Relationship between the Deposition of Phenolic Acids in the Cell Walls and the Cessation of Rapid Growth in Internodes of Floating Rice

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Abstract: We examined the involvement of \(p\)-coumaric, ferulic and 5-5-coupled diferulic acids ester-linked to cell walls in determining the elongation rate of internodes of floating rice (\emph{Oryza sativa} L.). When floating rice stem segments were exposed to air after 2 days of submergence, the elongation rate of internodes was reduced and the degree of reduction was greater in the light than in the dark, while the internodes of stem segments submerged further for a comparable period continued rapid elongation. The amounts of ferulic and 5-5-coupled diferulic acids in the cell walls in the elongation zone of internodes significantly increased during the first day after exposure to air either in light or darkness. The increase of these phenolics in the cell walls after exposure to air was also observed on the second day in light, but not in darkness. On the other hand, the amount of \(p\)-coumaric acid increased only slightly on the first day after exposure to air, but rapidly on the second day in light. This pattern of change in the amounts of \(p\)-coumaric acid resembled that in the cell-wall mass (dry weight). The application of sucrose to the segments in darkness increased the amounts of phenolics in the cell walls of internodes to almost the same amount as those in light. These results indicate that the accumulation of ferulic and 5-5-coupled diferulic acids in cell walls may be related to the cessation of internodal elongation in floating rice and that the synthesis of phenolics in the cell wall is caused partially by the provision of sugar in light.

Key words: \(p\)-Coumaric acid, Deepwater rice, Diferulic acid, Ferulic acid, Floating rice, Growth cessation, Internode, Submergence.

Internodes of floating or deepwater rice elongate rapidly in response to submergence (Catling, 1992; Kende et al., 1998). For such a rapid growth in plant tissues, cell-wall loosening is indispensable. In fact, rapidly elongating submerged internodes have much higher cell wall extensibility than air-grown internodes (Kutschera and Kende, 1988). The cell walls in rapidly elongating submerged internodes are much thinner than those in air-grown internodes and show a relatively low ratio of cellulosic to noncellulosic polysaccharides (Azuma et al., 1996). These properties of the cell wall may be contribute to the high extensibility of the cell walls of submerged internodes. In addition to these polysaccharides, the cell walls of \emph{Poaceae} 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).

We previously investigated the distributions of phenolic acids bound to cell walls in the internodes of submerged floating rice stem segments. Our investigation showed that the amounts of ferulic and 5-5-coupled diferulic acids were lowest around the intercalary meristem, and increased as cells were displaced through the elongation zone above it (Azuma et al., 2005). This observation suggests that the deposition of ferulic and diferulic acids in the cell wall is related to the cessation of internodal elongation in floating rice. To further clarify the role of these phenolic acids in the cessation of internodal elongation, we investigated the temporal relationship between the deposition of phenolic acids in the cell wall and the rate of internodal elongation in the floating rice stem segments that had been transferred from submergence to air.

Materials and Methods

1. Plant materials

Caryopses of floating rice (\emph{Oryza sativa} L. cv. Habiganj Aman II) were germinated, and the seedlings were grown as described previously (Azuma et al., 1990). Twenty-cm stem segments having the youngest elongating internodes were prepared from two- to three-month-old plants by the method of Raskin and Kende (1984). Segments in which the initial lengths of the youngest internodes were 3 to 5 cm were used for...
2. Treatment of stem segments

Stem segments were placed upright in a 100-mL beaker and fixed with glass beads to prevent them from floating upwards. The beaker was placed at the bottom of a one-liter, 50-cm-deep cylinder filled with distilled water. The submerged stem segments were incubated under fluorescent lights at 100 $\mu$mol m$^{-2}$ s$^{-1}$ at 27˚C. After a 2-day submergence, the stem segments were transferred to a 100-mL beaker containing 40 mL of distilled water, and the beaker was placed in a plastic cylinder (50 cm high, 5 cm in diameter) through which ethylene-free air at 100% relative humidity was passed at a rate of 200 mL min$^{-1}$ (Azuma et al., 2003). The segments were incubated at 27˚C in the dark or under fluorescent lights at 100 $\mu$mol m$^{-2}$ s$^{-1}$. The total duration of the experiment was 4 days. On days 2, 3 and 4, the internodal elongation of the stem segments was measured, and 2-cm sections of the elongation zone of the internodes, 0.5-2.5 cm from the basal end of the internodes, were excised for analysis of wall-bound phenolic acids. The sections were fixed in boiling methanol and then stored in fresh methanol at 4˚C until use.

When the effect of sugar on the amount of phenolic acids bound to the internodal cell walls was examined, stem segments that had been submerged for 2 days were placed upright in a 100 mL beaker containing 40 mL of 0.1 M sucrose solution or distilled water, and they were incubated in air for an additional 2 days under dark or light conditions. After the incubation, the internodal elongation of the stem segments was measured, and the 2-cm sections of the elongation zone of internodes were prepared, fixed and stored by the method described above.

