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Susanne Hoffmann-Benning

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Dr. Hans Kende

Major professor

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REGULATION OF GROWTH IN DEEPWATER RICE

By

Susanne Hoffmann-Benning

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ABSTRACT

REGULATION OF GROWTH IN DEEPWATER RICE

By

Susanne Hoffmann-Benning

Submergence induces rapid elongation of rice coleoptiles (*Oryza sativa* L.) and of deepwater rice internodes. The growth response of deepwater rice plants is mediated by ethylene and gibberellin (GA). Submergence and treatment with ethylene led to a 75% reduction in the level of ABA in the intercalary meristem and the growing zone of deepwater rice internodes, while the level of GA_1 increased fourfold. An interaction between GA and ABA could also be shown by application of these hormones. My results indicate that the growth rate of deepwater rice internodes is determined by the ratio of an endogenous growth promoter (GA) and a growth inhibitor (ABA).

Fluridone, an inhibitor of ABA biosynthesis, and submergence led to a reduction of the level of ABA and a promotion of growth in rice coleoptiles, indicating an involvement of ABA in determining the growth rate of rice coleoptiles.

The epidermis limits internodal growth in rapidly growing deepwater rice. Osmiophilic particles with a diameter of 80 nm in rice, 200 nm in cucumber, and up to 300 nm in corn appear between the plasma membrane and the outer epidermal wall. They are associated with rapid growth and, in deepwater rice, with the zone of cell elongation. Monensin inhibits the appearance of these particles, indicating that they are derived from the Golgi apparatus. Results of enzyme-gold labeling and

digestion experiments using proteinase K indicate that the osmiophilic particles are, at least in part, proteinaceous. Antibodies against lipid transfer protein, an extensin-like protein, an arabinogalactan protein, and "expansin" did not bind to the osmiophilic particles. Staining for peroxidase showed that enzyme activity is at least in close proximity to the osmiophilic particles.

Further experiments showed that in GA-treated, rapidly growing rice stem sections the cuticle is a growth-limiting structure. GA treatment leads to an increase in the incorporation of [^{14}C]palmitic acid and [^{14}C]oleic acid into the cuticle of growing internodes and to increased levels of at least one cuticular component, a dihydroxyhexadecanoic acid. I conclude that the activities of enzymes that catalyze hydroxylation of fatty acids may be enhanced.

To

Christoph and Urs

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I thank my parents for allowing me to go my way and for their support even against the advice of friends who said: "She is only a girl and will be married and housewife some day, so why doesn't she get a real job for now?" And - most of all - I thank my husband Christoph and my son Urs for their love, patience and support in trying to prove these friends wrong.

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ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AGP	arabinogalactan protein
AMO 1618	4-hydroxy-5-isopropyl-2-methylphenyltri-methylammonium chloride 1-piperidine
BSA	bovine serum albumine
CCC	(2-chloroethyl)trimethylammonium chloride
ELISA	enzyme-linked immunosorbent assay
fluridone	1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]-4(4H)pyridonone
GA	gibberellin
GC	gas chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid (auxin)
LTP	lipid transfer protein
PBS	phosphate buffered saline
PCIB	*p*-chlorophenoxy-isobutyric acid
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
TCH	thiocarbohydrazide
TEM	transmission electron microscope
THRGP	threonine hydroxiproline-rich glycoprotein
TLC	thin layer chomatography

CHAPTER 1

INTRODUCTION

DEEPWATER RICE AND ITS GROWTH RESPONSE

Rice (*Oryza sativa*) is grown on 11% of the world's arable land and almost exclusively for human consumption. There are three types of rice: upland rice, which is grown in high-lying areas and is rainfed; lowland rice, which is grown as paddy-rice, and deepwater rice. Deepwater rice is grown predominantly in Southeast Asia in areas that are flooded during the monsoon season. It has developed several adaptive mechanisms that allow it to survive flooding: first, it has an aeration system which transports O_2 from the atmosphere via the leaves and the internodal cavities all the way to the roots to maintain aerobic metabolism in submerged organs (Raskin and Kende, 1985). Second, submergence induces rapid internodal growth. Planted at least one month before the monsoon rains start, plants begin to elongate as they become partly submerged in the rising floodwaters. Growth rates of up to 25 cm per day have been observed, and plants can reach a total hight of up to 7 m (Vergara *et al.*, 1976). Flowering in deepwater rice is under photoperiodic control to ensure that panicle emergence occurs after the water has stopped rising. This is important since plants lose the ability to elongate once panicles have formed. Seeds are harvested from boats or after the water has receded. The ability of deepwater rice plants to survive flooding makes it a subsistence crop in areas like Bangladesh, where severe floods occur

frequently.

Deepwater rice is not the only rice variety that elongates when submerged. All rice varieties show an increase in coleoptile elongation when their seedlings are submerged, and an increase in stem growth once the internodal elongation has started prior to flowering. However, the capacity for rapid elongation starts in non-deepwater rice cultivars at approximately ten weeks of age while deepwater rice is responsive when it is ca. four weeks old (Keith *et al.*, 1986). Some other semiaquatic plants also show an induction of growth after submergence. One is *Callitriche platycarpa* in which stem elongation is controlled by ethylene and gibberellin (GA, Musgrave *et al.*, 1972). In *Ranunculus sceleratus* (Smulders and Horton, 1991) and some *Rumex* species, waterlogging and ethylene promote petiole growth (Voesenek and Blom, 1989; Voesenek *et al.*, 1990a; Voesenek *et al.*, 1990b).

The inducibility and rapid elongation of deepwater rice makes it a good system to study internodal growth. Elongation occurs predominantly in the youngest internode and can also be seen in isolated deepwater rice stem sections (Raskin and Kende, 1984a). Submergence leads to a decrease in the endogenous oxygen tension (as low as 2% during the dark period) and an increase in endogenous CO_2 level (to ca. 6%; for a review see Kende, 1987). The low O_2 tension leads to an increase in 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase activity (Cohen and Kende, 1987) and, thus, to an increase in the ethylene biosynthesis (Raskin and Kende, 1984a). Ethylene by itself promotes growth of non-submerged plants. (Métraux and Kende, 1983). Two enzymes of polyamine biosynthesis, S-adenosyl-L-methionine decarboxylase and arginine decarboxylase, are also stimulated within 4 h of

submergence and appear to be related to accelerated cell division in the intercalary meristem (Cohen and Kende, 1986).

The submergence response can also be induced by GA. Raskin and Kende (1984c) have shown that ethylene increases the responsiveness of the internodal tissue to GA. This, in turn, leads to internodal elongation and is associated with an enhancement of amylolytic activity and increased translocation of photosynthates in the growing zone (Raskin and Kende, 1984b; Smith *et al.*, 1987). This rise in the growth rate can be measured 180-220 min after submergence, 60 min after treatment with a gas mixture containing 3% O_2, 6% CO_2, 91% N_2, 1 μl/l ethylene, and 40-60 min after treatment with GA_3 (Rose-John and Kende, 1985). Growth is based on an approximately three-fold increase in the production of new cells in the intercalary meristem and on a three- to fourfold increase in the final size of cells emerging from the meristem into the cell elongation zone (Métraux and Kende, 1984; Bleecker *et al.*, 1986). Recent findings of Sauter and Kende (1992a) indicate that GA first promotes cell elongation in the intercalary meristem and that cell growth triggers an increase in the rate of cell divisions. In parallel, there is a decreased incorporation of Si into the cell wall (Rose-John and Kende, 1984), a delay in lignification (Sauter and Kende, 1992b), and a delay in cellulose microfibril reorientation from transverse to oblique (Sauter and Kende, 1993). In addition, Kutschera and Kende (1989) observed the appearance of osmiophilic particles between the plasma membrane and the outer epidermal wall of rapidly growing, submerged plants. The chain of events leading from submergence to internodal elongation, as it was known at the beginning of this thesis, is summarized in Figure 1.1.

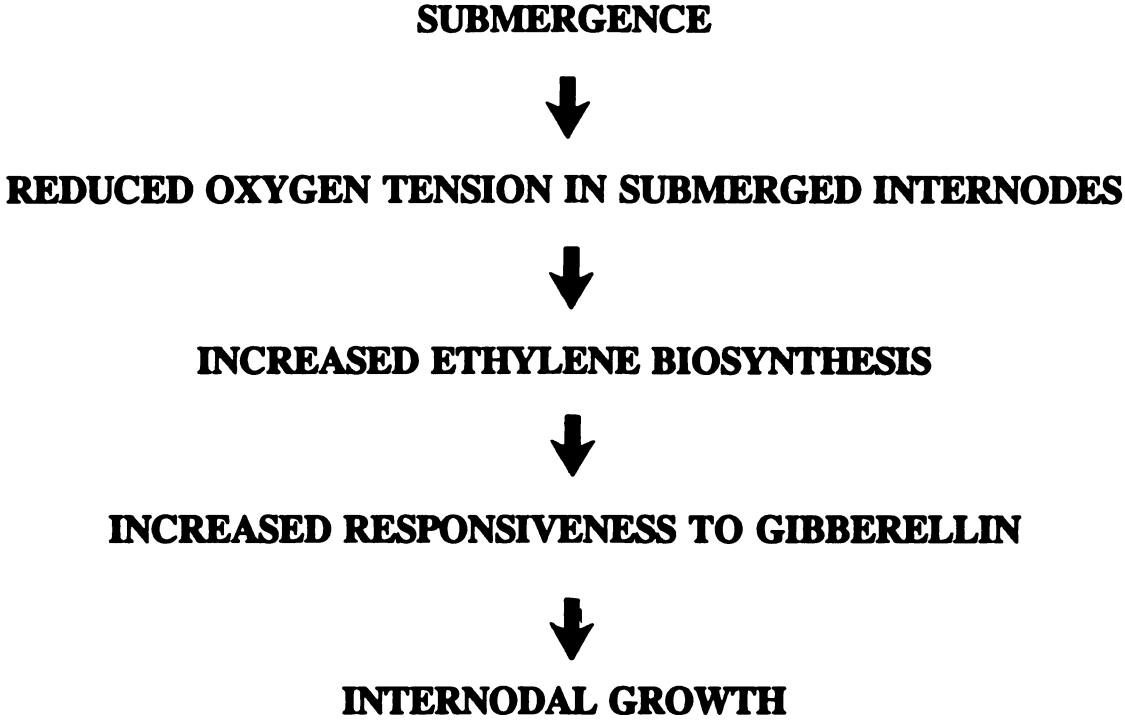


Figure 1.1. Chain of events linking submergence to accelerated growth of deepwater rice as known in 1988.

The questions asked in this thesis were:

1. How does ethylene change the responsiveness of the growing tissue to GA?
2. What are the osmiophilic particles and how are they associated with rapid growth?
3. Do cuticle biosynthesis and composition change in rapidly growing deepwater rice internodes?

THE ROLE OF THE PLANT GROWTH SUBSTANCES ABSCISIC ACID (ABA) AND GA IN GROWTH OF DEEPWATER RICE

What are plant hormones? "A plant hormone is an organic compound synthesized in one part of the plant and translocated to another part, where in very low concentrations it causes a physiological response." (From Salisbury and Ross, 1992). Plant hormones that promote growth are auxin and GA, those that inhibit growth are ABA and ethylene. Usually, ethylene is considered an inhibitor of growth in terrestrial plants. However, in some semiaquatic plants, it has been shown to induce stem or petiole elongation. Applied GA also induces growth in deepwater rice (Raskin and Kende, 1984c; Suge, 1985, Suge and Türkan, 1990). Yamaguchi (1973) and Türkan and Suge (1991) found, using a bioassay, that the level of biologically active GAs is higher in submerged rice than in non-submerged rice. When plants were pretreated with inhibitors of GA biosynthesis, neither submergence not ethylene induced internodal growth (Raskin and Kende, 1984c; Suge, 1985). Raskin and Kende (1984c) showed that ethylene increases the responsiveness of the internodal tissue to GA.

How does ethylene change the responsiveness of the internodal tissue to GA? Zeevaart (1983) showed that applied ethylene increases ABA metabolism and, thereby, causes a decrease of biologically active ABA in leaves of *Xanthium strumarium*. Ethylene could have a similar effect in deepwater rice. It could cause a decrease in the endogenous content of ABA, which is a potent inhibitor of GA action in many systems. With reduced ABA levels, endogenous or applied GA would be more effective in promoting growth. In other words, the rate of growth could be determined by the ratio of a growth inhibitor (ABA) to a growth promoter (GA). A similar antagonistic action of ABA and GA occurs e.g., in cereal seeds where GA enhances amylolytic activity, while ABA inhibits it (see Jacobson and Chandler, 1988). The possibility of ABA regulating internodal growth in deepwater rice is addressed in chapters 2 and 3 and summarized in Hoffmann-Benning and Kende (1992a).

ULTRASTRUCTURAL CHANGES IN DEEPWATER RICE IN RESPONSE TO SUBMERGENCE

Submergence of deepwater rice leads to an increase in internodal growth. Along with this increased growth, several ultrastructural changes appear: cell division and cell elongation increase (Sauter and Kende, 1992a), lignification is retarded (Sauter and Kende, 1992b), the reorientation of cellulose microfibrils is delayed (Sauter and Kende, 1993), and the internodes exhibit tissue tension (Kutschera and Kende, 1988). Tissue tension has been described by Sachs (1865) as follows: "the

thick outer epidermal wall maintains the thin inner walls in a state of compression and is the expansion-limiting structure of the whole organ". It is displayed as a rapid, elastic outward bending upon transfer of the stem sections into water. Kutschera and Kende (1988) suggested that an increase in the plastic extensibility of the cell wall takes place at the growth-limiting outer epidermal wall and examined the ultrastructure of the epidermis. They observed the appearance of osmiophilic particles between the plasmamembrane and the outer epidermal wall of submerged deepwater rice internodes (Kutschera and Kende, 1989); this was subsequently also seen in ethylene- and GA-treated internodes (Hoffmann-Benning and Kende, 1992b). These particles are 80-120 nm in diameter in rice and have been described previously in rapidly growing corn coleoptiles (Kutschera *et al.*, 1987). Similar but smaller and less distinct particles have been observed by Robards (1969) and Olesen (1980). In these cases, they were thought to be linked to cell wall growth. However, Barckhausen and Rosenstock (1973) saw similar particles in wounded potato tubers, where they appeared to be associated with suberization. Since the osmiophilic particles appear only on the outer epidermal wall, they could either be linked to cell wall or cuticle biosynthesis.

Thus, the following questions were addressed in chapter 4 of this thesis:

- Where are the osmiophilic particles localized and how are they related to submergence-induced growth?
- Are they transported through the secretory pathway?
- What is their composition (polysaccharides, fatty acids, proteins)?

WHAT IS THE CUTICLE AND HOW DOES GROWTH AFFECT ITS BIOSYNTHESIS AND COMPOSITION ?

How does GA affect internodal growth? Usually the epidermis is thought of as the growth-limiting tissue with the outer epidermal wall playing a major role in restraining growth (i.e. Kutschera and Kende, 1988). In promoting growth, GA should, therefore, have an effect on the outer epidermis and, particularly, on the outer epidermal wall. However, the cuticle on the outer epidermal wall could also contribute to tissue tension and play a role in limiting growth, and GA could influence cuticle biosynthesis and/or composition. Enhancement of cuticle biosynthesis at the level of its monomers has already been observed in GA-treated peas by Bowen and Walton (1988).

The plant cuticle is a lipid layer on the outside of the outer wall of epidermal cells. Its structural component is cutin, a wax composed of a family of C_{16} and C_{18} fatty acid monomers. These monomers often contain one or more hydroxy- or epoxy-groups. The major C_{16} component of cutin is dihydroxypalmitic acid; the major C_{18} components are 18-hydroxyoctadecanoic acid, 9,10-epoxy-hydroxyoctadecanoic acid, 9,10,18-trihydroxy-octadecanoic acid, and their Δ^{12} unsaturated counterparts (Kolattukudy, 1980). It appears that about half the monomers are cross-linked through hydroxy groups (Deas and Holloway, 1977; Kolattukudy, 1977; Kolattukudy, 1984). Small amounts of phenolic acids and possibly other types of ligands are covalently attached to the hydroxy groups of the polymer (Riley and Kolattukudy, 1975).

The existence of a cuticle is not restricted to higher plants. Frey-Wyssling and

Mühlethaler (1965) proposed that the cuticle of terrestrial plants replaces the mucilage layer of aquatic plants. It can be found in angiosperms, gymnosperms, psilophytes, liverworths, bryophytes and some pteridophyta, with ferns and lycopods seeming to have the chemically simplest cutin (for review see Holloway, 1980). Even though the cuticle of higher plants has been presumed to be an evolutionary adaptation to a nonaquatic environment, cutin-like surface compounds have been found in some bacteria and fungi (Caldicott and Eglinton, 1973), and in some higher aquatic plants such as *Zoestra marina* (Kolattukudy, 1978).

