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Cuticle Biosynthesis in Rapidly Growing Internodes of Deepwater Rice[1]

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Submergence induces rapid elongation of deepwater rice (*Oryza sativa* L.) internodes. This adaptive feature allows deepwater rice to grow out of the water and to survive flooding. The growth response of submerged deepwater rice plants is, ultimately, elicited by gibberellin (GA). Little attention has been given to the synthesis and role of the cuticle during plant growth. We investigated two questions regarding the cuticle in rapidly elongating deepwater rice internodes: (a) how does cuticle formation keep pace with internodal growth, which can reach rates of up to 5 mm/h; and (b) does the cuticle contribute to tissue stress in rice internodes? Treatment with GA for 48 h caused an up to 60-fold increase in the incorporation of [^{14}C]palmitic acid and an up to 6-fold increase in the incorporation of [^{14}C]oleic acid into the cuticle of growing internodes. GA also caused a qualitative change in the incorporation pattern of palmitic acid into several cutin monomers, the most prominent of which was tentatively identified by thin-layer chromatography as a derivative of dihydroxyhexadecanoic acid. Rapidly growing plant organs exhibit longitudinal tissue stress: the epidermal cell layer is under tension with a tendency to contract, whereas the internal cells are under compression with a tendency to expand. As a result of tissue stress, longitudinally sliced sections of elongating internodes bend outward upon isolation from the plant. Treating rapidly growing rice internodes with cutinase reduced such outward bending, indicating that the cuticle contributes to tissue stress. Based on these results, we propose that rapidly elongating structures such as deepwater rice internodes constitute an excellent system to study cuticle formation at the biochemical and cellular level.

Deepwater rice (*Oryza sativa* L.) is grown predominantly in areas of Southeast Asia that experience frequent flooding during the monsoon season. Among the physiological and metabolic adaptations that favor survival of this rice is its capacity to elongate rapidly when it becomes submerged. This feature helps deepwater rice plants to emerge from the water and to avoid drowning (Vergara et al., 1976). Plants respond to the altered gas composition within their submerged internodes, namely to reduced partial pressure of O_2, to increased partial pressure of CO_2, and to the accumulation of ethylene (Métraux and Kende, 1983; Raskin and Kende, 1984a). Reduced O_2 tensions promote ethylene biosynthesis, and ethylene increases the responsiveness of the internodal tissue to GA, at least in part by causing a rapid decrease in the level of endogenous ABA (Raskin and Kende, 1984a, 1984b; Hoffmann-Benning and Kende, 1992). Although both low, partial pressures of O_2 and applied ethylene enhance elongation of deepwater rice internodes, GA is the hormone that ultimately promotes growth (Métraux and Kende, 1983; Raskin and Kende, 1984a, 1984b).

Rapidly growing plant organs, e.g. internodes and coleoptiles, exhibit longitudinal tissue stress (for a review, see Kutschera, 1989). This stress arises from the fact that the epidermal cell layer is under tension with a tendency to contract, while the interior parenchymal cells are under compression with a tendency to expand. Tissue stress can be demonstrated by splitting isolated stem segments longitudinally. The combined effects of epidermal cell shrinkage and parenchymal cell expansion cause the two halves of the section to bend outward. In slowly growing organs, tissue stress is lower than in rapidly growing organs, and little or no outward bending of longitudinally split sections can be observed. It has been proposed that tissue stress arises from the difference in thickness and architecture of the outer epidermal cell wall and the walls of the inner parenchymal cells (Kutschera, 1989). The outer epidermal wall is thicker than the walls of the interior cells and has transversely and longitudinally arranged cellulose microfibrils, whereas the direction of microfibrils in parenchymal cell walls is largely transverse. Similar observations have been made with respect to the dimensions and architecture of cell walls in deepwater rice internodes (see Table I and Sauter et al., 1993), which also develop tissue stress when they are induced to grow rapidly (Kutschera and Kende, 1988).