3. Preparation of cell walls

After rehydration, the sections of the elongation zone of internodes were homogenized in a mortar with a pestle. Starch in the homogenate was removed by extraction with dimethyl sulfoxide and by treatment with porcine pancreatic $\alpha$-amylase (Azuma et al., 1996). Cell wall material was then washed with water, acetone, a methanol:chloroform mixture (1:1, v/v), methanol and water. The pellet thus obtained was frozen, lyophilized and weighed.

4. Determination of phenolic acids

The cell wall material prepared as above was extracted with 20 mM ammonium oxalate (pH 4.0) at 70˚C for 2 h to remove pectic substances. The residue was suspended in a 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 sulfate, evaporated under vacuum and then stored at –30˚C. The samples were analyzed by reversed-phase HPLC using a Shimadzu model SCL-6B system equipped with a Mightysil RP-18 GP column (250 x 4.6 mm, Kanto Chemical Co., Tokyo, Japan) and a UV detector (Shimadzu, model SPD-6A), as described previously (Azuma et al., 2005). Phenolic acids were monitored by absorbance at 320 nm and identified by retention time using $p$-coumaric, ferulic and 5-5-coupled-
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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 to the yield of the internal standard.

Results

1. Growth of internodes

Submergence of the stem segments of floating rice induces rapid internodal elongation in light but not in darkness (Raskin and Kende, 1984). Therefore, in order to induce internodal elongation, submergence of stem segments was carried out under light conditions. The increase of internodal length elicited by submergence was 52 mm on the 2nd day and 120 mm on the 4th day (Fig. 1). The rate of internodal elongation was considerably reduced when stem segments submerged for 2 days were exposed to ethylene-free air. The reduction was more conspicuous in light than in darkness.

Fig. 2 shows the dry weight of cell walls in the 2-cm sections (0.5-2.5 cm from the basal end) of the internodes, which corresponds to the elongation zone of internodes of submerged plants (Bleecker et al., 1986). There was no difference in the cell-wall weight of the elongation zone among stem segments submerged for 2, 3 and 4 days. Exposure of submerged segments to air did not lead to a significant change in cell-wall weight in either in light or in darkness on the 1st day, but significantly increased on the 2nd day in light.

2. Phenolic acids in cell walls

Figs. 3, 4 and 5 show the respective amounts of \( \beta \)-coumaric, ferulic and 5-5-coupled-diferulic acids bound to the cell walls in basal 0.5-2.5 cm region of the internodes in the stem segments that had been submerged for 2 days and then exposed to air. In submerged stem segments, the amount of \( \beta \)-coumaric acid remained almost constant during the 2nd to 4th day, while the amounts of ferulic and 5-5-coupled-diferulic acids significantly increased on the 4th day. Exposure of submerged segments to air increased the amounts of all the phenolic acids in both light and
darkness on the 1st day. On the 2nd day after exposure to air, the amounts of all the phenolic acids increased further in the light, but not in darkness.

3. Effect of sucrose on the amounts of phenolic acids

The application of sucrose had no significant effects on the elongation of internodes and cell-wall mass of the basal 2 cm sections of internodes in the stem segments transferred from submergence to air either in darkness or light (Fig. 6). In the stem segments kept in light, sucrose increased the amounts of p-coumaric acid bound to the cell wall in the basal sections of the internodes (Fig. 7) but not the amounts of ferulic (Fig. 8) and 5-5-diferulic acids (Fig. 9). On the other hand, in the stem segments kept in darkness, the application of sucrose significantly increased the amounts of all these phenolic compounds (Figs. 7-9).

Discussion

When the rapid elongation induced by submergence was retarded on the 1st day after exposure of submerged stem segments to air either in light or darkness (Fig. 1), the cell-wall mass per unit length of the elongation zone was not significantly changed (Fig. 2); on the other hand, the amounts of ferulic and 5-5-diferulic acids bound to the cell walls in the elongation zone were significantly increased by exposure to air (Figs. 4 and 5). These findings suggest that the deposition of ferulic and diferulic acids in the cell walls is related to the depression of rapid elongation of internodes. In the stem segments exposed to air in light, the amounts of ferulic and 5-5-diferulic acids bound to the cell walls in the elongation zone of internodes increased further on the 2nd day after exposure to air. Therefore, two factors, exposure to air and that to light, appear to be involved in the increase in the deposition of ferulic and diferulic acids in the internodal cell walls of floating rice plants transferred from submergence to

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Fig. 5. Changes in the amount of 5-5-coupled diferulic acid in the cell walls of floating rice internodes. For growth conditions, see Fig. 1. Measurements were made for 2-cm sections (0.5-2.5 cm from the basal end) of the youngest internodes. Data points indicate amounts of 5-5-coupled diferulic acid per section (A) and per gram of cell wall (B). Each value is the mean ± SE of results from three experiments. The vertical bars representing the SE are given only when they exceed the size of the symbols.