How is the cuticle synthesized? According to an early hypothesis, "procutin" is secreted through the outer wall of the differentiating epidermal cell as semisolid material (i.e. unsaturated fatty acids) where it gradually polymerizes (Frey-Wyssling and Mühlethaler, 1965). A more refined picture is shown in Figure 1.2 (from Holloway, 1980): Synthesis of monomeric fatty acid derivatives takes place in the cytoplasm. These are then transported through the cell wall to the outermost surface of the cell where polymerization takes place. Enzymes for monomer synthesis have been characterized by Kolattukudy and coworkers (C16: hydroxylase, Soliday and Kolattukudy, 1977, 1978; mixed-function oxidase, Walton and Kolattukudy, 1972; Soliday and Kolattukudy, 1978; C18: mixed-function oxidase, Croteau and Kolattukudy, 1975a; epoxide hydratase, Croteau and Kolattukudy, 1975b). In addition, Croteau and Kolattukudy (1974) found that a 1000 *g* fraction from *Vicia faba* leaves incorporated labeled hydroxy acids when ATP and CoA were present. This hydroxyacyl transferase enzyme seems to be responsible for the polymerization of cutin from monomers,which is thought to occur outside the epidermal cells. A role

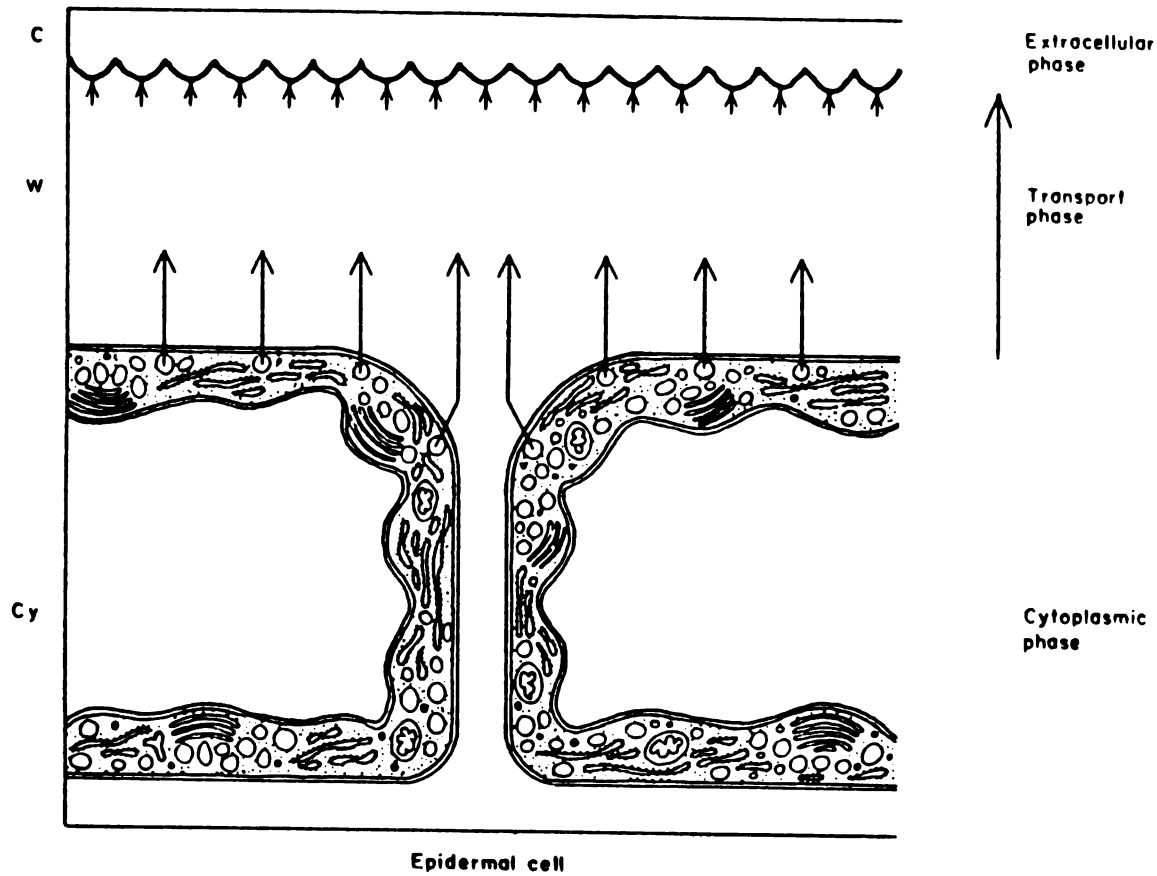


Figure 1.2. Proposed path of cutin biosynthesis (from Holloway, 1980). Monomer synthesis occurs in the cytoplasm (Cy). These predominantly fatty acid derivatives are then transported through the cell wall (W) and polymerized either during transport or at the cuticular matrix (C).

for peroxidases in the polymerization of phenolic compounds to cutin and suberin has also been proposed (Ferrer *et al.*, 1991; Roberts and Kolattukudy, 1989). Polymerization of cutin could take place on the inner face of the outer epidermal wall (Roberts *et al.*, 1959) or is, more likely, associated with the cuticular matrix (Kolattukudy and Espelie, 1985).

The form in which the cuticle precursors are transported through the epidermal wall is unknown. Some investigators have seen osmiophilic droplets migrate through the outer epidermal wall but provided no convincing evidence that these are composed of cutin precursors (C_{16} and C_{18} fatty acids and their hydroxy- and epoxy derivatives) rather than of cell wall components (Bollinger, 1959; Heide-Jorgensen, 1978, 1991). Paul and McWorther (1990) observed osmiophilic particles associated with wax filament formation in *Sorghum halepense* L. Thus, the osmiophilic particles in rice may, indeed, be involved in cuticle biosynthesis, especially since formation of the cuticle appears to be associated with active growth in other plants (Hull *et al.*, 1978; Martin and Juniper, 1970).

What is the role of the plant cuticle? It has been shown to constitute a major diffusion barrier for water and gas exchange (Schönherr, 1976) and is also thought to play a role as barrier between plants and microbes (van den Ende and Linskens, 1974). Kolattukudy (1965, 1970) found that cutin biosynthesis occurs most rapidly in expanding *Vicia faba* and *Brassica oleracea* leaves. The relative composition of the cuticle appears to change under various environmental conditions and at different physiological ages of the plant (Skoss, 1955; Kolattukudy *et al.*, 1974).

Chapter 5 is an account of investigations on cuticle biosynthesis in GA-treated,

rapidly growing deepwater rice internodes and the potential role of the cuticle as a growth-limiting structure.

REFERENCES

Barckhausen, R., Rosenstock, G. (1973) Feinstrukturelle Beobachtungen zur traumatogenen Suberinisierung beim Knollenparenchym von *Solanum tuberosum* L. *Z. Pflanzenphysiol.* **69**, 193-203.

Bleecker, A.B., Schuette, J.L., Kende, H. (1986) Anatomical analysis of growth and developmental patterns in the internode of deepwater rice. *Planta* **169**, 490-497.

Bolliger, R. (1959) Entwicklung und Struktur der Epidermisaussenwand bei einigen Angiospermenblättern. *J. Ultrastruct. Res.* **3**, 105-130.

Bowen, D.J., Walton, T.J. (1988) Cutin composition and biosynthesis during gibberellic acid-induced stem extension of *Pisum sativum* var. Meteor. *Plant Sci.* **55**, 115-127.

Caldicott, A.B., Eglinton, G. (1973) Surface waxes. In: Miller, L.P., ed. *Phytochemistry*, Vol. III, Van Nostrand Reinhold Company, New York, pp. 162-194.

Cohen, E., Kende, H. (1986) The effect of submergence, ethylene and gibberellin on polyamines and their biosynthetic enzymes in deepwater rice internodes. *Planta* **169**, 498-504.

Cohen, E., Kende, H. (1987) *In vitro* 1-aminocyclopropane-1-carboxylate synthase activity in internodes of deepwater rice. *Plant Physiol.* **84**, 282-286.

Croteau, R., Kolattukudy, P.E. (1974) Biosynthesis of hydroxyfatty acid polymers. Enzymatic synthesis of cutin from monomer acids by a cell-free preparation from the epidermis of *Vicia faba* leaves. *Biochemistry* **13**, 3193-3202.

Croteau, R., Kolattukudy, P.E. (1975a) Biosynthesis of hydroxy fatty acid polymers. Enzymatic epoxidation of 18-hydroxyoleic acid to 18-hydroxy-*cis*-9,10-epoxystearic acid by a particulate preparation from spinach (*Spinacia oleracea*). *Arch. Biochem. Biophysis.* **170**, 61-72.

Croteau, R., Kolattukudy, P.E. (1975b) Biosynthesis of hydroxy fatty acid polymers. Enzymatic hydration of 18-hydroxy-*cis*-9,10-epoxystearic acid to *threo*-9,10,18-trihydroxystearic acid by a particulate prepartion from apple (*Malus pumila*). *Arch. Biochem. Biophys.* **170**, 73-81.

Deas, A.H.B., Holloway, P.J. (1977) The intermediate structure of some plant cutins. In: Tevini, M., Lichtenthaler, H.K., eds. *Lipids and lipid polymers in higher plants*. Springer Verlag Berlin pp. 293-30

Ferrer, M.A., Muñoz, R., Ros Barcelló, A. (1991) A biochemical and cytological study on cuticle-associated peroxidases in *Lupinus*. *Ann. Bot.* **67**, 561-568.

Fry-Wyssling, A., Mühlethaler, K. (1965) Incrustation and Adcrustation: Cutinisation. In: *Ultrastructural Plant Cytology*. Elsevier Publishing Company, Amsterdam-London-New York. pp. 313-316.

Heide-Jorgensen, H.S. (1978) Xeromorphic leaves of *Hakea suaveolens* R.BR. II. Structure of epidermal cells, cuticle development and ectodesmata. *Bot. Tidsskrift* **72**, 227-244.

Heide-Jorgensen, H.S. (1991) Cuticle development and ultrastructure: evidence for a procuticle of high osmium affinity. *Planta* **183**, 511-519.

Hoffmann-Benning, S., Kende, H. (1992a) On the role of abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol.* **99**, 1156-1161.

Hoffmann-Benning, S., Kende, H. (1992b) Characterization of osmiophilic particles associated with rapid growth in corn and rice. *Plant Physiol.* **99**, Abstract # 177.

Holloway, P.J. (1980) The chemical constitution of plant cutins. In: Cutler, D.F., Alvin, K.L., Price, C.E., eds. *The Plant Cuticle*. Academic Press pp. 45-85.

Hull, H.M., Morton, H.L., Wharrie, J.R. (1975) Environmentel influences on cuticle development and resultant foliar penetration. *Bot. Rev.* **41**, 421-452.

Jacobson, J.V., Chandler, P.M. (1988) Gibberellin and abscisic acid in germinating cereals. In: Davies, P.J., ed. *Plant Hormones and their Role in Plant Growth and Development*. Kluwer Academic Publishers, Dordrecht-Boston-London, pp 164-193.

Keith, K.A., Raskin, I., Kende, H. (1986) A comparison of the submergence response of deepwater and non-deepwater rice. *Plant Physiol.* **80**, 479-482.

Kende, H. (1987) Studies on internodal growth using deepwater rice. In: Cosgrove, D.J., Knievel, D.P., eds. *Physiology of Cell Expansion during Plant Growth.* The American Society of Plant Physiologists. pp 227-238.

Kolattukudy, P.E. (1965) Biosynthesis of wax in *Brassica oleracea. Biochem.* **4,** 1844.

Kolattukudy, P.E. (1970) Cutin biosynthesis in *Vicia faba* leaves. *Plant Physiol.* **46,** 759-760.

Kolattukudy, P.E. (1977) Lipid polymers and associated phenols, their chemistry, biosynthesis, and the role in pathogenesis. In: F.A. Loewus, V.C. Runeckles, eds. *The Structure, Biosynthesis, and Degradation of Wood.* Plenum Publishing Corp., New York, pp. 185-246.

Kolattukudy, P.E. (1978) Chemistry and biochemistry of aliphatic components of suberin. In: Kahl, G. ed. *Biochemistry of Wounded Plant Tissues.* De Gruyter Verlag, Berlin, pp 680.

Kolattukudy, P.E. (1980) Cutin, suberin, and waxes. In: P.K. Stumpf, ed. *The Biochemistry of Plants. Vol.4 Lipids: Structure and Function.* Academic Press, New York, pp. 571-645.

Kolattukudy, P.E. (1981) Structure, Biosynthesis, and Biodegradation of Cutin and Suberin. In: *Ann. Rev. Plant Physiol.* **32,** 536-567.

Kolattukudy, P.E. (1984) Biochemistry and function of cutin and suberin. *Can. J. Bot.* **62,** 2918-2933.

Kolattukudy, P.E., Croteau, R., Brown, L. (1974):Structure and biosynthesis of cuticular lipids. *Plant Physiol.* **54,** 670-677.

Kolattukudy, P.E., Espelie, K.E., Soliday, C.L. (1981): Hydrophobic layers attached to cell walls, cutin, suberin, and associated waxes. *Encycl. Plant Physiol. New Ser.* **13B,** 225-254.

Kolattukudy, P.E., Espelie, K.E. (1985) Biosynthesis of cutin, suberin, and associated waxes. In: *Biosynthesis and Biodegradation of Wood Components.* Academic Press, pp.161-207.

Kutschera, U., Berfeld, R., Schopfer, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. *Planta* **170,** 168-180.

Kutschera, U., Kende, H. (1988) The biophysical basis of elongation growth in internodes of deepwater rice. *Plant Physiol.* **88,** 361-366.

Kutschera, U., Kende, H. (1989) Particles associated with the outer epidermal wall in internodes of deepwater rice. *Ann.Bot.* **63,** 385-388.

Martin, J.T., Juniper, B.E. (1970) In: *The Cuticles of Plants.* St. Martins, New York.

Métraux, J.-P., Kende, H. (1983) The role of ethylene in the growth response of submerged deep water rice. *Plant Physiol.* **72,** 441-446.

Métraux, J.-P., Kende, H. (1984) The cellular basis of the elongation response in submerged deep-water rice. *Planta* **160,** 73-77.

Musgrave, A., Jackson, M.B., Ling, E. (1972) *Callitriche* stem elongation is controlled by ethylene and gibberellin. *Nature New Biol.* **236,** 93-94.

Olesen, P. (1980) The visualization of wall-associated granules in thin sections of higher plant cells: occurence, distribution, morphology and possible role in cell wall biogenesis. *Z. Pflanzenphysiol.* **96,** 35-48.

Paul, R.N., McWorther, C.G.(1990) Correlation of ultrastructure with the mechanism of wax filament production in corl cells in johnsongrass (*Sorghum halepense* L. Pers.). In: *Proceedings of the XIIth International Congress for Electron Microscopy.* San Francisco Press, San Francisco, pp 676-677.

Raskin, I., Kende, H. (1984a) Regulation of growth in stem sections of deepwater rice. *Planta* **160,** 66-72.

Raskin, I., Kende, H. (1984b) Effect of submergence on translocation, starch content and amylolytic activity in deepwater rice. *Planta* **162,** 556-559.

Raskin, I., Kende, H. (1984c) Role of gibberellin in the growth response of submerged deep water rice. *Plant Physiol.* **76,** 947-950.

Raskin, I., Kende, H. (1985) Mechanism of aeration in rice. *Science* **228,** 327-329.

Riley, R.G., Kolattukudy, P.E. (1975): Evidence for covalently attached *p*-coumaric and ferulic acid in cutins and suberins. *Plant Physiol.* **56,** 650-654.

Robards, A.W. (1969) Particles associated with developing cell walls. *Planta* **88,** 376-379.

Roberts, E., Kolattukudy, P.E. (1989) Molecular cloning, nucleotide sequence, and abscisic acid induction of a suberinization-associated highly anionic peroxidase. *Mol. Gen. Genet.* **217,** 223-232.

Roberts, M.F., Batt, R.F., Martin, J.T. (1959) Studies on plant cuticle II. The cutin component of the cuticles of leaves. *Ann. Appl. Biol.* **47**, 573-582.

Rose-John, S., Kende, H. (1984) Effect of submergence on the cell wall composition of deepwater rice internodes. *Plant Physiol.* **76**, 106-111.

Rose-John, S., Kende, H. (1985) Short-term growth response of deep-water rice to submergence and ethylene. *Plant Sci.* **38**, 129-134.

Rumpho, M.E., Kennedy, R.A. (1981) Anaerobic metabolism in germinating seeds of *Echinochloa cruz-galli* (Barnyard grass). *Plant Physiol.* **68**, 165-168.

Sachs, J. (1965) *Handbuch der Experimentalphysiologie der Pflanzen.* Engelmann Verlag, Leipzig.

Salisbury, F.B., Ross, C.W. (1992) *Plant Physiology*, Wadsworth Publishing Company, Belmont.

Sauter, M., Kende, H. (1992a) Gibberellin-induced growth and regulation of the cell division cycle in deepwater rice. *Planta* **188**, 362-368.

Sauter, M., Kende, H. (1992b) Level of ß-glucan and lignin in elongating internodes of deepwater rice. *Plant Cell Physiol.* **33**, 1089-1097.

Sauter, M., Kende, H. (1993) Internodal elongation and orientation of cellulose microfibrils and microtubules in deepwater rice. *Planta* submitted.

Schönherr, J. (1976) Water permeability of isolated cuticular membranes: the effect of cuticular waxes on diffusion of water. *Planta* **131**, 159-164.

Skoss, J.D. (1955) Structure and composition of plant cuticle in relation to environmental factors and permeability. *Bot. Gazette* pp 55.

Smith, M.A., Jacobsen, J.V., Kende, H. (1987) Amylase activity and growth in internodes of deepwater rice. *Planta* **172**, 114-120.

Smulders, M.J.M., Horton, R.F. (1991) Ethylene promotes elongation growth and auxin promotes radial growth in *Ranunculus sceleratus* petioles. *Plant Physiol.* **96**, 806-811.

Soliday, C.L., Kolattukudy, P.E. (1977) Biosynthesis of cutin. -Hydroxylation of fatty acids by a microsomal preparation from germinating *Vicia faba. Plant Physiol.* **59**, 1116-1121.

Soliday, C.L., Kolattukudy, P.E. (1978) Midchain hydroxylation of 16-hydroxy-palmitic acid by the endoplasmic reticulum fraction from germinating *Vicia faba*. *Arch. Biochem. Biophys.* **188**, 338-347.

Suge, H. (1985) Ethylene and gibberellin: regulation of internodal elongation and nodal root development in floating rice. *Plant Cell Physiol.* **26**, 607-614.

Suge, H., Türkan, I. (1990) Endogenous gibberellin relationships in internode elongation in floating rice: a genetic study. *Japan. J. Genet.* **65**, 183-191.

Türkan, I., Suge, H. (1991) Localization of high concentration of gibberellins in elongating internodes of floating rice. *Japan. J. Breed.* **41**, 553-559.

Van den Ende, G., Linskens, H.F. (1974): Cutinolytic enzymes in relation to pathogenesis. *Annu. Rev. Phytopathol.* **12**, 247-258.