The outer epidermal wall of internodes is distinct not only because of its thickness and arrangement of its cellulose microfibrils. It is also covered by the cuticle, whose structural component is cutin, a polyester composed mainly of C_{16} and C_{18} hydroxy and epoxy fatty acids (Kolattukudy, 1980). The effect of the cuticle on tissue stress has not been investigated, and cuticle formation during rapid internodal growth has received much less attention than has synthesis of the cell wall proper. In fact, Bowen and Walton (1988) appear to be the only authors who have assessed the influence of a plant hormone on cuticle formation by measuring incorporation of [^{14}C]palmitic acid into cutin monomers of GA-treated pea stem sections. In this paper, we report on the promotion of cuticle biosynthesis in GA-treated deepwater rice internodes

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and on the potential role of the cuticle in creating tissue stress.

MATERIALS AND METHODS

Growth of Plants

Deepwater rice (*Oryza sativa* L., cv Habiganj Aman II) plants were grown as described by Stünzi and Kende (1989), except that the day temperature was 27°C for 11 h centered within a 13-h photoperiod.

Treatment of Whole Plants

Eight- to 12-week-old adult plants were submerged in 300-L plastic tanks as described by Métraux and Kende (1983) in the same growth chamber where they had been grown. After 2 d, the basal 1-cm portion of the youngest internode containing the intercalary meristem and part of the elongation zone above it was excised and frozen in liquid N_2.

Isolation and Treatment of Stem Sections

Stem sections containing the youngest internode were excised from 9- to 11-week-old plants as described by Raskin and Kende (1984a). They were placed in 100-mL beakers containing 30 mL of water (control) or 10 μM GA_3 solution and incubated at 25°C under fluorescent lights (35 μmol m^{-2} s^{-1}) in 2.5-L plastic cylinders through which humidified air was passed at a rate of 80 mL/min.

Treatment with Cutinase

Pure *Pseudomonas putida* cutinase (Sebastian and Kolattukudy, 1988), produced by expression in *Escherichia coli*, was obtained from Dr. P.E. Kolattukudy (Ohio State University). Stem sections containing the youngest internode (Raskin and Kende, 1984a) were grown in either water or GA_3 for a total of 24 h as described above. At the times specified, they were injected between the leaf sheath and the base of the youngest internode with 50 μL of either buffer (0.05 M $NaHCO_3$/Na_2CO_3, pH 9.0, 0.05% Tween 20) or 40 μg/mL cutinase in the above buffer, and incubation in water or GA_3 was continued for the remainder of the 24-h period. One set of water- and GA-treated stem sections were not injected as further controls. Twenty-four hours after the start of GA treatment, the leaf sheaths were peeled off and the lowest 3-cm zone of the internode was excised and cut longitudinally into quarters. These segments were incubated in distilled water for about 15 min and photocopied, and the angle formed by the two lines drawn between the ends and the center of each section was determined. This angle was 180° in straight sections and declined as sections bent outward.

Labeling of the Rice Cuticle

For labeling of the cuticle, 0.5 μCi of [1-^{14}C]palmitic acid (57.1 mCi/mmol, ICN, Irvine, CA) or [1-^{14}C]oleic acid (50 mCi/mmol, ICN) in 25 μL of aqueous solution were injected between the leaf and the youngest internode of stem sections at various times of incubation in water or GA_3 solution. Two hours later, the leaf sheath was removed and the lowest 1- or 2-cm portion of the internode was excised, rinsed with water, and frozen in liquid N_2. During this 2-h time period, incorporation of radioactivity into the cuticular fraction was linear. For large-scale cuticle preparations to be used for TLC, the cuticle was labeled for 20 h prior to excision. Uptake of [^{14}C]palmitic acid and [^{14}C]oleic acid was not affected by GA_3.