Fig. 6. Effects of light and sucrose on the elongation (A) and the cell wall mass (B) of internodes in stem segments transferred from submergence to air. Stem segments submerged for 2 days in light were placed upright in a beaker containing 40 mL of distilled water or a 0.1 M sucrose solution. The stem segments were incubated in air for 2 days under light or dark conditions. As a control, segments submerged for 2 days were submerged for an additional 2 days. Measurements for cell-wall mass (dry weight) were made for 2-cm sections (0.5-2.5 cm from the basal end) of the youngest internodes. Each value is given as the mean ± SE (elongation of internode, n = 15; cell wall mass, n = 5).
air. On the other hand, the increase in the amount of 
\( \beta \)-coumaric acid was slight in the segment exposed to 
air for 1 day, although the amount obviously increased 
in light on the 2nd day. This pattern of changes in 
amount of \( \beta \)-coumaric acid resembled that in cell-
wall mass (Fig. 2). In earlier work, we showed that the 
distribution of \( \beta \)-coumaric acid bound to cell walls in 
the highest internodes of submerged and air-grown 
stem segments of floating rice closely resembled that of 
cell-wall mass (Azuma et al., 2005). These observations 
suggest that the deposition of \( \beta \)-coumaric acid in cell 
walls is related to the formation of secondary cell walls 
in floating rice internodes.

In rice seedlings, the amounts of ferulic and 
diferulic acids bound to cell walls were larger in 
coleoptiles grown in air than in submerged coleoptiles 
(Tan et al., 1991). The exposure of submerged rice 
seedlings to air increases the amounts of ferulic 
and diferulic acids in the cell walls of coleoptiles 
(Kawamura et al., 2000), as is the case in the floating 
rice internodes in the present study. When the 
seedlings exposed to air were submerged again, the 
rates of the increases in the amounts of ferulic and 
diferulic acids were reduced (Kawamura et al., 2000). 
The mechanism by which the environmental change 
from submergence to air condition induces the 
deposition of these phenolic acids in cell walls of rice 
tissues, such as coleoptiles and internodes, is unknown. 
The increase in the amounts of these phenolic acids 
duced by the environmental change seem to be 
accompanied by increases in cell wall mass in rice 
coleoptiles (Tan et al., 1991; Kawamura et al., 2000). 
On the other hand, in floating rice internodes, the 
change from submergence to air condition induced an 
increase in the amounts of the phenolic acids initially 
without increasing the dry weight of the cell wall (Figs. 
2, 4 and 5). We previously examined the amounts of 
phenolic acids ester-linked to cell walls in the different 
regions of the highest internodes of submerged 
stem segments, and observed that the increase in the 
amounts of ferulic and 5,5-diferulic acids preceded 
the increase in the dry weight (mass) of cell walls.
Therefore, at least in floating rice internodes, the deposition of ferulic and 5-5-ferulic acids in the cell wall is not necessarily related to the synthesis of the cell walls.

Light irradiation has been reported to increase the amounts of ferulic and diferulic acids in the cell walls of gramineous coleoptiles and such an increase has been considered to decrease the cell-wall extensibility, resulting in a suppression of growth (Tan et al., 1992; Miyamoto et al., 1994; Parvez et al., 1997). Unlike the case of coleoptiles, the rapid growth of floating rice internodes induced by light must be due, at least in part, to an increased supply of photosynthetic assimilates because the floating rice-stem segments used in our experiments had leaf sheaths with chloroplasts.

The results of the present investigation show that the increase in the amounts of ferulic and diferulic acids in the cell wall probably participate in the cessation of rapid elongation of submerged floating rice internodes after exposure to air. The diferulic acid cross-linkages formed by dimerization of feruloylated polysaccharides may be a factor causing a decrease in the cell wall extensibility in floating rice internodes. In this study, we only measured the amounts of 5-5-coupled diferulic acid. However, it has been reported that grass walls have 8-5-, 8-8-, 8-o-4- and 4-o-5-coupled diferulic acids in addition to 5-5-coupled diferulic acid and that the 5-5-coupled isomer occupies only a small portion of the diferulic acids in the grass wall (Harfield et al., 1999; MacAdam and Grabber, 2002). Therefore, the actual level of diferulic bridge formation in the internodal cell wall of floating rice must be much higher than that assessed from the amount of the 5-5-coupled isomer measured in the present study.

Acknowledgements

We thank Prof. S. Kamisaka, Toyama University, Japan, for helpful suggestions and the gift of authentic diferulic acid. We also express our thanks to Prof. T. Hoson and Dr. K. Wakabayashi, Osaka City University, for valuable suggestions.

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