Vergara, B.S., Jackson, B., DeDatta, S.K. (1976) Deep-water rice and its response to deep-water stress. In: *Climate and Rice.* International Rice Research Institute, Los Baños, Philippines, pp. 310-319.

Voesenek, L.A.C.J., Blom, C.W.P.M. (1989) Growth responses of *Rumex* species in relation to submergence and ethylene. *Plant Cell Environ.* **12**, 433-439.

Voesenek, L.A.C.J., Perik, P.J.M., Blom, C.W.P.M., Sassen, M.M.A. (1990a) Petiole elongation in *Rumex* species during submergence and ethylene exposure: The relative contributions of cell division and cell expansion. *J.Plant Growth Regul.* **9**, 13-17.

Voesenek, L.A.C.J., Harren, F.J.M., Bögemann, G.M., Blom, C.W.P.M., Reuss, J. (1990b) Ethylene production and petiole growth in *Rumex* plants induced by soil waterlogging. *Plant Physiol.* **94**, 1071-1077.

Walton, T.J., Kolattukudy, P.E. (1972): Enzymatic conversion of 16-hydroxypalmitic acid into 10,16-dihydroxypalmitic acid in *Vicia faba* epidermal extracts. *Biochem. Biophys. Res. Commun.* **46**, 16-21.

Yamaguchi, T. (1973) Studies on the floating rice. VIII. Effects of raising water level on the amounts of endogenous gibberellin-like substances. 147-153.

Zeevaart, J.A.D. (1983) Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. *Plant Physiol.* **71**, 477-481.

CHAPTER 2

THE ROLE OF ABA IN THE GROWTH OF RICE COLEOPTILES

ABSTRACT

In contrast to other cereals, rice seedlings and adult plants of some rice varieties can survive flooding. In seedlings, submergence promotes coleoptile growth, a response that can also be induced by a gas mixture containing 3% O_2, 15% CO_2, and 1 μl/l ethylene in N_2. I investigated whether abscisic acid (ABA) is involved in regulating the growth of rice coleoptiles. Rice seedlings were grown on solutions containing fluridone, an inhibitor of carotenoid and, indirectly, of ABA biosynthesis. Treatment with fluridone reduced the level of ABA in coleoptiles and first leaves by more than 75% and promoted coleoptile growth by more than 60%. Little or no enhancement of coleoptile growth by fluridone was observed in barley, oat, or wheat. Applied ABA counteracts fluridone-induced growth. Seedlings grown in the above gas mixture or in air containing 3 to 5 μl/l ethylene as well as submerged or fluridone-treated seedlings show not only increased coleoptile growth but also a reduction in the level of endogenous ABA. This indicates that ABA may be involved in regulating rice coleoptile growth. The involvement of ABA in determining the growth rate of rice coleoptiles may be related to the semiaquatic growth habit of this plant.

INTRODUCTION

Rice (*Oryza sativa,* L.) has a number of physiological and metabolical adaptations that enhance its chances for survival under conditions of temporary flooding. One of these is the capacity of plants to elongate rapidly when they become submerged. This feature helps rice plants to emerge from the water and to avoid drowning. In adult deepwater rice plants, submergence stimulates elongation of the internode (Métreaux and Kende, 1983; Vergara *et al.*, 1976). In seedlings, it promotes coleoptile growth and reduces root growth (Turner *et al.*, 1981; Wada, 1961; Yamada, 1954). These morphological changes in response to submergence can also be seen with seedlings grown under low partial pressures of O_2 (Kordan, 1977; Ohwaki, 1967; Ranson and Parija, 1955) or in air containing low concentrations of ethylene (Ku *et al.*, 1970; Miller and Miller, 1974; Suge, 1971). Atwell *et al.* (1982) have suggested that accumulation of ethylene is responsible for the stimulation of rice coleoptile elongation in stagnant water and that CO_2, an antagonist of ethylene, inhibits coleoptile growth. However, Ishizawa and Esashi (1984a) proposed a stimulatory role of CO_2 and ethylene in rice coleoptile growth. Raskin and Kende (1983) showed that coleoptile growth is promoted independently and to the same extent by low levels of O_2 (3%), high levels of CO_2 (15%), and by 1 μl/l ethylene. This regulatory mechanism allows rice to grow out of shallow water where O_2 is limiting and where CO_2 and ethylene accumulate in the submerged organs of the plant.

How do these conditions alter growth of rice seedlings? One possibility is that

submergence leads to an increase in the endogenous level of plant growth promoters. Both auxin (indole-3-acetic acid, IAA) and gibberellin (GA) have been examined for their role in inducing rice coleoptile growth. However, no clear picture concerning the role of GA and IAA in submergence-induced growth has emerged so far. Imaseki and Pjon (1970) found that ethylene enhances auxin-induced growth in rice coleoptile segments. Similarly, Ishizawa and Esashi (1983, 1984) suggested that auxin is at least necessary to maintain long-term growth of rice coleoptile segments and that ethylene enhances the IAA-induced growth response. However, Katsura and Suge (1979) concluded that ethylene may act independently from auxin.

Kefford (1962) found that *p*-chlorophenoxy-isobutyric acid (PCIB), a presumed antiauxin, inhibited rice coleoptile growth in seedlings grown under various light conditions, while IAA and GA promoted it. Inhibition by PCIB could be overcome by IAA. In addition, PCIB reduced GA-induced growth. Kefford concluded that there is an interaction between IAA and GA in the control of rice coleoptile growth and that the depth of submergence might influence the endogenous auxin concentration. This suggestion is consistent with a later observation of Mapelli (1986) who detected an increase in auxin levels in rice seedlings under anaerobiosis. But Hoson *et al.* (1992) detected lower IAA levels in submerged rice coleoptiles than in air-grown ones. However, Gopalakrishnan and Sircar (1974) found no effect of GA on the growth of rice shoots, and Suge (1974) observed only a very small effect of applied GA on coleoptiles in dwarf rice but a strong growth promotion of first leaf sheaths. Murakami (1962) also observed a small induction of coleoptile growth in rice seedlings cultured on GA for six days. Horton (1991) reported that application of IAA

and especially of GA in air promoted coleoptile growth of rice seedlings which had been irradiated with a short pulse of light. The results described above are contradictory and do not establish the role of plant hormones in regulating elongation of rice coleoptiles. Even so, it appears that applied IAA and GA may stimulate rice coleoptile growth under certain conditions. However, their role with respect to submergence-induced growth is not yet clear. Up to now, the possibility that reduction in the level of a growth inhibitor during submergence could lead to increased rice coleoptile growth has not been considered. In maize seedlings, the abscisic acid (ABA) content is increased and shoot elongation is reduced at low water potential (Saab *et al.*, 1990). Inhibition of ABA biosynthesis with fluridone relieved the inhibitory effect of water stress on growth. Thus, increased growth of submerged rice coleoptiles may result from a decrease in the level of ABA. Promotion of rice coleoptile growth by ethylene may also be related, at least in part, to a reduced ABA content as Zeevaart (1983) has found that treatment with ethylene reduces the level of biologically active ABA in leaves of *Xanthium strumarium.*

This chapter is a report of experiments on the role of ABA in the growth response of rice coleoptiles. ABA levels were either increased by application of the hormone or decreased by environmental factors (submergence; gas composition of the ambient atmosphere) or by treatment with fluridone, an inhibitor of carotenoid biosynthesis. The role of ABA in influencing the elongation of wheat, oat, and barley coleoptiles has also been determined for comparative purposes. Results of this work have been reported earlier (Hoffmann and Kende, 1990; Hoffmann-Benning and Kende, 1991).

MATERIALS AND METHODS

Growth of plant material. Seeds of rice (*Oryza sativa* L., cv. M-9), wheat (*Triticum vulgare*, cv. Ionia), oat (*Avena sativa* L., cv. Korwood), and barley (*Hordeum vulgare* L., cv. Lakeland) were allowed to imbibe in darkness at 29° C in a Petri dish containing 15 ml sterile distilled water. Germinated seeds were transferred to sterile 1-liter glass jars containing 150 ml of vermiculite wetted with 80 ml of sterile distilled water, varying concentrations of ABA (Sigma, St. Louis, MO), IAA (Sigma, St.Louis, MO), gibberellin A_3 (GA_3, Calbiochem-Behring, CA), 30 μM fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]-4(4H)pyridonone, a gift of Eli Lilly, Indianapolis, IN), 150 mg/l AMO 1618 (4-hydroxy-5-isopropyl-2-methylphenyltri-methylammonium chloride 1-piperidine carboxylate, from Calbiochem-Behring, CA), or 250 mg/l CCC [(2-chloroethyl) trimethylammonium chloride, from American Cyanamide, Princeton, NJ]. Fluridone was dissolved in acetone, which, upon dilution, had a final concentration of 0.5%; in these experiments, the water used for control treatment and treatment with the other chemicals also contained 0.5% acetone. The jars, which had gas inlet and outlet ports, were sealed, and the seedlings were grown in the dark at 29° C under a continuous flow (60 ml/min) of humidified air with or without 3 to 5 μl/l ethylene or in a gas mix containing 3.5% O_2, 19.3% CO_2, and 5-6 μl/l ethylene in N_2. To prepare ethylene-free air, the air was passed through a 25-cm-long column (7 cm I.D.) packed with purafil (Purafil Inc., Atlanta, GA). Ethylene was determined by gas chromatography (GC) of 1-ml samples (Kende and Hanson, 1976). For submergence,

the seedlings were grown in the same jars containing 600 ml sterile distilled water. Four days after start of imbibition, growth of the seedlings was measured, and the coleoptiles or coleoptiles plus first leaves were harvested and frozen in liquid N_2 for further analysis.

ABA extraction and determination. ABA from coleoptiles was extracted according to Walker-Simmons (1987), and either phase partitioned with ethyl acetate for ELISA (enzyme-linked immunosorbant assay) or purified by HPLC (high performance liquid chromatography) and measured by GC (Zeevaart *et al.*, 1989). The ELISA was performed as described by Walker-Simmons (1987) with the monoclonal antibody of Mertens *et al.* (1983), which was purchased from Idetek (San Bruno, CA). The ABA-4'-conjugate was prepared according to Weiler (1980). Belefont and Fong (1989) observed that organic acids may interfere with the ELISA. I verified that the ABA determinations made by ELISA were consistent with those made by HPLC/GC (not shown). The amounts of ABA determined by either method showed the same changes after the various treatments.

RESULTS AND DISCUSSION

Submerged rice seedlings develop a much longer coleoptile than do air-grown rice seedlings (see Fig. 2.5). Since information on the role of IAA and GA on rice coleoptile growth is contradictory, I re-examined this question by determining the effects of applied IAA and GA_3 and of two inhibitors of GA biosynthesis, AMO 1618

and CCC, on the elongation of intact rice coleoptiles. Under my conditions, applied IAA had no effect on coleoptile growth (Table 2.1). This indicates that IAA levels do not limit growth of intact rice coleoptiles and that addition of IAA does not mimick the response to submergence. Treatment with 100 μM GA_3 lead to a small but significant increase in coleoptile length (17%) which, by itself, cannot explain the submergence-induced growth increase (72%, see, e.g., Table 2.3). Treatment with 10 μM GA_3 and the GA-biosynthesis inhibitors CCC or AMO 1618 did not affect coleoptile growth (Table 2.2). Therefore GA does not seem to have a regulatory function in coleoptile growth.

Because two growth promoters, IAA and GA, did not promote rice coleoptile growth, it may be the decrease in the amount of an endogenous growth inhibitor, e.g. of ABA that is responsible for the enhanced elongation of submerged rice coleoptiles.

To investigate the possible role of ABA as an inhibitor of coleoptile growth, I compared the effect of fluridone on rice, oat, barley, and wheat seedlings. There is strong evidence that ABA is synthesized via an indirect pathway from carotenoids (Gage et al., 1989; Li and Walton, 1990; Rock and Zeevaart, 1991). Because fluridone is an inhibitor of carotenoid biosynthesis (Bartels and Watson, 1978; Gamble and Mullet, 1986), its effect on growth in rice coleoptiles can be explained in terms of reduced ABA formation from carotenoids. Treatment with fluridone for four days promoted the growth of wheat, oat, and barley coleoptiles by 14%, 22%, and 14% above control, respectively (Fig. 2.1). In the same experiment, rice grown on fluridone for four days showed a 67% increase in coleoptile length. When fluridone was added during the last two days of incubation, it had no effect on the growth of

Table 2.1. Effect of IAA (100 μM) or GA_3 (100 μM) on growth of intact rice coleoptiles. The values represent the mean of the lengths of 42 (IAA/Control) and 54 (GA_3/Control) coleoptiles ± S.E. determined four days after start of imbibition.

	Coleoptile length (mm)			
Treatment	Control	IAA or GA_3	n	p
IAA	17.4 ± 0.7	16.9 ± 0.8	42	n.s.
GA_3	16.1 ± 0.6	18.8 ± 0.7	54	0.05

Table 2.2. Effect of GA_3 (10 μM) and of CCC (250 mg/l) and AMO 1618 (150 mg/l), inhibitors of GA biosynthesis, on the growth of rice coleoptiles. The values represent the mean length of 21 coleoptiles ± S.E. determined four days after start of imbibition.

	Coleoptile length (mm)					
Exp	Control	GA_3	CCC	CCC + GA_3	AMO	AMO + GA_3
I	17.0 ± 0.8	18.3 ± 1.2	15.4 ± 0.8	16.6 ± 1.3	n.d.	n.d.
II	17.9 ± 0.8	18.1 ± 1.2	n.d.	n.d.	15.8 ± 1.0	17.7 ± 1.1

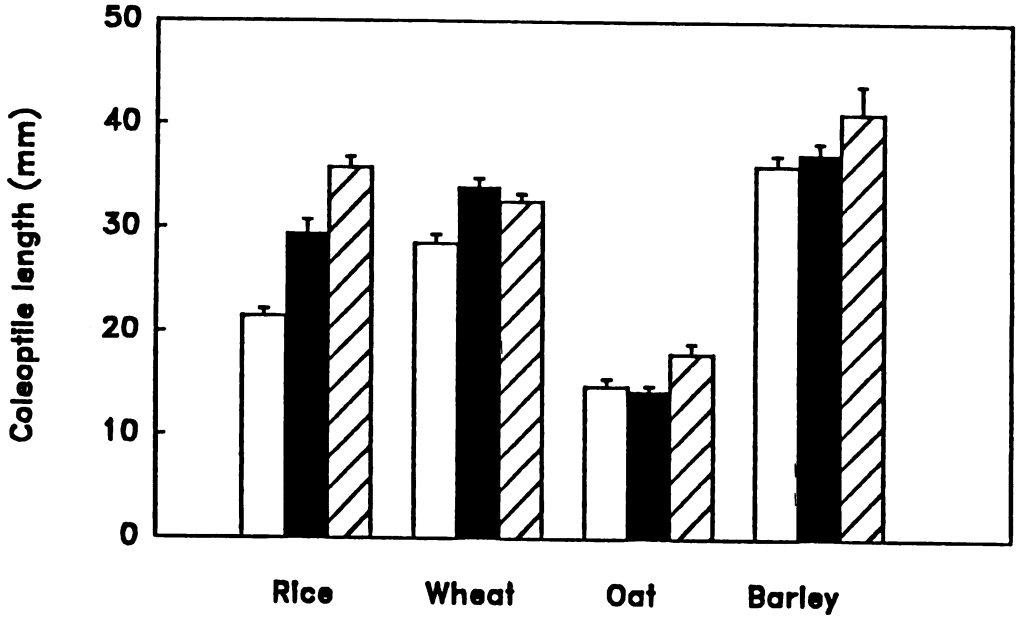


Figure 2.1: Growth of rice, wheat, oat, and barley coleoptiles in response to treatment with 30 μM fluridone. Water control (□); fluridone present during the last two days of growth (■); fluridone present during four days of growth (▨). The length of coleoptiles was measured four days after the start of imbibition. The bars represent the means ± S.E. of 20 (control and two days of treatment) and 15 (four days of treatment) coleoptiles.

oat and barley coleoptiles, and the effect on wheat was the same as that observed after four days. Over a two-day period, fluridone promoted rice coleoptile elongation by 37%. Fluridone inhibited mesocotyl as well as root growth in rice seedlings.

To investigate further the role of ABA in regulating the growth of rice coleoptiles, seedlings were grown on 1 μM ABA, 30 μM fluridone, or fluridone plus ABA (Fig. 2.2). ABA treatment reduced growth of the rice coleoptile by 40% when compared with the control, whereas fluridone promoted coleoptile elongation as found before. When ABA and fluridone were added simultaneously, fluridone-induced growth was eliminated. The response of the mesocotyl was the opposite of that of the coleoptile. Its growth was promoted by ABA as reported earlier by Takahashi (1972, 1973) and was reduced by fluridone (Fig. 2.2). Rice mesocotyls are the only known organs of non-stressed, wild type plants whose growth is stimulated by ABA. However, ABA has been shown to have a growth-promoting effect on ABA deficient mutants, e.g. of *Arabidopsis* (Koornneef *et al.*, 1982) and a growth-maintaining effect on the roots of water-stressed maize (Saab et al., 1990).