Preparation of Rice Cutin

Extraction of cutin was modified from the method of Kolattukudy (1970). Internodal tissue was ground in liquid N_2. Ten to 20 mL of distilled water were added to the ground tissue, and the resulting slurry was mixed and centrifuged at 27,000*g* for 10 min at 4°C. The pellet was extracted several times in chloroform:methanol (2:1, v/v) until it was totally white and all membrane lipids had been removed. The residue contained predominantly cell wall material and cuticle. It was dried and weighed, and the radioactivity was determined by liquid scintillation counting. For cutin analysis, the dried sample was refluxed under N_2 for 24 h in distilled tetrahydrofuran containing $LiAlH_4$. The amount of $LiAlH_4$ was three times the sample dry weight. After 24 h, some drops of water were carefully added to react with the remaining $LiAlH_4$, and the pH of the solution was lowered to approximately 3. At this point, the cuticle was depolymerized and its fatty acid components were reduced to fatty alcohols. These products remained in solution while the cell wall, which retained trace amounts of radioactivity only, formed a precipitate. The solution was filtered through Whatman No. 4 filter paper, 40 mL of distilled water were added to the filtrate, and this aqueous solution was extracted three times with ethyl acetate. The ethyl acetate phases were collected, evaporated, and resuspended in a small volume of chloroform:methanol (2:1, v/v).

TLC

TLC was performed according to Kolattukudy (1970). For the separation of hydroxylated derivatives of fatty acids, silica TLC plates were activated at 110°C for about 30 min. The chromatographic solvent was ethyl ether:hexane:methanol:acetic acid (80:20:10:1.5, v/v). The TLC plates were stained with iodine or sulfuric acid, and radioactivity associated with individual bands was determined using a phosphor imager (Molecular Dynamics, Sunnyvale CA).

EM

To measure the thickness of the cell wall and the cuticle, internodal tissue was fixed as described (Kutschera and Kende, 1989). Ultrathin sections were viewed in a Phillips 201 transmission electron microscope, and measurements were made at the center of cells on photographic prints.

RESULTS

Since the growth rate of submerged and GA-treated rice internodes can be as high as 5 mm/h (Stünzi and Kende, 1989), the question arose whether cell wall and cuticle synthesis keep pace with such rapid extension or whether there

is a "thinning out" of the cuticle and the cell wall. To examine this, we determined by EM the thickness of the epidermal cell walls and of the cuticle in the growing region of control internodes and of internodes that had been submerged or treated with GA_3 (Table I). Our measurements confirmed that the outer epidermal cell wall is considerably thicker than the lateral and the inner tangential walls. Neither the epidermal cell wall nor the cuticle became thinner as a result of submergence- or GA-promoted, rapid growth. If anything, the cuticle of GA_3-treated internodes may even have gained in thickness. These results indicate that both cell-wall and cuticle biosynthesis must have increased considerably in rapidly growing rice internodes.

To examine the rate of cuticle synthesis, rice stem sections were incubated in GA_3 solution or in water as control, and either [^{14}C]palmitic acid or [^{14}C]oleic acid was applied to the internode during the last 2 h of incubation. Incorporation of radioactivity into the cuticular fraction of the basal part of the internode was determined. GA-induced growth commences in the intercalary meristem at the very base of the internode and progresses with time to the internodal region above the meristem (Sauter et al., 1993). Treatment with GA_3 greatly increased the rate of [^{14}C]palmitate incorporation into the cuticle (Fig. 1, A and B). After 48 h of incubation, the rate of [^{14}C]palmitate incorporation into the cuticle of the basal 1-cm region of the internode was 60-fold higher in GA_3-treated than in control internodes (Fig. 1A). GA_3 also enhanced incorporation of [^{14}C]oleic acid into the cuticle of deepwater rice internodes (Fig. 2). The level of oleic acid incorporation into the cuticle of the basal 1-cm internodal region was 6-fold above the control level at 48 and 72 h of incubation.

To compare the incorporation pattern of [^{14}C]palmitic acid and [^{14}C]oleic acid, the products of reductive hydrolysis of the cuticle from GA-treated and control internodes were separated by TLC. In the hydrolysate of internodes labeled with palmitate, the most prominent band was near the solvent front and corresponded to the alcohol derivative of palmitic acid (Fig. 3A). The second-most prominent band (R_F = 0.54) contained over three times more radioactivity in the cuticle hydrolysate of GA-treated internodes than in the cuticle hydrolysate of control internodes. Based on the chro-

Table I. *Thickness of the epidermal cell walls and the cuticle*

Tissue was isolated from the internodal region 5 mm above the second highest node of deepwater rice plants that had been air-grown, submerged, or treated with 10 μM GA_3. The measurements were taken at the center of each cell. The values represent the means of 13 measurements ± SE.

