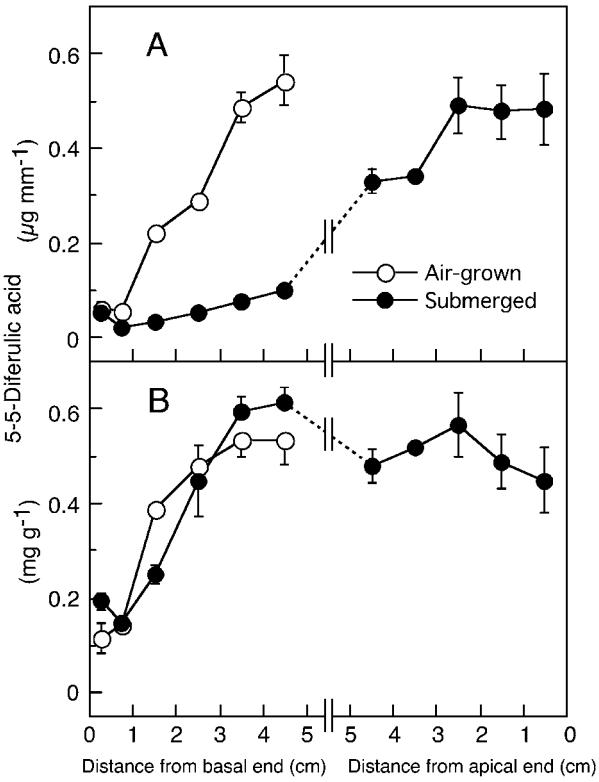

Fig. 6. The amount of 5-5-coupled diferulic acid per millimeter length (A) and per gram of cell wall (B) in cell walls in each section of the highest internodes of floating rice stem segments. Each value is the mean ± SE of results from four experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

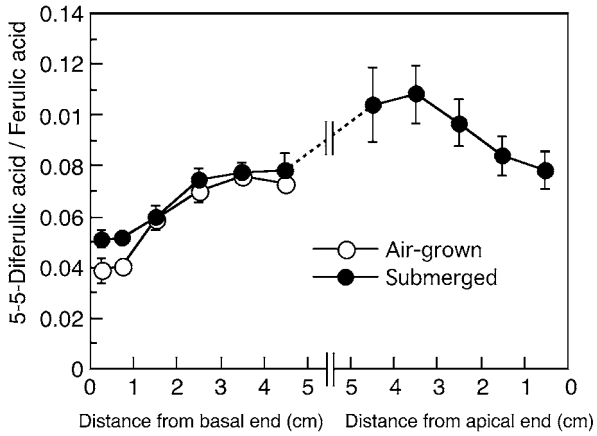

Fig. 7. The ratio of 5-5-coupled diferulic acid to ferulic acid in cell walls in each section of the highest internodes of floating rice stem segments. Each value is the mean ± SE of results from four experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

non-growing region as mentioned above (Figs. 1 and 3), the cell wall deposition of ferulic and 5-5-coupled-diferulic acids that are involved in the cessation of elongation should precede secondary cell wall deposition or lignification.

Fig. 7 shows the ratio of 5-5-coupled-diferulic acid to ferulic acid content in cell walls of the internodes of submerged and air-grown stem segments. The ratio was lowest in the basal 0-1 cm region and increased with increasing distance from the basal end in both stem segments in a similar manner. Ferulic acid bound to cell walls undergoes dimerization through peroxidase-mediated oxidative coupling to produce diferulic acid, which cross-links matrix polysaccharides (Markwelder and Neukom, 1976). This reaction can be controlled at several levels such as feruloylation of matrix polysaccharides, peroxidase activities and oxidant formation (Kamisaka et al., 1990). The ratio of 5-5-coupled-diferulic acid to ferulic acid contents has been reported to be almost constant in oat coleoptiles at different ages grown in the dark (Kamisaka et al., 1990) and in rice coleoptiles grown in air and under water (Tan et al., 1991). However, the ratio has been reported to decrease in light in coleoptiles of oat (Miyamoto et al., 1994) and maize (Parvez et al., 1997), although it was almost constant in rice coleoptiles either in the dark or in light (Tan et al., 1992a). These facts suggest that either the step of feruloylation of matrix polysaccharides or the step of peroxidase-catalyzed coupling reaction to produce diferulic bridges determines the rate of diferulic acid formation due to differences in plant materials and growth conditions. In floating rice internodes, however, the diferulic acid level in the cell wall may be controlled by both the feruloylation and the coupling, because the levels of ferulic acid and the ratio of 5-5-coupled-diferulic acid to ferulic acid increased with increasing distance from the base of the internode (Fig. 7).

In addition to 5-5-coupled diferulic acid, which was measured in this study, grass walls have 8-5-, 8-8-, 8-*O*-4- and 4-*O*-5-coupled diferulic acids (Hartfield et al., 1999). Furthermore, the 5-5-coupled isomer represents a small portion of the diferulic acids in grass walls (Harfield et al., 1999; MacAdam and Grabber, 2002). Therefore, assessment of diferulic bridges by the level of the 5-5-coupled isomer alone will lead to underestimation. However, the level of the 5-5-coupled isomer may still be a marker of the degree of overall diferulic cross-linking, because the level of the 5-5-coupled isomer did not show a distinctive behavior compared to those of the other isomers (MacAdam and Grabber, 2002).

In conclusion, the increase in the ferulic and diferulic acids contents in the cell walls may be involved in the cessation of cell elongation in floating rice internodes as it is in coleoptiles of graminaceous plants. The regulation of feruloylation and coupling of ferulic acid in the cell walls of internodes is under

investigation to clarify further the roles of ferulic and diferulic acids in internodal elongation of submerged floating rice.

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