It had been shown that the level of endogenous ABA decreases during seed germination, e.g. in wheat caryopses, while the growth rate of the seedling increases (Morris and Walker-Simmons, 1989). I determined the ABA content of dry rice seeds and seedlings that were grown for up to four days on water, 30 μM fluridone or that were submerged on day two as described in Materials and Methods (Fig. 2.3). In control plants grown in air, the level of ABA dropped during the first day by 45% and continued to decline over the next three days to 20% of the original level in dry

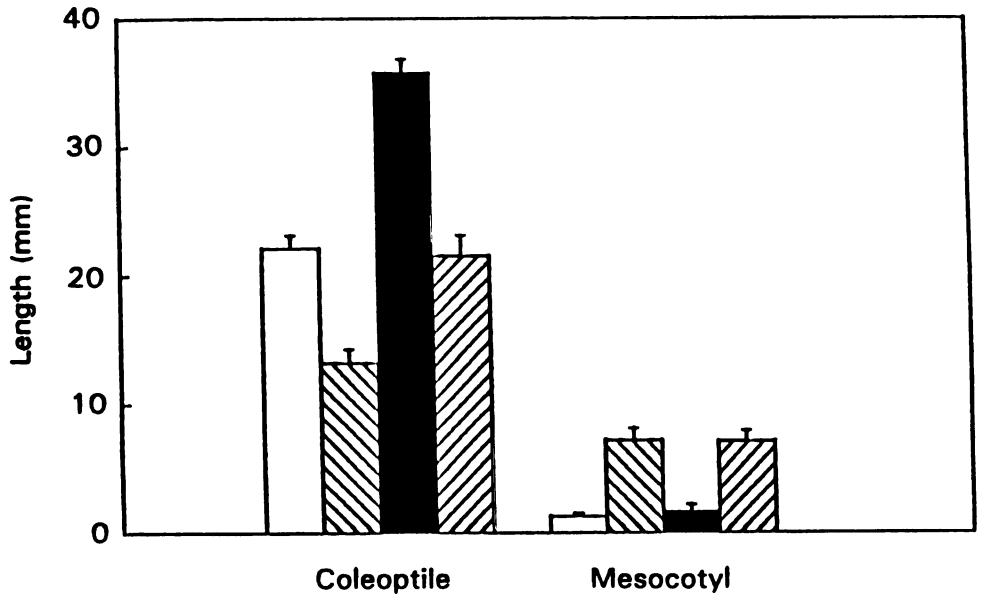


Figure 2.2. Response of rice seedlings to ABA and fluridone. Treatment was started at imbibition, and measurements were taken after four days. Water control (□); 1 μM ABA (▨); 30 μM fluridone (■); 30 μM fluridone and 1 μM ABA (▨). The bars represent the means of 15 seedlings ± S.E.

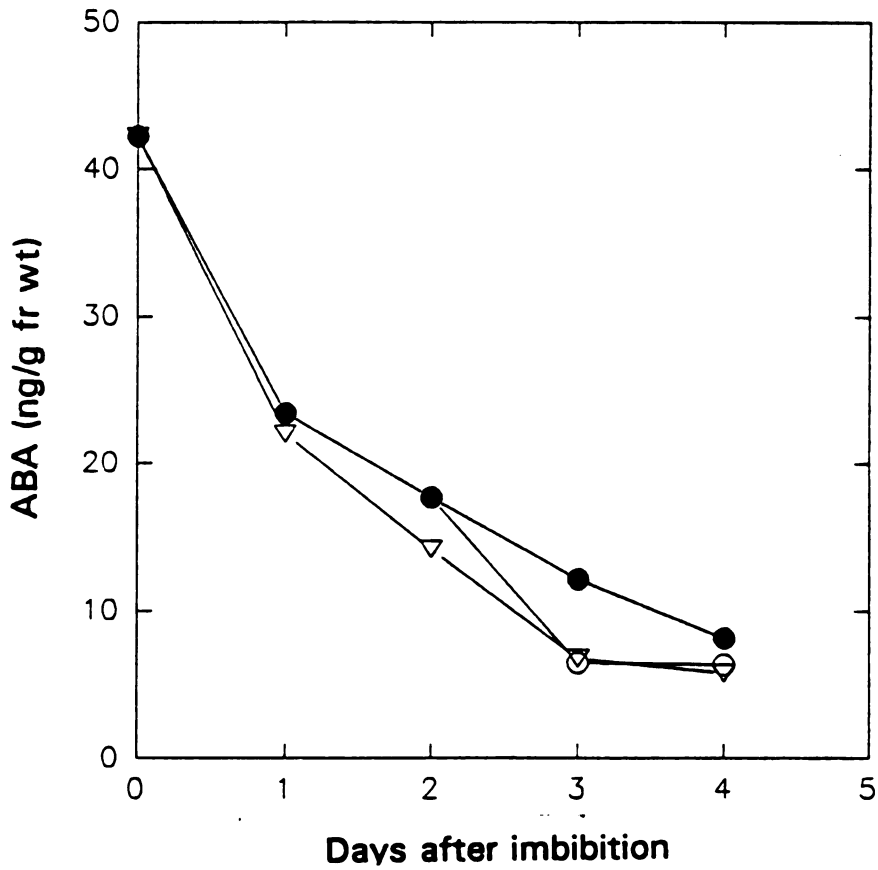


Figure 2.3. ABA levels in rice seedlings at various times after imbibition. Seedlings were grown on vermiculite moistened with water (air grown, ●) or 30 μM fluridone (▽), or they were submerged two days after start of imbibition (○). The points represent the means ± S.E. of three (fluridone, submergence) and five (air grown) experiments, respectively, with approximately 40 seedlings per experiment.

seeds. The ABA content of fluridone-treated seedlings was slightly lower than that of control plants on days one and two, and approximately 60 % that of control plants on days three and four. The concentration of ABA was not reduced to zero with fluridone, probably because of carry-over of ABA from the dry seeds. The ABA level in seedlings that were submerged two days after start of imbibition decreased within one day to that of the fluridone-treated plants. The change in the ABA concentration was reflected in increased coleoptile growth in both submerged and fluridone-treated seedlings (Fig. 2.4).

Figure 2.5 shows rice seedlings that had been submerged, treated with fluridone, or with ethylene. Both, Figure 2.5 and Table 2.3 demonstrate that treatment with fluridone leads to a growth response similar to that caused by submergence, 3-5 μl/l ethylene, or a gas mixture containing 3.5% O_2, 19% CO_2, 5-6 μl/l ethylene in N_2. All four treatments increased elongation of coleoptiles and reduced root growth, with submergence having the most pronounced effect.

Table 2.3 also shows that the levels of ABA were reduced in coleoptile plus primary leaf and coleoptile alone as a result of the above treatments. In rice seedlings grown in 30 μM fluridone, coleoptile growth was stimulated by 63%, and the ABA content was reduced in both the coleoptile and the coleoptile plus primary leaf by 66% and 77%, respectively. Even though fluridone did not elicit the largest growth response in coleoptiles, it did cause the most prominent reduction of endogenous ABA levels. A possible reason for this may be that, besides reducing ABA levels, fluridone may also have a secondary inhibitory effect on coleoptile growth. Submergence and treatment with ethylene or the a gas mixture consisting of 3.5% O_2, 19% CO_2,and 5-6

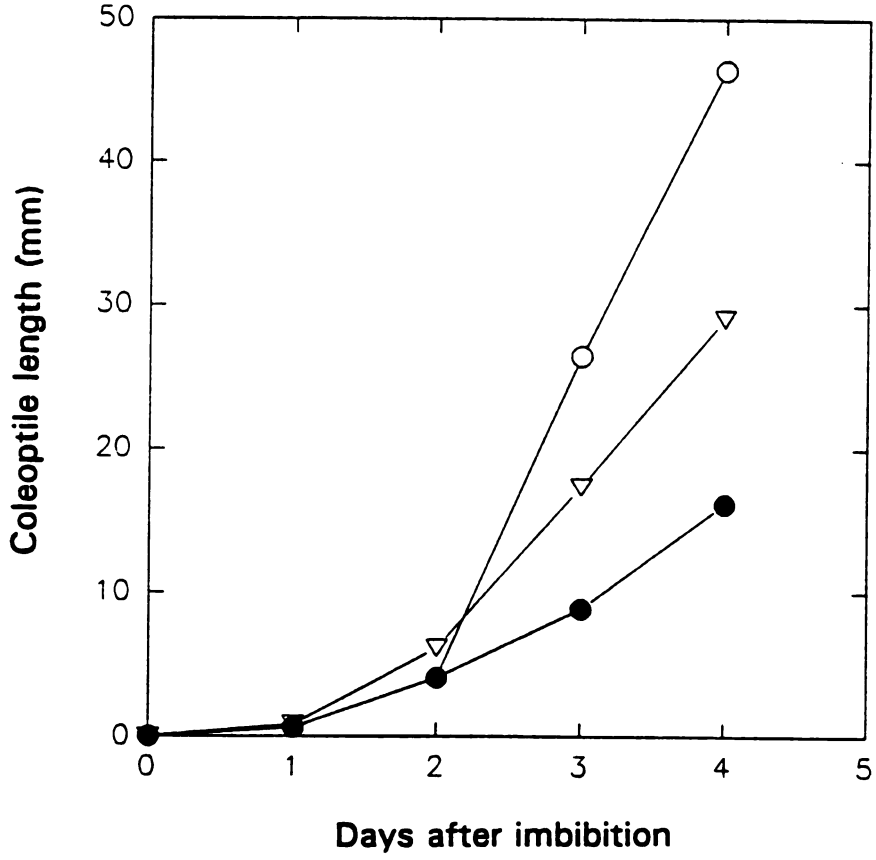


Figure 2.4. Coleoptile length of rice seedlings grown as described in Figure 2.3. The symbols correspond to the ones used in Figure 2.3.

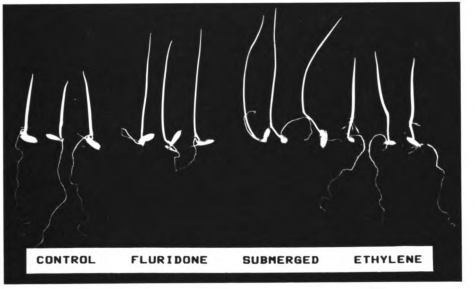


Figure 2.5. Rice seedlings grown on vermiculite moistened with water (air control), with 30 μM fluridone, submerged, or treated with 4 μl/l ethylene. The picture was taken four days after start of imbibition.

Table 2.3. Length of coleoptile, mesocotyl, and root (top) and ABA level in rice coleoptiles plus primary leaves (middle) and in rice coleoptiles alone (bottom). Seedlings were grown on water in air (C), on 30 μM fluridone in air (F), submerged in water (SM), in air containing 3-5 μl/l ethylene, or in a gas mixture containing 3.5% O_2, 19% CO_2, and 5-6 μl/l ethylene in N_2 (Mix). The comparison of control and gas mixture in the two columns on the right was done in a separate experiment. The values for length represent the mean ± S.E. of 45 coleoptiles. The values for the ABA levels represent the mean ± S.E. of three experiments (each with 50-100 seedlings) plus % control. (*) one determination.

	Treatment					
	C	F	SM	Ethylene	C	Mix
Length (mm)						
Coleoptile	16.3 ± 0.4	26.7 ± 0.6	44.3 ± 1.2	26.8 ± 0.6	13.0 ± 0.5	26.1 ± 0.8
Mesocotyl	3.2 ± 0.4	2.6 ± 0.2	7.2 ± 0.4	5.1 ± 0.5	2.3 ± 0.2	8.3 ± 0.4
Roots	50.7 ± 3.7	30.4 ± 1.3	8.7 ± 0.7	51.5 ± 1.8	39.4 ± 1.8	19.5 ± 0.9
ABA in coleoptile						
+ prim. leaf	9.2	2.1	2.9	4.8	9.4	5.1
(ng/g fr wt)	± 0.9	± 0.2	± 0.6	(*)	± 1.6	± 0.5
% of control	100.0	22.8	31.5	52.2	100.0	54.3
ABA in						
coleoptiles	7.0	2.4	4.3	5.5	6.7	4.2
(ng/g fr wt)	± 0.7	± 1.2	± 1.8	± 0.7	± 0.2	± 0.4
% of control	100.0	34.3	61.4	78.6	100.0	62.7

μl/l ethylene in N_2 led to a reduction in ABA content and to a promotion of coleoptile growth.

Ethylene alone caused only a 21% reduction of ABA content in coleoptiles which was far less than the reduction with fluridone or submergence. This can be explained by the fact that the full submergence response in rice coleoptiles requires an atmosphere of low O_2, high CO_2 and ethylene, with low O_2, high CO_2 and ethylene each contributing one third of the response (Raskin and Kende, 1983).In an atmosphere of 3.5% O_2, 19% CO_2, and 5-6 μl/l ethylene, the ABA content of rice coleoptiles was reduced by 37% compared to the control. This is comparable to the 38% reduction in submerged coleoptiles.

In rice seedlings, submergence promotes growth, in part, through the action of ethylene. It is important to know whether fluridone induces growth of rice seedlings by promoting ethylene biosynthesis. Fluridone leads to an approximately 2.5-fold increase in ethylene biosynthesis, namely from 16.9 pmol ethylene $(g\ FW)^{-1}\ h^{-1}$ in contol plants to 42.5 pmol ethylene $(g\ FW)^{-1}\ h^{-1}$ in fluridone-treated plants. To test whether fluridone acts via stimulating ethylene biosynthesis, I measured the length of rice coleoptiles of seedlings grown for four days on water in air, on water in 10 μl/l ethylene, on 30 μM fluridone in air, and on fluridone in ethylene. Table 2.4 shows the combined result of two independent experiments. Both, treatment with ethylene and fluridone led to increased coleoptile growth (36% and 78%, respectively). The increase in the presence of ethylene and fluridone was 116%. Therefore, the effects of ethylene and fluridone on growth are additive, and fluridone does not act by increasing ethylene synthesis. In addition, the effect of fluridone on

Table 2.4. Ethylene (3-5 μl/l) and fluridone (30 μM) act independently on rice coleoptile growth. The values represent the means of ten measurements (2 exp.) $\pm$ S.E.

	Control	Fluridone	Ethylene	Ethylene + Fluridone
Coleoptile length (mm)	18.0 $\pm$ 1.0	32.1 $\pm$ 1.6	24.4 $\pm$ 1.2	38.8 $\pm$ 1.9
Growth above control (mm)		14.1	6.4	20.8
Growth above control (%)		78	36	116

growth was comparable in closed systems, where ethylene accumulated (as above), and in open systems, where ethylene-free air was continuously circulated through the incubation flask, thereby removing ethylene produced by the seedlings. It is possible that ethylene acts on ABA metabolism, while fluridone acts on ABA biosynthesis. Indeed, Zeevaart (1981) has shown that in *Xanthium* ethylene increases ABA metabolism to phaseic acid and ABA-glucose ester, both of which are biologically inactive.

CONCLUSIONS

Fluridone, an inhibitor of carotenoid biosynthesis, induces coleoptile growth in rice as does submergence (Fig 2.1, Table 2.3). The effect of fluridone can be reversed by ABA (Fig. 2.2). This indicates that ABA may be involved in the regulation of rice coleoptile growth and is additional evidence that ABA is synthesized from carotenoids. Figure 2.3 shows that the level of endogenous ABA drops during germination of rice. This coincides with an increase in coleoptile length (Fig. 2.4). Submergence and fluridone hasten this decline in ABA content. Our data indicate that submergence- and possibly ethylene-induced coleoptile growth is based on a reduction in ABA levels, and that ABA plays a role in regulating the growth of rice coleoptiles. Promotion of shoot growth by fluridone was also described by Saab et al. (1990) for water-stressed corn seedlings. In that case, fluridone appears to prevent accumulation of ABA at low water potentials.

I could not show an involvement of GA in the regulation of rice coleoptile

growth either by applying GA_3 or by examining the effect of gibberellin biosynthesis inhibitors (Table 2.1, 2.2). I also could not find an effect of IAA on the growth of intact seedlings, which indicates that endogenous auxin levels do not limit growth of intact coleoptiles.

Therefore, submergence may only in part stimulate growth by increasing the level of a growth promoter, e.g. auxin, but mostly by reducing the level of a growth inhibitor, e.g. ABA. The rate of growth in rice coleoptiles may be determined by the ratio of the promoter (IAA) and the inhibitor, (ABA).

The submergence response of rice seedlings may thus be regulated in the following way:

$$\text{submergence} \longrightarrow \begin{matrix} \text{reduced } O_2 \\ \text{increased } C_2H_4 \\ \text{increased } CO_2 \end{matrix} \longrightarrow \text{reduced ABA} \longrightarrow \text{coleoptile growth}$$

REFERENCES

Atwell, B.J., Waters, I., Greenway, H. (1982) The effect of oxygen and turbulence on elongation of coleoptiles of submergence-tolerant and -intolerant rice cultivars. *J. Exp. Bot.* **33**, 1030-1044.

Bartels, P.G., Watson, C.W. (1978) Inhibition of carotenoid biosynthesis by fluridone and norflurazone. *Weed Sci.* **2**, 198-203.

Belefant, H., Fong, F. (1989) Abscisic acid ELISA: organic acid interference. *Plant Physiol.* **91**, 1467-1470.

Gage, D.A., Fong, F., Zeevaart, J.A.D. (1989) Abscisic acid biosynthesis in isolated embryos of *Zea mays* L. *Plant Physiol.* **89**, 1039-1041.

Gamble, P.E., Mullet, J.E. (1986) Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated, dark-grown barley. *Eur.J.Biochem.* **160**, 117-121.

Gopalakrishnan, S., Sircar, S.M. (1974) A comparative study of the effects of some growth regulators on the biochemistry of *Oryza sativa* L. *Ann. Bot.* **38**, 115-120.

Hoffmann, S., Kende, H. (1990) The role of ABA in the growth of rice coleoptiles. (Abstract No. 409). *Plant Physiol.* **93**, S-71.

Hoffmann-Benning, S., Kende, H. (1991) On the role of abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol.* **99**, 1156-1161.

Horton, R.F. (1991) The effect of ethylene and other regulators on coleoptile growth of rice under anoxia. *Plant Sci.* **79**, 57-62.

Hoson, T., Masuda, Y., Pilet, P.E. (1992) Auxin content in air and water grown rice coleoptiles. *J. Plant Physiol.* **139**, 685-689.

Imaseki, H., Pjon, C.-J. (1970) The effect of ethylene on auxin-induced growth of excised rice coleoptile segments. *Plant Cell Physiol.* **11**, 827-829.

Ishizawa, K., Esashi, Y. (1983) Cooperation of ethylene and auxin in the growth regulation of rice coleoptile segments. *J. Exp. Bot.* **34**, 74-82.