	Thickness		
	Control	Submerged	GA_3
	nm		
Cell wall			
Outer epidermal wall	112.5 ± 7.6	122.8 ± 4.2	116.6 ± 8.5
Lateral epidermal wall	20.4 ± 4.8	24.3 ± 2.7	20.0 ± 2.7
Inner epidermal wall	51.3 ± 4.2	55.2 ± 3.0	65.6 ± 2.8
Cuticle	37.7 ± 4.1	42.5 ± 3.9	52.2 ± 4.9

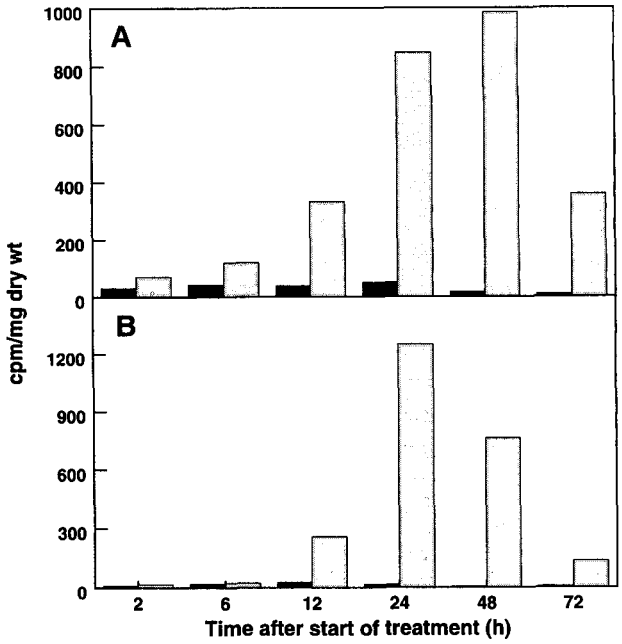

Figure 1. Incorporation of [^{14}C]palmitic acid into the cuticle of the lowest (A) and second-lowest (B) 1-cm zone of the youngest internode of deepwater rice. Plants were grown in water (closed bars) or in 10 μM GA_3 (shaded bars) for the indicated times. Labeling was performed during the last 2 h of each period. Incorporation is expressed on the basis of the dry weight of the cell wall-cuticle fraction following extraction with chloroform-methanol. Similar results were obtained when incorporation was expressed on a per section basis. This figure represents the results of one experiment. A similar promotion of [^{14}C]palmitate incorporation into the cuticle of GA-treated internodes was obtained in three other experiments, even though the uptake and incorporation of radioactivity varied between different lots of plants.

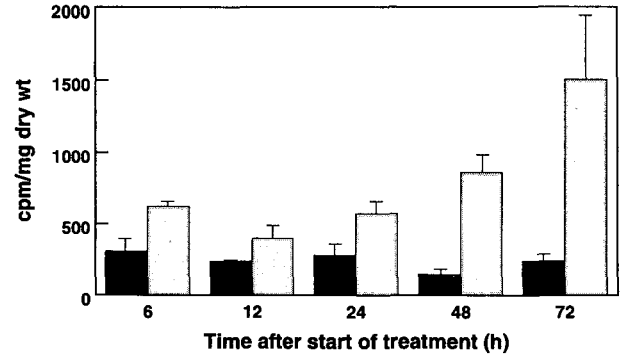

Figure 2. Incorporation of [^{14}C]oleic acid into the cuticle of the lowest 1-cm zone of the youngest internode of deepwater rice. Plants were grown in water (closed bars) or in 10 μM GA_3 (shaded bars) for the indicated times. Labeling was performed during the last 2 h of each time period. The results were similar when expressed on a per section basis. Values represent the means of three experiments ± SE.