Ishizawa, K., Esashi, Y. (1984a) Gaseous factors involved in the enhanced elongation of rice coleoptiles under water. *Plant, Cell, Environment* **7**, 239-245.

Ishizawa, K., Esashi, Y. (1984b) Osmoregulation in rice coleoptile elongation as promoted by cooperation between IAA and ethylene. *Plant Cell Physiol.* **25,** 495-504.

Katsura, N., Suge, H. (1979) Does ethylene induce elongation of the rice coleoptile through auxin? *Plant Cell Physiol.* **20,** 1147-1150.

Kefford, N.P. (1962) Auxin-gibberellin interaction in rice coleoptile elongation. *Plant Physiol.* **37,** 380-386.

Kende, H., Hanson, A.D. (1976) Relationship between ethylene evolution and senescence in morning-glory flower tissue. *Plant. Physiol.* **57,** 523-527.

Kordan, H.A. (1977) Coleoptile emergence in rice seedlings in different oxygen environments. *Ann. Bot.* **41,** 1205-1209.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C., Karssen, C.M. (1982) The isolation of abscisic acid (ABA)-deficient mutants by selection of induced revertants in non-germinating gibberellin-sensitive lines of *Arabidopsis thaliana* (L.) Heyn. *Theor. Appl. Genet.* **61,** 385-393.

Ku, H.S., Suge, H., Rappaport, L., Pratt, H.K. (1970) Stimulation of rice coleoptile growth by ethylene. *Planta* **90,** 333-339.

Li, Y., Walton, D.C. (1990) Violaxanthin is an abscisic acid precursor in water-stressed dark-grown bean leaves. *Plant Physiol.* **92,** 551-559.

Mapelli, S., Rocchi, P., and Bertani, A. (1986) ABA and IAA in rice seedlings under anaerobic conditions. *Biol. Plant.* **28,** 57-61.

Mertens, R., Deus-Neumann, B., Weiler, E.W. (1983) Monoclonal antibodies for the detection and quantification of the endogenous plant growth regulator, abscisic acid. *FEBS Letters* **160,** 269-272.

Miller, J.H., Miller, P.M. (1974) Ethylene and the responses to light of rice seedlings. *Physiol. Plant.* **30,** 206-211.

Morris, C.F., Walker-Simmons, M. (1989) ABA-inducible cDNAs in embryos of dormant wheat grain. (Poster No.1020) *Plant Physiol.* **89,** p.171.

Murakami, Y. (1962) The promoting effect of gibberellin on the elongation of rice radicle. *The Bot. Mag.* **75,** 102-106.

Ohwaki, Y. (1967) Growth of rice coleoptiles in relation to oxygen concentrations. *Sci. Rep. Tôh. Univ.* **33,** 1-5.

Ranson, S.L., Parija, B. (1955) Experiments on growth in length of plant organs. II. Some effects of depressed oxygen concentrations. *J. Exp. Bot.* **6**, 80-93.

Raskin, I. and Kende, H. (1983) Regulation of growth in rice seedlings. *J. Plant Growth Regul.* **2**, 193-203.

Rock, C.D., Zeevaart, J.A.D. (1991) The aba mutant of *Arabidopsis* is impaired in epoxy-carotenoid biosynthesis. *Proc. Natl. Acad. Sci. USA* **88**, 7496-7499.

Saab, I.N., Sharp, R.E., Pritchard, J., and Voetberg, G.S. (1990) Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. *Plant Physiol.* **93**, 1329-1336.

Suge, H. (1971) Stimulation of oat and rice mesocotyl growth by ethylene. *Plant Cell Physiol.* **12**, 831-837.

Suge, H. (1974) Synergistic action of ethylene with gibberellins in the growth of rice seedlings. *Crop. Sci. Soc. Japan. Proc.* **43**, 83-87.

Takahashi, K. (1972) Abscisic acid as a stimulator of rice mesocotyl growth. *Nature New Biol.* **238**, 92-93.

Takahashi, K. (1973) Interaction between ethylene, abscisic acid and gibberellic acid in elongation of rice mesocotyl. *Planta* **109**, 363-364.

Turner, F.T., Chen, C.-C., McCauley, G.N. (1981) Morphological development of rice seedlings in water of controlled oxygen levels. *Am. J. Bot.* **29**, 721-738.

Vergara, B.S., Jackson, B., DeDatta, S.K. (1976) Deep-water rice and its response to deep-water stress. *In: Climate and Rice.* International Rice Research Institute, Los Baños, Philippines, pp 301-319.

Wada, S. (1961) Growth patterns of rice coleoptiles grown on water and under water. *Sci. Rep. Tôhoku Univ.* **27**, 199-207.

Walker-Simmons, M. (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars *Plant Physiol.* **84**: 61-66.

Weiler, E.W. (1980) Radioimmunoassay for the differential and direct analysis of free and conjugated abscisic acid in plants. *Planta* **148**, 262-272.

Yamada, N. (1954) Auxin relationships of the rice coleoptile. *Plant Physiol.* **29**, 92-96.

Zeevaart, J.A.D. (1983) Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. *Plant Physiol.* **71**, 477-481.

Zeevaart, J.A.D., Heath, T.G., Gage, D.A. (1989) Evidence for a universal pathway of abscisic acid biosynthesis in higher plants from ^{18}O incorporation patterns. *Plant Physiol.* **91**, 1594-1601.

CHAPTER 3

THE ROLE OF ABSCISIC ACID AND GIBBERELLIN IN THE REGULATION OF GROWTH IN DEEPWATER RICE INTERNODES

ABSTRACT

Submergence induces rapid elongation in rice coleoptiles (*Oryza sativa* L.) and of deepwater rice internodes. This adaptive feature helps rice to grow out of the water and to survive flooding. Earlier, we found that the growth response of submerged deepwater rice plants is mediated by ethylene and gibberellin (GA). Ethylene promotes growth, at least in part, by increasing the responsiveness of the internodal tissue to GA. In the present work, I examined the possibility that increased responsiveness to GA was based on a reduction in endogenous abscisic acid (ABA) levels. Submergence and treatment with ethylene led, within three hours, to a 75% reduction in the level of ABA in the intercalary meristem and the growing zone of deepwater rice internodes. The level of GA_1 increased fourfold during the same time period. An interaction between GA and ABA could also be shown by application of these hormones. ABA inhibited growth of submerged internodes, and GA counteracted this inhibition. Our results indicate that the growth rate of deepwater rice internodes is determined by the ratio of an endogenous growth promoter (GA) and a growth inhibitor (ABA).

INTRODUCTION

Rice (*Oryza sativa* L.) has a number of physiological and metabolic adaptations that enhance its chances for survival under conditions of temporary flooding. One of these is the capacity of plants to elongate rapidly when they become submerged. This feature helps rice to emerge from the water and to avoid drowning. In seedlings, submergence promotes coleoptile growth (Kordan *et al.*, 1977; Turner *et al.*, 1981; Yamada, 1959) and in adult deepwater rice plants, elongation of the internode (Métraux and Kende, 1983; Vergara *et al.*, 1976). In both instances, the plants respond to the altered gas composition of their submerged organs, namely to reduced partial pressure of O_2, to increased partial pressure of CO_2, and to the accumulation of ethylene (Ku et al., 1970; Ohwaki, 1967; Ranson and Parija, 1955; Raskin and Kende, 1984 a,b; for a review, see Jackson and Pearce, 1991). In deepwater rice, low partial pressures of O_2 promote ethylene biosynthesis (Raskin and Kende, 1984a) by enhancing the activity of the ethylene-biosynthetic enzyme 1-aminocyclopropane-1-carboxylate (ACC) synthase (Cohen and Kende, 1987). Ethylene promotes growth, at least in part, by increasing the responsiveness of the internodal tissue to GA (Raskin and Kende, 1984b). This is very different from what has been found earlier in rice seedlings (see Chapter 2; Hoffmann and Kende, 1990), where ethylene also promotes coleoptile growth. GA does not seem to be involved in regulating rice coleoptile growth, while rice internodal growth is induced by GA and inhibited by GA-biosynthesis inhibitors (Raskin and Kende, 1984b).

In the studies described below, I investigated one possible mechanism by

which ethylene may modulate the responsiveness of deepwater rice to GA. Zeevaart (1983) found that applied ethylene reduced the level of ABA in leaves of *Xanthium strumarium*. Thus, increased responsiveness to GA in deepwater rice may be based on an ethylene-mediated reduction in the level of endogenous ABA, a potent inhibitor of growth in rice. To examine this possibility, I measured the level of ABA in the intercalary meristem and the growing zone of deepwater rice plants that had been induced to grow rapidly by submergence or treatment with ethylene. I also determined the level of GA_1 and GA_{20} in the same tissue. These experiments were designed to test my working hypothesis that the rate of internodal growth in deepwater rice is determined by the balance of GA, a growth promoter, and of ABA, a growth inhibitor. Earlier, I had shown that coleoptile growth in rice seedlings is inhibited by ABA and stimulated by fluridone, an inhibitor of ABA biosynthesis. In addition, both, ethylene treatment and submergence led to a reduction of the endogenous ABA content in rice coleoptiles (Hoffmann and Kende, 1990; chapter 2).

MATERIALS AND METHODS

Growth of plants. Rice plants (*Oryza sativa* L., cv. Habiganj Aman II) 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. Adult plants were submerged as described by Métraux and Kende (1983). For ethylene treatment, they were placed in plastic cylinders through which air containing 3 to 5 μl/ml ethylene was passed at a rate of 400 ml/min. These treatments were performed in the same growth chamber in which the plants had been grown. At various times, the basal 1-cm portion of the youngest internode containing the intercalary meristem and part of the elongation zone above it was harvested and frozen in liquid N_2. Submergence and treatment with ethylene were performed with 8- to 12-week-old plants.

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 either submerged in glass cylinders containing 4 l of water with or without GA_3 and/or ABA or kept in plastic cylinders through which humidified air was passed at a rate of 80 ml/min. The increase in internodal length was measured after three days of growth under continuous light.

ABA extraction and determination. ABA from the basal 1-cm portion of internodes was extrated and an ELISA performed as described by Walker-Simmons (1987) using the monoclonal antibody of Mertens *et al.* (1983) which was purchased from Idetek (San Bruno, CA). The ABA-4'-conjugate was prepared according to Weiler (1980).

GA extraction and determination. The basal 1-cm portions of internodes from submerged and air-grown plants were collected over a period of several weeks and frozen. Tissue from 40 to 100 plants was lyophilized yielding 1 to 3 g (dry wt) of material. GAs were extracted, purified, and seperated according to Talon and Zeevaart (1990). In brief, GAs from rice tissue were extracted overnight, first in 100% and then in 80% (v/v) methanol. They were purified by phase partitioning with hexanes and ethyl ether, by absorption chromatography with Charcoal:Celite (1:2), another phase partitioning with ethyl acetate, and chromatography on a QAE-Sephadex A-25 column. They were separated by reverse-phase HPLC. Fractions corresponding to GA_1 and GA_{20} were analyzed by GC-selected ion monitoring as described by Talon and Zeevaart (1990). The ions monitored were for $GA_1/[^2H_2]GA$-methyl ester trimethylsilyl ether (m/z 508, 506, 450, 448) and for $GA_{20}/[^2H_2]GA$-methyl ester trimethylsilyl ether (m/z 420, 418, 377, 375). GA concentrations were quantified as in Talon and Zeevaart (1990).

Statistical analysis. The data shown in Figures 3.3 and 3.4 were subjected to analysis of variance for a complete randomized block design with three (submergence) or four (treatment with ethylene) replicates (Steele and Torrie, 1960). One block consisted of one series of ABA determinations from plants that were air grown, submerged, or treated with ethylene for various time periods.

RESULTS AND DISCUSSION

Stem sections isolated from adult rice plants were either kept in air or submerged in cylinders containing water and one of several concentrations of ABA (Fig. 3.1). At a concentration of 1 μM, ABA reduced submergence-induced growth by 30%; at 100 μM, it eliminated it completely. This inhibition was reversed by the addition of GA_3 to sections submerged in 3 μM ABA (Fig. 3.2).

Two sets of experiments were performed to assess the effect of submergence and ethylene treatment on the ABA content of the intercalary meristem and the elongation zone of deepwater rice internodes. In one set, plants were immersed in 300-l plastic tanks filled with water or were kept in air in the same growth chamber. In the second set, deepwater rice plants were placed in plastic cylinders through which ethylene-free air or air containing 3 to 5 μl/l ethylene was passed. Within 1 h of submergence, the ABA level in the intercalary meristem and the cell elongation zone above it decreased by more than 50% (Fig. 3.3). After 3 h, it was reduced by 75% and it decreased further during the next 21 h. In air-grown control plants, the ABA content remained at its original level. These data were subjected to analysis of variance for a randomized complete block design with three replicates (Table 3.1). The differences in ABA levels between different treatments were highly significant (F test; $P < 0.005$) with a highly significant effect of submergence (F test; $P < 0.005$).

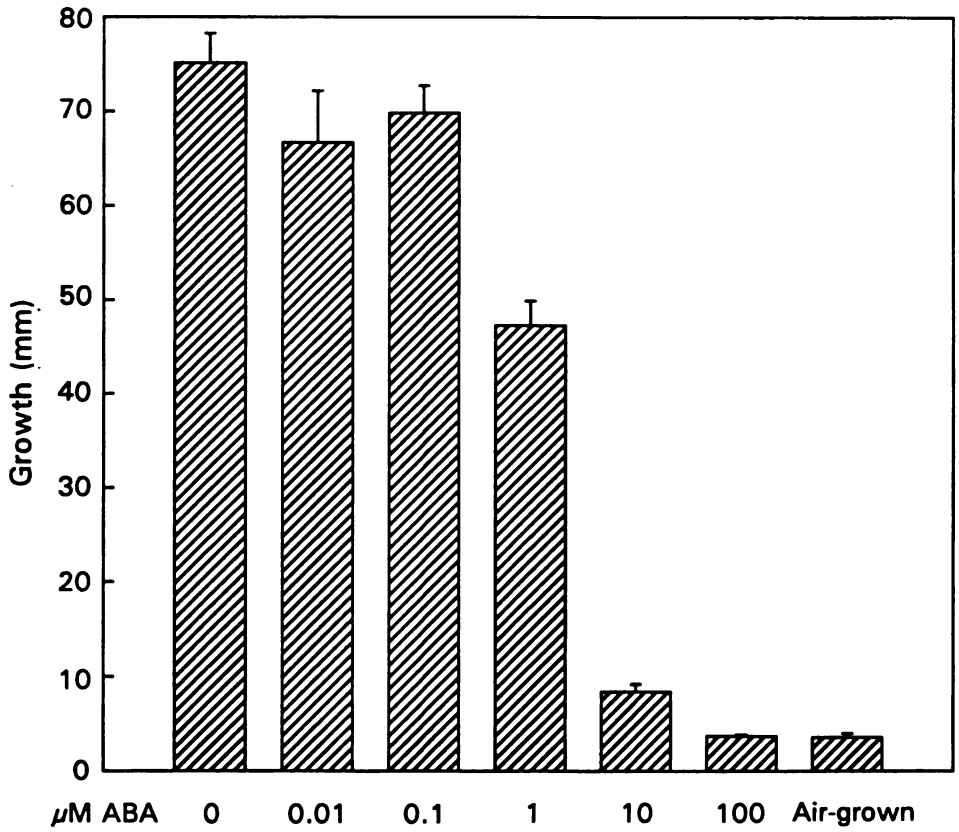


Figure 3.1. Growth of deepwater rice stem sections submerged for three days in water containing one of several concentrations of ABA. The air-grown sections were kept in a glass cylinder with an air flow of 80 ml/min. The values represent the means ± S.E. of 22 to 30 sections.

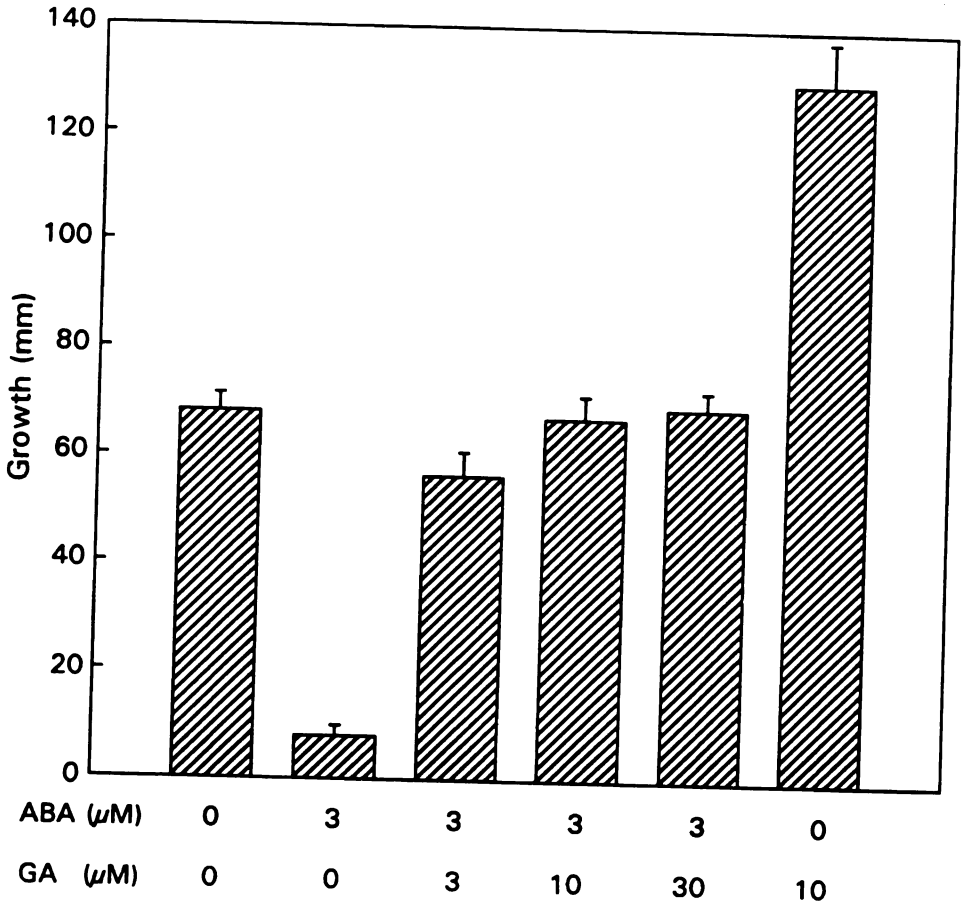


Figure 3.2. Growth of deepwater rice stem sections submerged for three days in water, in 3 μM ABA, in 3 μM ABA plus one of several concentrations of GA_3, or in 10 μM GA_3 alone. The values represent the means $\pm$ S.E. of 22 to 30 sections.

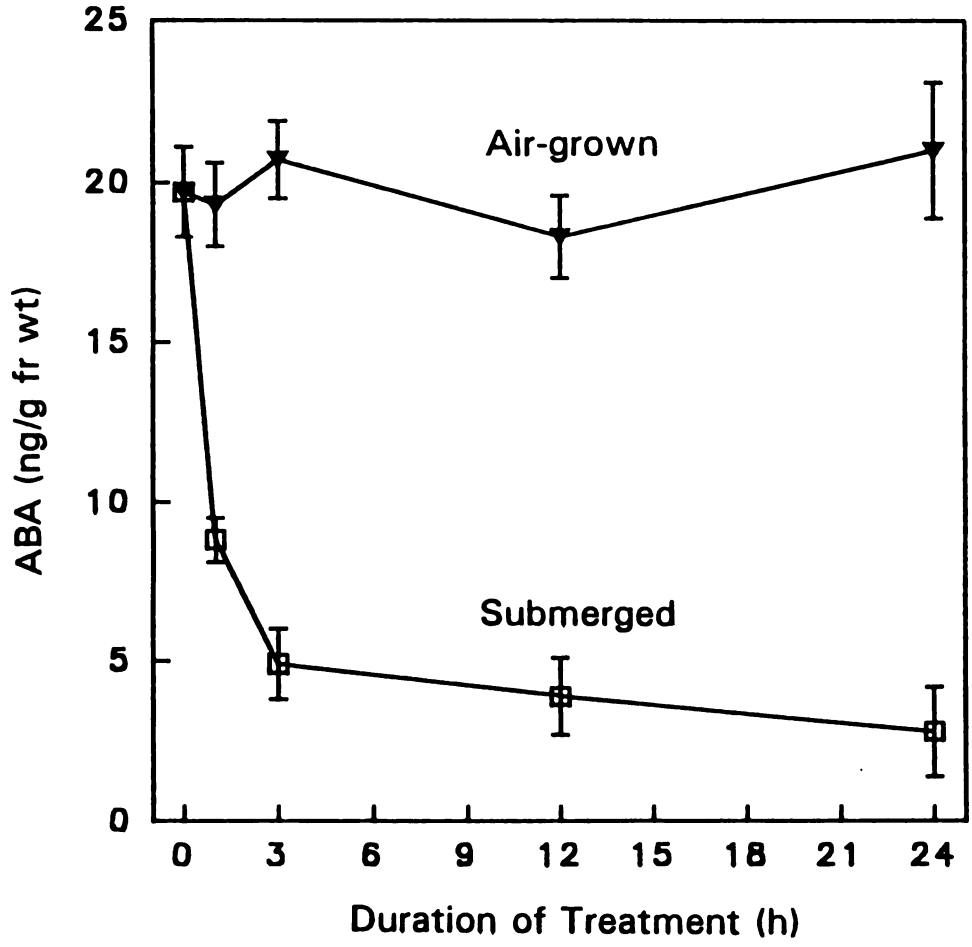


Figure 3.3. Effect of submergence on the level of ABA in deepwater rice. Plants were submerged in 300-l tanks filled with water (▾) or kept in air as control (□). One-centimeter portions containing the intercalary meristem and part of the cell elongation zone above it were excised for extraction from the internodes at the times indicated. Each point represents the mean ± S.E. of three independent experiments.

Table 3.1. Analysis of Variance on the effect of submergence on ABA levels in deepwater rice internodes (Data from Fig.3.3). df Degrees of freedom; ** Significant at the 0.01 level; *** Significant at the 0.005 level.

Source	df	Mean squares
Replicates	2	
Treatments	7	197.0 (***)
a (submergence)	1	1303.9 (***)
b (time)	3	15.0 (**)
a x b	3	15.4 (**)
Error	13	2.7

In deepwater rice plants treated for 3 h with ethylene, the ABA level in the intercalary meristem and the cell elongation zone was 75% lower than that of the corresponding control plants at the same time (Fig. 3.4). This difference in the ABA concentration was maintained over the next 21 h. Again, the data were evaluated by analysis of variance for a randomized complete block design with four replicates (Table 3.2). There were significant differences in ABA content between different treatments (F test; $P = 0.025$) with a highly significant effect of ethylene (F test; $P < 0.005$).

I determined the amount of the endogenous GA_1 and GA_{20} in the intercalary meristem and internodal elongation zone of deepwater rice plants that had been submerged for 0, 1, 3, and 24 h (Table 3.3). The level of GA_1 increased fourfold within 3 h of submergence. The level of GA_{20}, which is the immediate precursor of GA_1, also increased but more slowly than that of GA_1. After 24 h of submergence, it was 3 times higher than the original value. The GA extractions and quantifications were repeated with a second batch of plants and obtained similar results.

CONCLUSIONS

The results described above support the notion that the growth rate of deepwater rice internodes is determined not only by a growth-promoting plant hormone, GA, but also by a growth inhibitor, ABA. Elongation of deepwater rice internodes, like that of rice coleoptiles, is stimulated by submergence (Métraux and Kende, 1983; Raskin and Kende, 1984a; Vergara et al., 1976). There are, however,

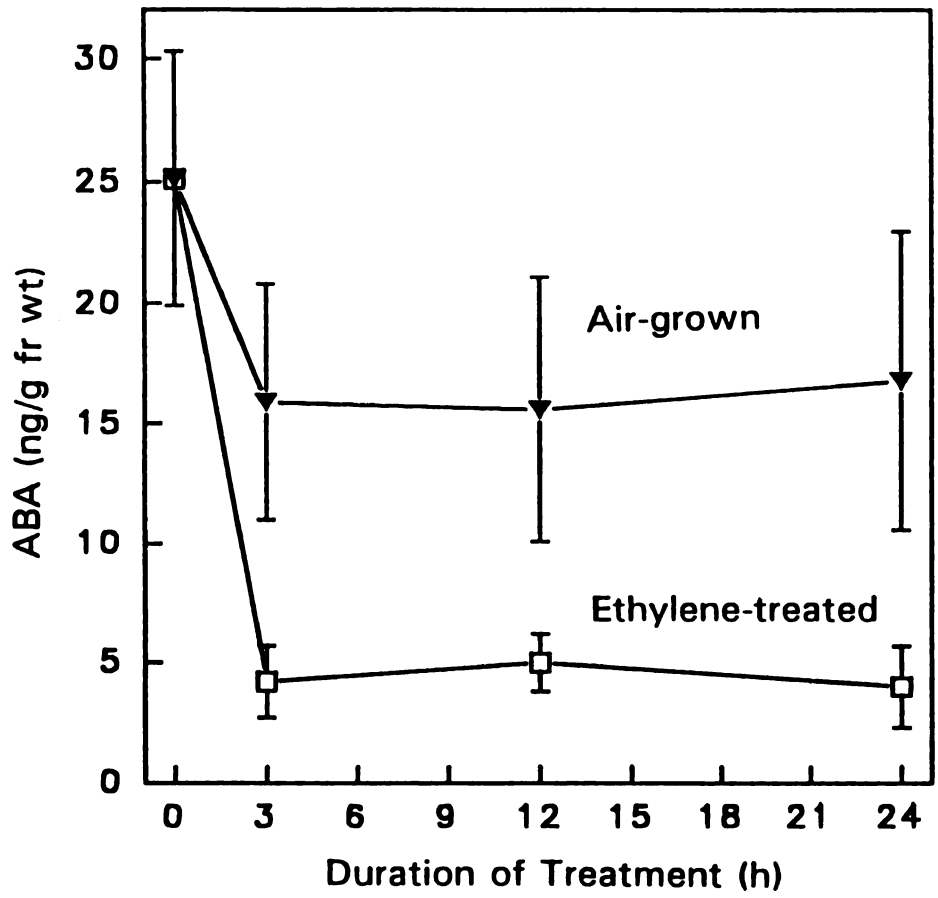


Figure 3.4. Effect of ethylene on the level of ABA in deepwater rice. Plants were grown in plastic cylinders through which ethylene-free air (▾) or air containing 3 to 5 μl/l ethylene (□) was circulated at a flow rate of 400 ml/min. One-centimeter portions containing the intercalary meristem and part of the cell elongation zone above it were excised for extraction from the internodes at the times indicated. Each point represents the mean ± S.E. of four independent experiments.

Table 3.2. Analysis of Variance on the effect of ethylene on ABA levels in deepwater rice internodes (Data from Fig. 3.4.). df Degrees of freedom;

* Significant at the 0.025 level; *** Significant at the 0.005 level.

Source	df	Mean squares
Replicates	3	
Treatments	5	132.5 (*)
a (submergence)	1	586.1 (***)
b (time)	2	14.1
a x b	2	24.2
Error	15	29.8

Table 3.3. Levels of GA_1 and GA_{20} in the intercalary meristem and cell elongation zone of internodes of adult deepwater rice plants grown in air or submerged for various times. Tissue for this experiment was harvested over a period of several weeks (ca. 60 plants per time point). A repetition of the GA_1 determinations with a second batch of tissue lead to similar increases with similar amounts of GA_1.
n.d. not determined.

Duration of treatment (h)	GA_1 (ng/g dry wt)	Relative level	GA_{20} (ng/g dry wt)	Relative level
0	15.2	1.0	12.5	1.0
1	18.9	1.2	n.d.	n.d.
3	62.3	4.1	19.4	1.6
24	63.0	4.1	40.9	3.3

distinct differences in the growth response of seedlings and adult plants. Both are stimulated to grow at low partial pressures of O_2, high partial pressures of CO_2, and in the presence of ethylene (Raskin and Kende, 1983; Raskin and Kende, 1984a). In coleopiles, these gases act independently of each other, each eliciting about one-third of the overall response (Raskin and Kende, 1983). In adult plants, on the other hand, there is an interaction between O_2, CO_2, and ethylene (Raskin and Kende, 1984a). The hormonal basis for coleoptile and internodal growth appears to be different as well. Elongation of rice coleoptiles is not reduced by inhibitors of GA biosynthesis (Hoffmann and Kende, 1990; Hoffmann-Benning and Kende, 1992) and is thought to require auxin (Jackson and Pearce, 1991). In contrast, internodal growth is completely dependent on the presence of GA (Raskin and Kende, 1984b). I hypothesized that ethylene may cause a reduction in endogenous ABA levels, as it does in *Xanthium* leaves (Zeevaart, 1983) and that responsiveness to GA may be a function of ABA content. My data support this view. The level of ABA decreased in the the intercalary meristem and the cell elongation zone of submerged and ethylene-treated internodes by 75% (Figs. 3.3 and 3.4), and the inhibition of internodal growth by ABA was fully reversed by GA (Fig. 3.2). The lag phase for the induction of growth by submergence is between 3 and 4 h (Stünzi and Kende, 1989); that for the reduction in ABA content is less than 3 h (Figs. 3.3 and 3.4). Therefore, the change in ABA levels precedes the growth response.

The level of GA_1 in the intercalary meristem and cell elongation zone increases fourfold within 3 h of submergence, and that of GA_{20} threefold within 24 h (Table 3.3). GA_1 is the active GA in rice, and GA_{20} is its immediate precursor (Kobayashi et

al., 1989). My results are in agreement with those of Suge (1985) and Türkan and Suge (1990) who, using a bioassay, found an increase in GA levels upon submergence of deepwater rice plants. It is not known whether submergence or ABA affect GA content directly or whether the level of GA increases as a function of enhanced growth, e.g. because of the formation of new cells in the intercalary meristem.

On the basis of the results reported above, I can postulate two new links in the chain of events that leads from submergence to accelerated growth in deepwater rice (Fig. 3.5). The reduced O_2 tension in submerged internodes promotes ethylene synthesis (Raskin and Kende, 1984a) by enhancing the activity of ACC synthase (Cohen and Kende, 1987). In addition, ethylene is physically trapped in submerged internodes because of its low diffusion rate in water. Ethylene inhances the responsiveness of deepwater rice internodes to GA (Raskin and Kende, 1984b), at least in part because of a reduction in ABA content. While the level of ABA decreases in submerged internodes, that of GA increases. Thus, rapid internodal growth of deepwater rice may result from an altered balance between a growth-promoting (GA) and a growth-inhibiting (ABA) hormone.

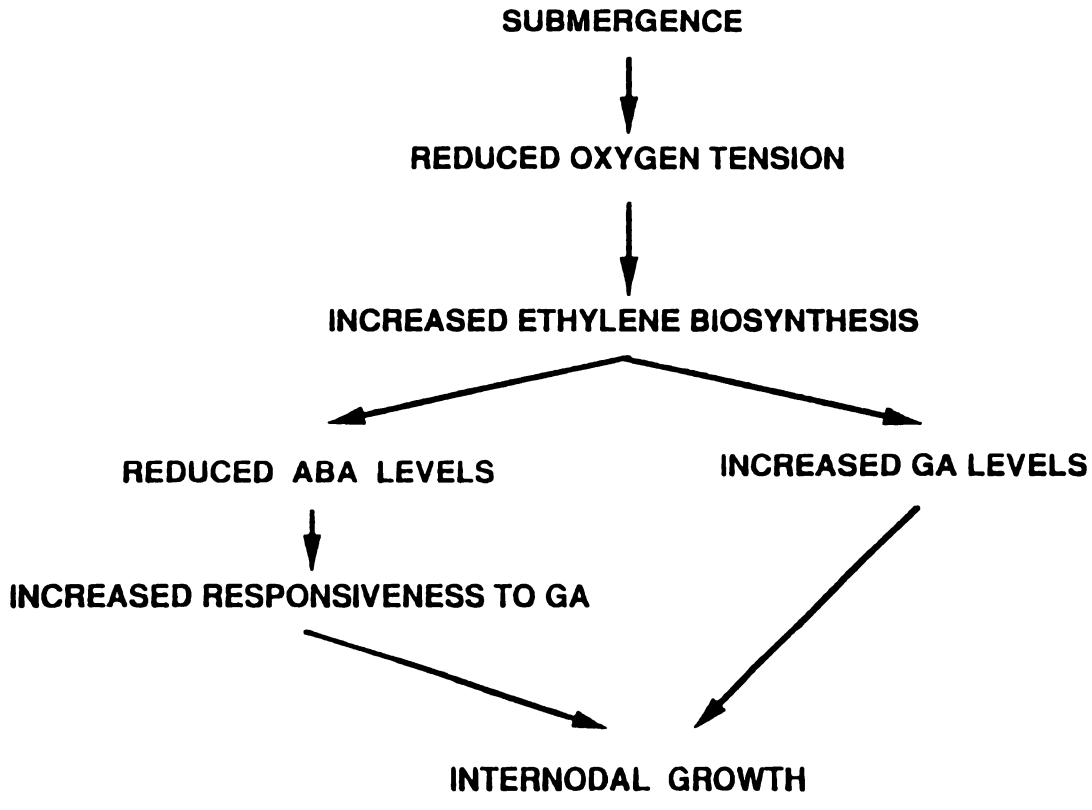


Figure 3.5. Chain of events linking submergence to accelerated growth of deepwater rice.

REFERENCES

Cohen, E., Kende, H. (1987) *In vivo* 1-aminocyclopropane-1-carboxylase synthase activity in internodes of deepwater rice. Enhancement by submergence and low oxygen levels. *Plant Physiol.* **84,** 282-286.

Hoffmann, S., Kende, H. (1990) The role of ABA in the growth of rice coleoptiles (Abstract No. 409). *Plant Physiol.* **93,** S-71.

Hoffmann-Benning, S., Kende, H. (1992) On the role of abscisic acid and gibberellin in the regulation of growth in deepwater rice. *Plant Physiol.* **99,** 1156-1161.

Jackson, M.B., Pearce, D.M.E. (1991) Hormones and morphological adaptation to aeration stress in rice. In: *Plant Live under Oxygen Deprivation.* M.B. Jackson, D.D. Davies, H. Lambers, eds, SPB Academic Publishing, The Hague, Netherlands, pp. 47-67.

Kordan, H.A. (1977) Coleoptile emergence in rice seedlings in different oxygen environments. *Ann. Bot.* **41,** 1205-1209.

Kobayashi, M., Sakurai, A., Saka, H., Takahashi, N. (1989) Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. *Plant Cell Physiol.* **30,** 963-969.

Ku, H.S., Suge, H., Rappaport, L., Pratt, H.K. (1970) Stimulation of rice coleoptile growth by ethylene. *Planta* **90,** 333-339.

Mertens, R., Deus-Neumann, B., Weiler, E.W. (1983) Monoclonal antibodies for the detection and quantification of the endogenous plant growth regulator, abscisic acid. *FEBS Lett.* **160,** 269-272.

Métraux, J.-P., Kende, H. (1983) The role of ethylene in the growth response of submerged deepwater rice. *Plant Physiol.* **72,** 441-446.

Ohwaki, Y. (1967) Growth of rice coleoptiles in relation to oxygen concentrations. *Sci. Rep. Tôh. Univ.* **33,** 1-5.

Ranson, S.L., Parija, B. (1955) Experiments on growth in length of plant organs. II. Some effects of depressed oxygen concentrations. *J. Exp. Bot.* **6,** 80-93.

Raskin, I. and Kende, H. (1983) Regulation of growth in rice seedlings. *J. Plant Growth Regul.* **2,** 193-203.

Raskin, I., Kende, H. (1984a) Regulation of growth in stem sections of deepwater rice. *Planta* **160**, 66-72.