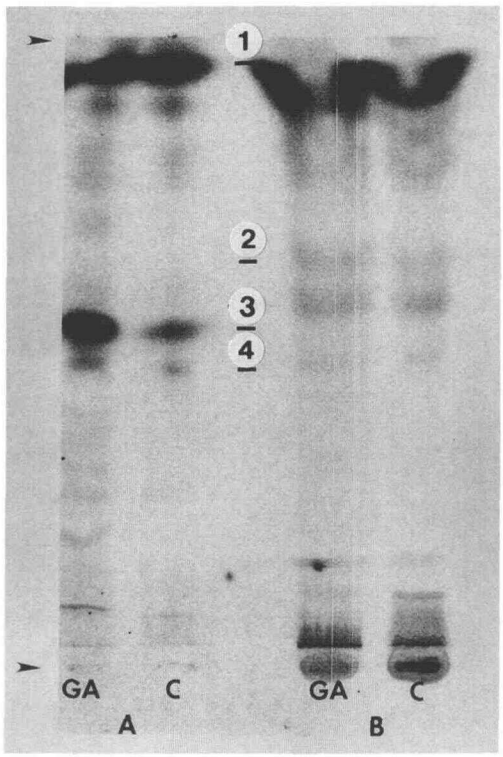

Figure 3. Phosphor-imager printout showing the radioactivity in cutin monomers prepared by reductive hydrolysis and separated by TLC. The cutin was obtained from stem sections grown in water (C) or 10 μM GA_3 (GA) for 48 h and had been labeled with [^{14}C]-palmitic acid (A) or [^{14}C]oleic acid (B) for the last 20 h of that time period. Both lanes of one set contain the same amount of radioactivity. Similar results were obtained when extract representing equal number of sections was loaded per lane. The arrowheads at the bottom and top mark the origin and the solvent front, respectively. The bars in the center indicate the positions of cutin monomers from apple, which had been obtained from Dr. P.E. Kolattukudy (Ohio State University), and which were co-chromatographed as standards (1: hexadecanol, R_F 0.96; 2: hexadecandiol, R_F 0.64; 3: hexadecantriol, R_F 0.54; 4: hexadecantetraol, R_F 0.48). Analysis of [^{14}C]palmitate incorporation was repeated six times and that of [^{14}C]oleate incorporation was repeated twice with similar results.

matographic behavior of cutin monomer standards, this band appears to be a hexadecantriol. Since reductive hydrolysis converts fatty acid components of the cuticle to their corresponding alcohols, the compound at R_F 0.54 was probably derived from a dihydroxyhexadecanoic acid. Incorporation of [^{14}C]palmitic acid into several minor components of the cuticle was also enhanced by GA. There were no differences in the pattern of [^{14}C]oleic acid incorporation into the cuticle of control and GA_3-treated stem internodes (Fig. 3B).

To examine whether the cuticle contributes to tissue stress in rapidly growing internodes, control and GA_3-treated rice internodes were incubated for various times with cutinase or buffer. They were then sliced longitudinally and the curvature of bending was determined (Fig. 4). The sections not treated with cutinase (0 h) show the difference in tissue stress between slowly growing control sections and rapidly growing

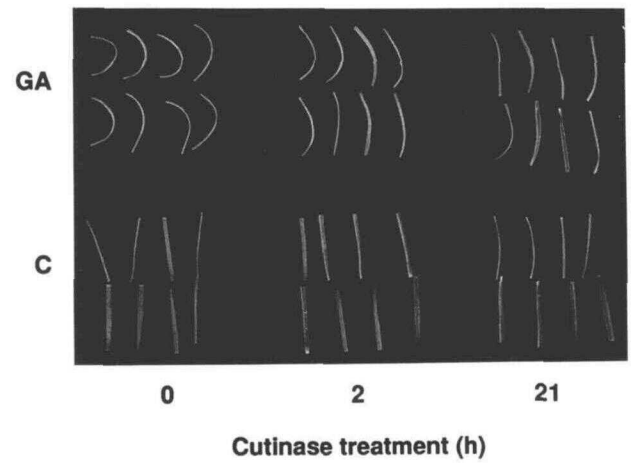

Figure 4. Effect of cutinase on tissue stress in the basal 3-cm portion of the youngest internode of deepwater rice. Stem sections were grown in water (C) or in 10 μM GA_3 (GA) for a total of 24 h. Treatment with cutinase took place during the last 2 or 21 h. The outer epidermis is on the concave side of each section.