Raskin, I., Kende, H. (1984b) Role of gibberellin in the growth response of submerged deep water rice. *Plant Physiol.* **76**, 947-950.

Steele, R.G.D., Torrie, J.H. (1960) Principles and procedures of statistics. McGraw-Hill, New York.

Stünzi, J.T., Kende, H. (1989) Gas composition in the internal air spaces of deepwater rice in relation to growth induced by submergence. *Plant Cell Physiol.* **30**, 49-56.

Suge, H. (1985) Ethylene and gibberellin: regulation of internodal elongation and nodal root development in floating rice. *Plant Cell Physiol.* **26**, 607-614.

Talon, M., Zeevaart, J.A.D. (1990) Gibberellins and stem growth as related to photoperiod in *Silene armeria* L. *Plant Physiol.* **92**, 1094-1100.

Türkan, I., Suge, H. (1991) Localization of high concentration of gibberellins in elongating internodes of floating rice. *Japan. J. Breed.* **41**, 553-559.

Turner, F.T., Chen, C.-C., McCauley, G.N. (1981) Morphological development of rice seedlings in water of controlled oxygen levels. *Am. J. Bot.* **29**, 721-738.

Vergara, B.S., Jackson, B., DeDatta, S.K. (1976) Deep-water rice and its response to deep-water stress. In: *Climate and Rice.* International Rice Research Institute, Los Baños, Philippines, pp. 310-319.

Walker-Simmons, M. (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resitant and susceptible cultivars. *Plant Physiol.* **84**, 61-64.

Weiler, E. (1980) Radioimmunoassay for the differential and direct analysis of free and conjugated abscisic acid in plants. *Planta* **148**, 262-272.

Yamada, N. (1954) Auxin relationships of the rice coleoptile. *Plant Physiol.* **29**, 92-96.

Zeevaart, J.A.D. (1983) Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. *Plant Physiol.* **71**, 477-481.

CHAPTER 4

CHARACTERIZATION AND PARTIAL IDENTIFICATION OF GROWTH-RELATED OSMIOPHILIC PARTICLES IN CORN AND DEEPWATER RICE

ABSTRACT

The epidermal cell layer appears to limit internodal growth in deepwater rice as it does in other rapidly growing plant organs. Ultrastructural examination of the growing zone of deepwater rice internodes showed the appearance of osmiophilic particles between the plasma membrane and the outer epidermal wall of plants induced to grow rapidly by submergence or by treatment with ethylene or gibberellin. The diameter of the osmiophilic particles in deepwater rice is about 80 nm, in cucumber 200 nm, and in auxin-treated corn coleoptiles up to 300 nm. In all cases, they are associated with rapid growth and, in deepwater rice, with the zone of cell elongation. Monensin inhibits the appearance of these particles, indicating that they are derived from the Golgi apparatus. In excised tissue and ultrathin sections treated with proteinase K, the number of osmiophilic particles was reduced, while glucanase and cutinase treatment were without effect. Cutinase-gold accumulated mostly in the layer on the inside of the cuticle. The osmiophilic particles became densely labeled after incubation with proteinase K-gold, indicating that they are, at least in part, proteinaceous. Antibodies against lipid transfer protein (LTP), an extensin-like

protein, an arabinogalactan protein, and "expansin" bound to the cell wall or plasma membrane but not to the osmiophilic particles.

INTRODUCTION

Deepwater rice is grown in Southeast Asia in areas that are flooded during the monsoon season. Its most distinct feature is its ability to grow very rapidly in response to submergence. This enables the plant to keep part of its foliage above water and to survive flooding (Vergara *et al.*, 1976). Submergence induces internodal growth by increasing ethylene biosynthesis which, in turn, causes a decrease in endogenous abscisic acid (ABA) level and an increase in sensitivity to gibberellins (GA; see Kende, 1987; Hoffmann-Benning and Kende, 1992a). Kutschera and Kende (1988) showed that, in rapidly growing internodes, the epidermis is under tension and suggested that the outer epidermal wall is a growth-limiting structure. They further showed by electronmicroscopy the appearance of osmiophilic particles between the plasma membrane and the outer epidermal wall of submerged internodes (Kutschera and Kende, 1989). Similar particles had been described by Kutschera *et al.* (1987) in rapidly growing corn coleoptiles. In both cases, the particles were thought to be associated with cell wall biosynthesis. Robards (1969, in *Salix* and *Fagus*) and Olesen (1980, in *Euphorbiaceae, Pedicularis, Phyllodoce, Batrachium, Samulus, Gentiana, Epilobium,* and *Salsola*) observed osmiophilic particles and reported that they are involved in cell wall biosynthesis. However, Barckhausen and Rosenstock (1973) considered similar osmiophilic particles in wounded potato tubers to be related to

suberization. Osmiophilic particles were thought to be associated with wax filament formation in *Sorghum* (Paul and McWorther, 1989).

Since the osmiophilic particles seem to be associated with rapid growth in deepwater rice internodes and appear only between the plasma membrane and outer epidermal wall of the outer epidermis, they could contain:

(1) cell wall components

(2) enzymes mediating growth of the cell wall

(3) cuticle components

(4) enzymes of cutin biosynthesis

I determined the time course of appearance and the localization of the osmiophilic particles along the rice internode and analyzed their composition by enzyme-gold labeling, selective digestion, staining, and immunocytochemistry.

MATERIALS AND METHODS

Growth of plants. Rice plants (*Oryza sativa* L., cv. Habiganj Aman II) 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.

Corn (*Zea mays* L.) kernels were soaked in aerated water overnight and planted in wet vermiculite. They were grown for four days in total darkness at 20° C. On the last two days, a red-light pulse was given for 10 min to reduce mesocotyl growth.

Treatment of whole plants. Nine- to 12-week-old adult deepwater rice plants were submerged as described by Métraux and Kende (1983). For ethylene treatment, they were placed in plastic cylinders through which air or air containing 3 to 5 μl/ml ethylene was passed at a rate of 400 ml/min. These treatments were performed in the same growth chamber where the plants had been grown.

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 water (control) or in 10 μM GA_3 solution and incubated in plastic cylinders through which humidified air was passed at a rate of 80 ml/min or were completely submerged (Raskin and Kende, 1984a). For most experiments, plants were treated for three days.

Treatment of coleoptiles. After four days of growth, the coleoptiles were separated from the first leaves, and 1-cm sections were isolated 2 to 3 mm below the tip. These sections were first incubated in water for 1 h and then in either water (control) or 10 μM IAA for an additional hour (Kutschera et al., 1987).

Monensin treatment. Deepwater rice stem sections were placed into a beaker containing water or 10 μM GA_3 with or without 20 μM monensin for the indicated times prior to fixation. Corn coleoptile sections were incubated in water or 10 μM IAA with or without monensin.

Preparation of tissue for electron microscopy. Rice stem segments of 2 mm length were excised, unless mentioned otherwise, 5 mm above the nodal septum of the youngest, growing internode and fixed in 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 1.5 h on ice. The tissue was subsequently washed three times in the same buffer on ice. Secondary fixation followed in 1% OsO_4 in the above buffer for 1.5 h at room temperature. The tissue was then washed twice in phosphate buffer, twice in water, dehydrated in a graduated series of acetone and embedded in Spurr's epoxy resin. Ultrathin sections ($\approx$ 80 nm) were cut, stained with uranyl acetate and lead citrate (Reynolds, 1963), and viewed on a Philips 201 transmission electron microscope (TEM).

One-mm corn coleoptile segments were excised from the center of the coleoptile sections and treated as described for rice stem sections.

High-pressure freezing and freeze substitution. Rice stem segments of less than 2 mm length were excised 5 mm above the nodal septum of the youngest internode, vacuum infiltrated in hexadecene, and frozen in a Balzers High Pressure Freezer at Miami University, Oxford, Ohio. The frozen tissue pieces were freeze substituted in 1% OsO_4 in acetone at -80° C for 3 days, at -20° C for 24 h, washed four times with acetone for 15 min each, and embedded in Spurr's epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and viewed on a Philips 201 transmission electron microscope.

Tissue pieces of less than 1 mm length were excised from the center of corn coleoptile sections and prepared as described for rice stem sections.

Digestion experiments. Tissue samples fixed chemically as described above were incubated in cutinase or proteinase solution (0.1 mg/ml) or 0.01 M Na_2HPO_4 (pH 9) for 1 h either between primary and secondary fixation or after embedding, sectioning, and etching with 0.5% sodium metaperiodate. Ultrathin sections were stained as described above. The experiments were done at least twice.

Enzyme-gold labeling. The method used was adapted from Bendayan *et al.* (1984). For the preparation of the enzyme gold complex, all steps were performed on ice. The pH of a 5-ml colloidal gold suspension (Ted Pella, Redding, CA) containing 5 to 10 drops of phosphate buffered saline (PBS) was adjusted to a value higher than the pI of the respective enzyme (for proteinase K, pH 10.5; for cutinase, pH 11). This suspension was added to 0.2 mg enzyme dissolved in 200 μl distilled water and mixed. Two drops of 1 % polyethylene glycol (PEG, MW 20000) were added, mixed, and the preparation centrifuged at 28000 rpm for 35 min at 4° C (Beckmann ultracentrifuge, Ti 50 rotor). The supernatant was discarded, and the pellet resuspended in 1.5 ml 0.01 M phosphate buffer (pH 9), containing 0.02% PEG and stored in a refrigerator.

For the labeling procedure, fixed tissue was sectioned and collected to nickel grids (silver to light gold sections), etched with sodium metaperiodate for 45 min, and washed thoroughly with water. The grids were then incubated for 20 min in PBS, for 1 h in enzyme-gold solution, washed with PBS and distilled water, stained for 1 h with uranyl acetate and viewed in the TEM. All labeling steps were performed at room temperature. The labeling with proteinase K gold plus controls was performed

five times with two independent sets of blocks. Control labeling was done at least three times.

Antibody-gold labeling. Ultrathin sections of conventionally fixed corn tissue mounted on nickel grids were etched for 45 min with 0.5 % sodium metaperiodate and washed thoroughly with water. For the detection of threonine hydroxyproline-rich glycoprotein (THRGP) they were blocked in 0.01 M PBS containing 2% bovine serum albumine (BSA) for 15 min. For the detection of lipid transfer protein (LTP), arabinogalactan protein (AGP) and peroxidase sections were blocked in 0.01 M PBS containing 2% BSA, 0.2% Triton X 100, and 0.2% Tween 20 for 15 min. Subsequently, grids were incubated in the appropriate antibody solution in the above buffers for 1 h, followed by a wash with buffer, and an incubation in protein A gold (1:50; EY Laboratories, San Mateo, CA) in PBS containing 1% BSA for 30 min. The sections were again washed in buffer and water, stained with uranyl acetate for 1 h, and viewed in the TEM. All labeling steps were performed at room temperature. The experiments were repeated three times.

Histochemical staining. Staining for peroxidase activity was performed according to Sexton and Hall (1991). Staining for glycol groups was adapted from Thiéry (1967) and Roland and Vian (1991). Ultrathin sections were collected on nickel or titanium grids, treated with 1% periodic acid for 25 min, washed, and then incubated in 0.2% thiocarbohydrazide (TCH) in 20% acetic acid for 24 h. They were washed in water with decreasing concentrations of acetic acid followed by a wash in

water. The grids were transferred to 1% silver proteinate, incubated in the dark for 30 min and, after several washes in water, viewed in the TEM.

RESULTS AND DISCUSSION

CHARACTERIZATION OF ULTRASTRUCTURAL CHANGES ASSOCIATED WITH RAPID GROWTH

Rapidly growing rice internodes display a phenomenon called "tissue tension" (Kutschera and Kende 1988). When they are cut longitudinally after one day of treatment with GA_3 and then floated on water for 10 min, they curve outwards with the epidermis on the concave side of the section (see Chapter 5). This curvature, which has also been seen in other rapidly growing tissues (Kutschera et al., 1987), is not seen in slowly growing rice. Tissue tension has been interpreted as the manifestation of two conflicting forces in a rapidly growing stem: the epidermis is under tension because it is growth limiting while the inner tissue does not limit growth and is under compression (Kutschera, 1989). To study the role of the epidermis in regulating rapid growth, its ultrastructure was examined.

Kutschera and Kende (1988) had observed the appearance of osmiophilic particles between the plasma membrane and the outer epidermal wall of submerged rice plants. These particles were also seen in GA_3-treated rice plants (Fig. 4.1) which also grew rapidly. They were 80 to 100 nm in size and appeared only in the outer epidermis at the interface betwee the plasma membrane and the outer epidermal wall.

They were not seen in other cells of the rice internodes.

Such osmiophilic particles had been described previously by Barckhausen and Rosenstock (1973) in suberizing potato tubers and by Kutschera et al. (1987) in rapidly growing corn coleoptiles. In corn coleoptiles that were induced to grow by treatment with auxin, the size of the particles could reach up to 300 nm (Fig. 4.2). They were visible between the plasma membrane and the outer epidermal wall and predominantly, but not exclusively, on the outside of the cell.

The conclusion that the osmiophilic particles are associated with growth is based on the following three observations:

(1) Corn coleoptiles induced to grow rapidly had 10-14 osmiophilic particles (Fig. 4.2, Table 4.3 on p. 84) per cell in cross sections while their number in slowly growing corn coleoptiles was 3 per cell in cross sections.

(2) Osmiophilic particles were also observed in internodes of deepwater rice (Fig. 4.1) which were induced to grow rapidly by submergence, treatment with GA_3 or ethylene, but they were not evident in slowly growing plants (Table 4.1, p. 75).

(3) Particles were also visible in the rapidly growing region of cucumber hypocotyls (0.5 cm below the hook; Fig. 4.3) but not in the slowly or non-growing regions of the hypocotyl (0.5 cm above the base).

To correlate further the presence of osmiophilic particles with the submergence-induced growth response of deepwater rice, their distribution and time course of appearance was analyzed. The number of osmiophilic particles per cell and section increased with time after submergence from 0.5 to 3.2 on day 3 (Fig. 4.4). This paralleled the growth of rice plants which began ca. 4 h after submergence.

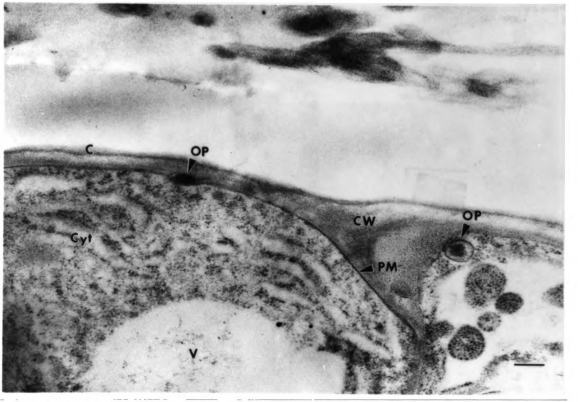


Figure 4.1. Transmission electron micrograph of a cross section through the stem of a GA_3-treated deepwater rice plant (high pressure frozen and freeze substituted). In this and all further electron micrographs the osmiophilic particles (OP) are indicated by arrowheads and following abbreviations are used: C = cuticle, CW = cell wall, PM = plasma membrane, Cyt = cytoplasm, V = vacuole. Bar = 200 nm.

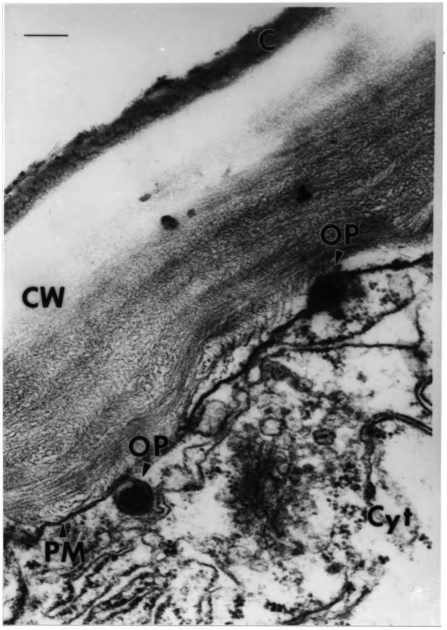


Figure 4.2. Transmission electron micrograph of a cross section through a corn coleoptile epidermis cell treated with 10 μM IAA for 1 h. Tissue used for this and all further electron micrographs and experiments has been chemically fixed. Bar = 200 nm.

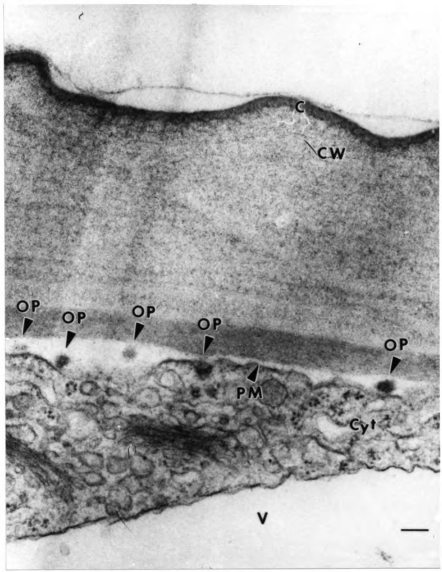


Figure 4.3. Transmission electron micrograph of a cross section through a cucumber hypocotyl epidermis cell. The tissue was taken from 5 mm below the hook. Bar = 200 nm.