GA-treated sections. The control sections remained straight while sections from rapidly growing internodes displayed strong outward bending upon transfer to water. Treatment with cutinase for 2 and 21 h progressively reduced the angle of bending. These observations were quantified by measuring the internal angle of bending (Table II). Application of cutinase had no effect on the bending of internodal sections from air-grown plants. However, incubation with cutinase for 2 h reduced bending of sections from GA-treated internodes considerably (Table II), whereas treatment with buffer alone had no effect on bending (Table III).

DISCUSSION

Our results show that the rate of cuticle biosynthesis was considerably higher in the elongation zone of rapidly growing, GA-treated deepwater rice internodes than in control internodes (Figs. 1 and 2). The observation that more palmitic than oleic acid was incorporated confirms the findings of Kolattukudy and Walton (1972) that the C_{16} family of cutin monomers predominates in growing plant tissues such as

Table II. *Effect of cutinase on tissue stress*

Stem sections were incubated for 24 h in water (control) or in 10 μM GA_3 and were treated with cutinase during the last 2 h of this time period. Tissue stress was determined by measuring the degree of outward bending of internodal sections that had been isolated from the basal 3-cm region of the youngest internodes. Each value represents the mean internal angle ± SE of 44 sections.

Treatment	Duration of Cutinase Treatment	
	0 h	2 h
	Degrees bending	
Water	166 ± 2	169 ± 1
GA_3	98 ± 4	149 ± 3

Table III. *Effect of cutinase and buffer on tissue stress*

Stem sections were incubated for 24 h in water (control) or in 10 μM GA_3 and were treated with cutinase or buffer during the last 2 or 4 h of this time period. Tissue stress was determined by measuring the degree of outward bending of internodal sections that had been isolated from the basal 3-cm region of the youngest internodes. Each value represents the mean internal angle ± SE of 32 sections (16 at 4 h).

Treatment	Duration of Treatment		
	0 h	2 h	4 h
	Degrees bending		
Buffer	97 ± 5	83 ± 4	89 ± 4
Cutinase	97 ± 5	123 ± 4	131 ± 5

expanding leaves. Treatment with GA_3 led to a change in the incorporation pattern of palmitic acid but not in that of oleic acid (Fig. 3). The cutin monomer whose level increased most in the cuticle of GA-treated internodes was tentatively identified as the hydroxylated derivative of a dihydroxyhexadecanoic acid. The chromatographic properties of the other cutin monomers showing increased incorporation of [^{14}C]-palmitic acid (Fig. 3) indicated that they were more polar than was the free fatty acid. They were, therefore, probably derived from hydroxylated or otherwise modified fatty acids. In addition to enhancing cutin biosynthesis, treatment with GA also appears to promote hydroxylation of cutin monomers.

Bowen and Walton (1988) examined the effect of GA_3 on cuticle biosynthesis and composition in pea stem sections by determining the incorporation of [^{14}C]palmitic acid into three selected cutin monomers. Palmitic acid incorporation was up to 2.5-fold higher in GA-treated than in control stem sections, but, in contrast to our results, no qualitative differences in incorporation were found. The fact that GA enhances cuticle biosynthesis in deepwater rice internodes to a much greater extent than in pea stem sections may reflect the difference in the growth rates of these two tissues.

Elongating rice internodes exhibit tissue stress (Fig. 4; Kutschera and Kende, 1988), as do other rapidly growing plant organs. This has been interpreted as showing that the epidermal cell walls are growth-limiting structures that keep the interior parenchymal cells under compression (for a review, see Kutschera, 1989). Treatment of the cuticle with cutinase reduced outward bending of longitudinal internodal sections (Fig. 4, Tables II and III). This is taken as evidence that the elastic properties of the cuticle may be part of the driving force that causes outward bending of longitudinally sliced sections of rapidly growing deepwater rice internodes.

In summary, little attention has been given to the synthesis and role of the cuticle during plant growth. GA-stimulated elongation of deepwater rice internodes is accompanied by a greatly accelerated rate of cutin biosynthesis, making this tissue a suitable object for the study of biochemical and cellular aspects of cuticle formation. Our results also indicate that the cuticle of rapidly growing rice internodes is under tension and that its shrinkage contributes to the outward bending of longitudinally cut sections. The cuticle may not be a growth-limiting structure, however, as has been postulated for the epidermal cell walls.

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