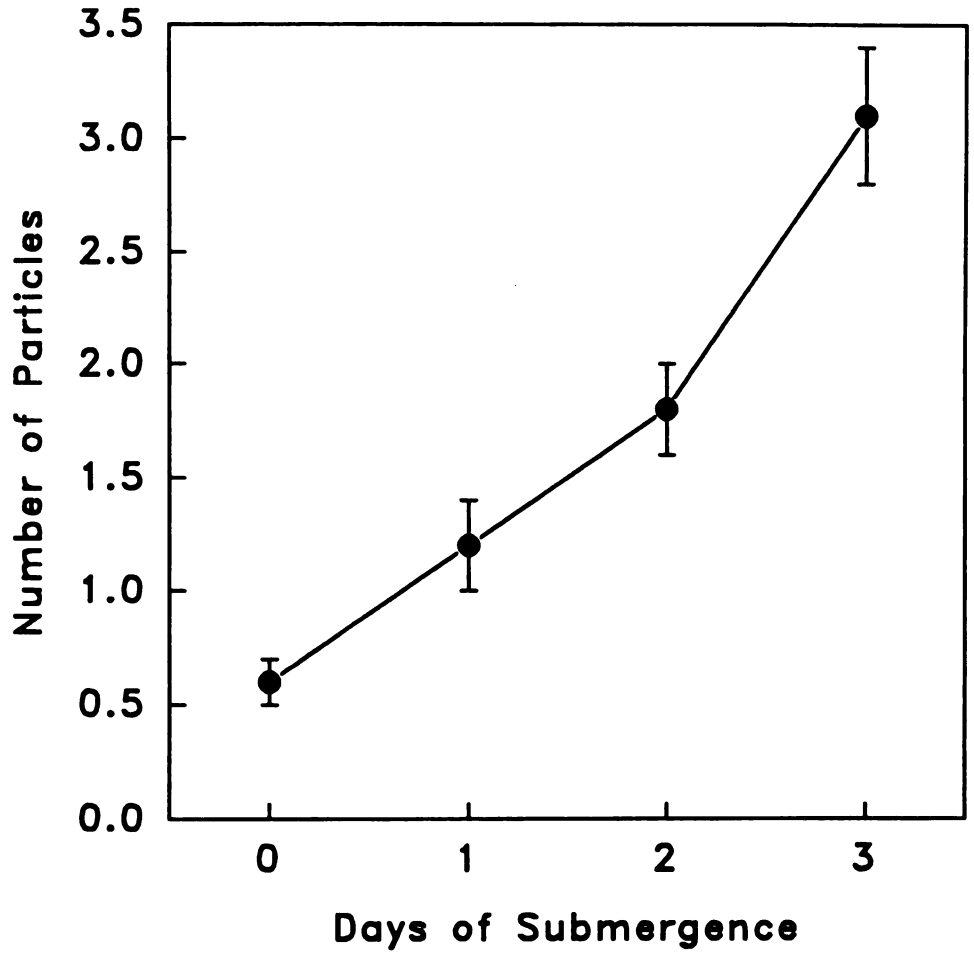


Figure 4.4. Time course of appearance of osmiophilic particles in the outer epidermal wall of submerged deepwater rice stems. The number of osmiophilic particles was determined 5 mm above the nodal septum. The values represent the means ± S.E. of osmiophilic particles in 30 cells.

The distribution of osmiophilic particles along rice internodal sections grown in water (control), 10 μM GA_3 or submerged for three days is given in Table 4.1. In control plants, the number of osmiophilic particles was less than one per cell and section. However, in both GA_3-treated and submerged plants, their number started to increase 4 mm above the nodal septum and reached more than three particles per cell and section between 5 and 15 mm above the nodal septum. At 30 mm above the nodal septum, their number was again reduced to the control level. The region between 4 and 15 mm above the nodal septum corresponds to the zone of increased cell elongation (Bleecker *et al.*, 1986). This indicates that the osmiophilic particles may play a role in cell elongation.

The number of osmiophilic particles in rice was approximately three per cell and cross section and 14 per cell and longitudinal section after three days of submergence. Treatment with ethylene (not shown) and GA_3 (Table 4.4), which also promote growth of deepwater rice plants, caused a similar increase in the number of osmiophilic particles as did submergence. In corn coleoptiles, where growth was induced by 10 μM auxin, the number of osmiophilic particles increased to about 12 per cell and cross section and to 30 per cell and longitudinal section. They were up to 300 nm in diameter, which made them easier to observe and analyze than the particles in rice.

Table 4.1. Distribution of osmiophilic particles along the rice stem. The tissue was taken three days after start of the indicated treatments. The values represent the means ± S.E. of 60 cells.

Distance from nodal septum (mm)	Number per cell and section		
	Control	Submerged	+ GA
2.5	0.4 ± 0.1	0.9 ± 0.1	
3	0.8 ± 0.1	1.0 ± 0.1	0.5 ± 0.1
4	0.8 ± 0.1	2.0 ± 0.2	2.3 ± 0.2
5	0.6 ± 0.1	3.2 ± 0.2	2.7 ± 0.2
6	0.9 ± 0.1	3.1 ± 0.1	3.1 ± 0.2
7	0.6 ± 0.1	2.1 ± 0.2	2.2 ± 0.2
15	0.9 ± 0.1	3.5 ± 0.2	
30	0.5 ± 0.1	0.6 ± 0.1	

ENZYME-GOLD LABELING

The method of enzyme-gold labeling was developed by Bendayan *et al.*(1984). It is based on the assumption that an enzyme may still recognize its substrate even in a fixed section. If the enzyme is bound to colloidal gold, one can localize the substrate by observing the distribution of the gold grains which are associated with the enzyme. Figure 4.5 shows a scheme of this binding as modified from Bendayan *et al.* (1984). To determine the general composition of the osmiophilic particles, I tested the binding of cutinase gold and of proteinase K gold to ultrathin sections of auxin-treated corn coleoptiles. Corn was used since the osmiophilic particles in corn are much larger and more abundant than in rice. Corn sections labeled with proteinase K gold showed dense labeling of the particles at all stages of secretion (Fig. 4.6). Particles appeared in vesicles within the cytoplasm (Fig. 4.6.A), between the plasma-membrane and the cell wall (Fig. 4.6.B-D), and within the cell wall (Fig. 4.6.E).

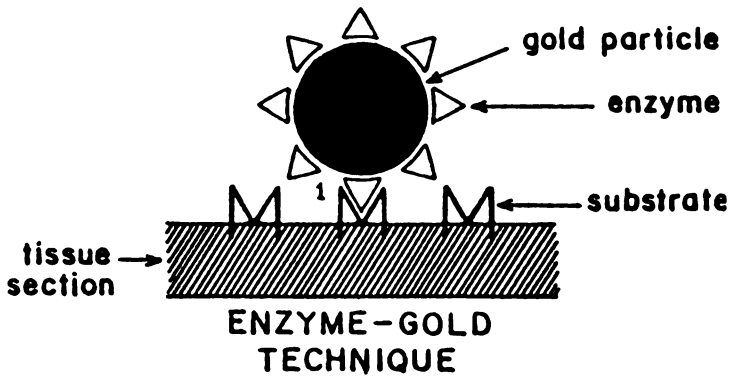


Figure 4.5. Scheme of enzyme-gold labeling as adapted from Bendayan *et al.* (1984).

Figure 4.6. Transmission electron micrograph of cross sections of auxin-treated corn coleoptile epidermal cells labeled with proteinase K gold. The particles occurred within the cytoplasm (A), at the plasmamembrane-cell wall interface (B-D), and within the cell wall (E). Bar = 200 nm.

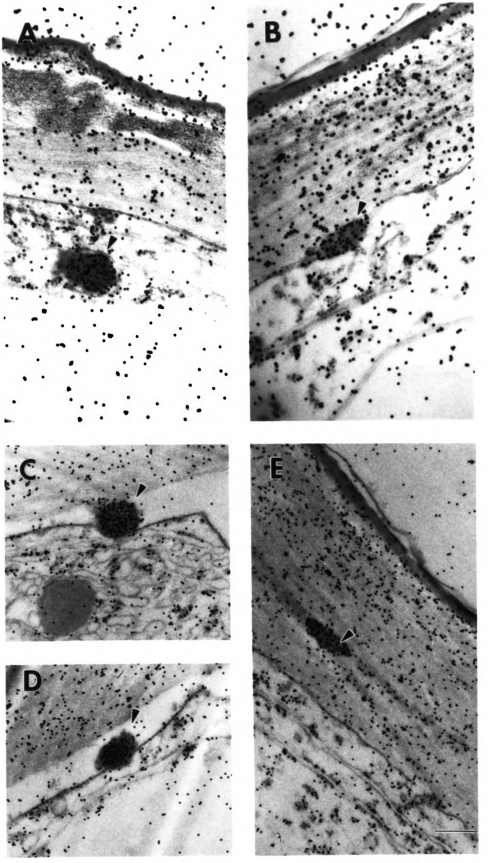

A
B
C
D
E

Table 4.2. Number of gold grains in osmiophilic particles and other cell compartments after labeling with proteinase K gold. The gold grains were counted in 13 areas of equal size for all cell components. Values followed by different letters are significantly different from each other ($LSD_{0.01}$).

Location	Gold grains per 10^4 nm^2
Osmiophilic particles	20.4 ± 2.0 a
Cell Wall	5.5 ± 0.4 b
Vacuole	1.4 ± 0.2 c
Cytoplasm/Organelles	3.6 ± 0.4 bc
Resin	1.2 ± 0.2 c

To confirm that the distribution of gold grains was more dense in the osmiophilic particles, I counted the number of gold grains within the particles and in areas of the same size in the cytoplasm, vacuole, cell wall, and resin. The number of gold grains in the osmiophilic particles was four times higher than in the cell wall, six times higher than in the cytoplasm, and approximately 20 times higher than in the resin (Tab. 4.2). In the vacuole, the number of gold grains was at background level, either because of loss of the vacuolar contents during fixation or because of the low level of protein in the vacuoles. I conclude that the gold grains accumulate in the osmiophilic particles, indicating that they are proteinaceous. To test whether the binding of proteinase K gold was specific, various controls were performed (Figure 4.7):

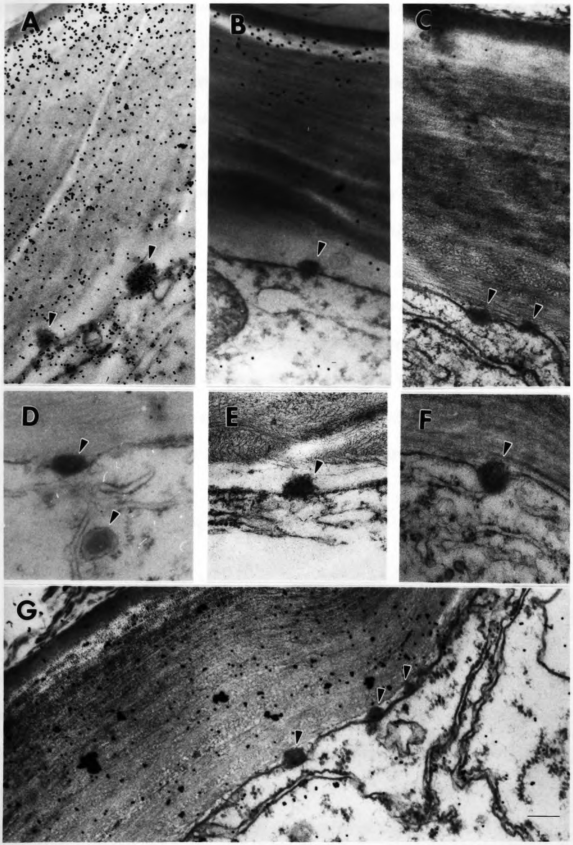
(1) Incubation with proteinase K gold plus BSA eliminated the labeling almost completely (Fig. 4.7 C). This was expected since BSA competes with the substrate in the cell.

(2) Incubation with proteinase K gold plus unlabeled proteinase K also eliminated gold labeling because free proteinase K competed with the gold-labeled enzyme (Fig. 4.7 E).

(3) When sections were incubated in proteinase K gold at pH 3, there was, as expected, no gold labeling because the enzyme is not active at this pH (Fig. 4.7 F).

(4) PMSF, an inhibitor of proteinase K that binds covalently to the active site of the enzyme, prevented binding of proteinase K to the osmiophilic particles (Fig. 4.7 G). Instead, there seemed to be an aggregation of the enzyme.

Figure 4.7. Transmission electron micrograph of corn coleoptile cross sections labeled with proteinase K gold (A), cutinase gold (B), proteinase K gold plus BSA (C), not labeled (D), proteinase K gold plus unlabeled proteinase K (E), proteinase K gold at pH 3 (F), proteinase K gold plus PMSF (G). The osmiophilic particles are indicated with arrowheads. Bar = 200 nm.


A
B
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G

(5) An unlabeled, unstained section showed again two osmiophilic particles (Fig. 4.7 D). One appeared to be within a vesicle in the cytoplasm. The other appeared to be in a vesicle fusing with the plasma membrane.

These controls, when compared to the proteinase K gold labeled section (Fig. 4.7 A), showed that the binding of proteinase K gold was indeed specific.

Labeling of sections with cutinase gold (Fig. 4.7 B) showed a distribution of gold grains different from that found with proteinase K gold (Fig. 4.7 A). Cutinase is an enzyme that degrades polymerized cutin. With cutinase gold, the label was most dense in the layer inside of the cuticle, with decreasing amounts of gold towards the inner part of the cell wall; no labeling of the osmiophilic particles was found with cutinase gold. This supports the hypothesis of Kolattukudy that the cuticle monomers become increasingly polymerized as they are transported across the cell wall. It also shows that the osmiophilic particles do not contain polymerized cutin.

DIGESTION EXPERIMENTS

To confirm the above result, digestion of the osmiophilic particles was attempted using proteinase K, cutinase, a combination of both, or glucanase. These digestions were done either between primary and secondary fixation or after embedding. In both, rice and corn, the number of osmiophilic particles was reduced to about 60% of the control when the sections were incubated in proteinase K after embedding (Table 4.3 A). Incubation with cutinase, buffer, or glucanase resulted in no change in the number of particles. A combination of proteinase K and cutinase

Table 4.3. Number of osmiophilic particles after digestion with proteinase K, cutinase, glucanase, or treatment with buffer (Control 1). Incubation occured for 1 h after embedding (A) or between primary and secondary fixation (B). Control 2 represents an untreated section. The values represent the means ± S.E. of 30 cells.

A	Number of osmiophilic particles per cell and section				
	Control 1	Proteinase K	Cutinase	Proteinase K plus cutinase	Glucanase
Corn	9.5 ± 0.4	5.8 ± 1.4	8.6 ± 5.3	5.3 ± 0.3	11.4 ± 0.8
Rice	4.3 ± 0.2	2.6 ± 0.2	4.2 ± 0.2	2.1 ± 0.2	

B	Number of osmiophilic particles per cell and section				
	Control 1	Proteinase K	Cutinase	Control 2	Glucanase
Corn	14 ± 1	3 ± 0.3	16 ± 1	14 ± 1	14 ± 1

reduced the number of osmiophilic particles to the same extent as did proteinase K alone. The decrease in the number of osmiophilic particles was probably not larger because the enzyme can digest only those particles localized on the surface of the resin section. Particles that are deeper in the resin are not exposed to the enzyme and remain undigested.

In corn coleoptiles incubated with proteinase K after primary fixation, the number of osmiophilic particles was reduced from 14 to 3 per cell and cross section, while incubation with buffer (control 1) or cutinase had no effect on their number (Table 4.3 B). A mixture of proteinase K plus cutinase showed the same reduction in the number of osmiophilic particles as did proteinase K alone. The fact that the osmiophilic particles were digested with proteinase K confirms that they are, at least in part, proteinaceous.

MONENSIN EXPERIMENTS

Monensin is an ionophore that inhibits Golgi transport. Because it appeared that the osmiophilic particles were transported in vesicles to the cell wall, I tested the effect of monensin on their appearance. After 12 h of monensin treatment, some vesicles of the Golgi apparatus in rice were larger in size than at 0 h, while others had retained their original size and shape (Fig. 4.8). It appeared that monensin was starting to affect Golgi transport. This was confirmed by the reduction of osmiophilic particles from 3 per cell and cross section to 0.5 per cell and cross section. After 17 h of monensin treatment, the Golgi apparatus appeared to deteriorate, its vesicles

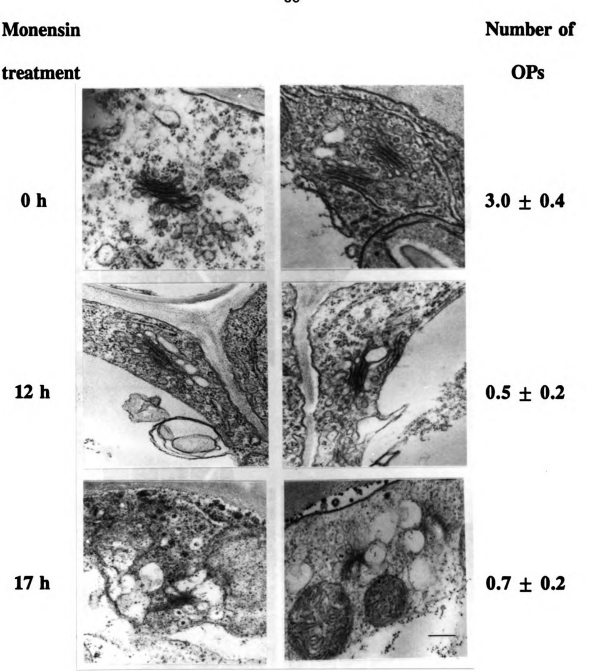


Figure 4.8. Transmission electron micrographs showing the effect of monensin on the Golgi apparatus. Deepwater rice stems were grown in 10 μM GA_3 for three days ± 20 μM monensin for the indicated times. Bar = 200 nm. Numbers accompanying the electronmicrographs indicate the number of osmiophilic particles counted in cross sections of internodes ± monensin (mean of 20 cells ± S.E).

were very large, and some cells showed a reduction in membrane structures. The number of osmiophilic particles was reduced to 0.7 per cell and section.

In rapidly growing rice internodes and corn coleoptiles, treatment with monensin for 12 h reduced the number of osmiophilic particles from 2.6 and 8.0 to 0.5 and 3.8, respectively. This resembles closely the number of particles in the slowly growing control plants (0.6 in rice; 2.1 in corn; Table 4.4). The results of the monensin experiments indicate that the osmiophilic particles are transported through the Golgi secretory pathway and that their contents may be glycosylated.

ENZYME ASSAYS AND HISTOCHEMICAL STAINS

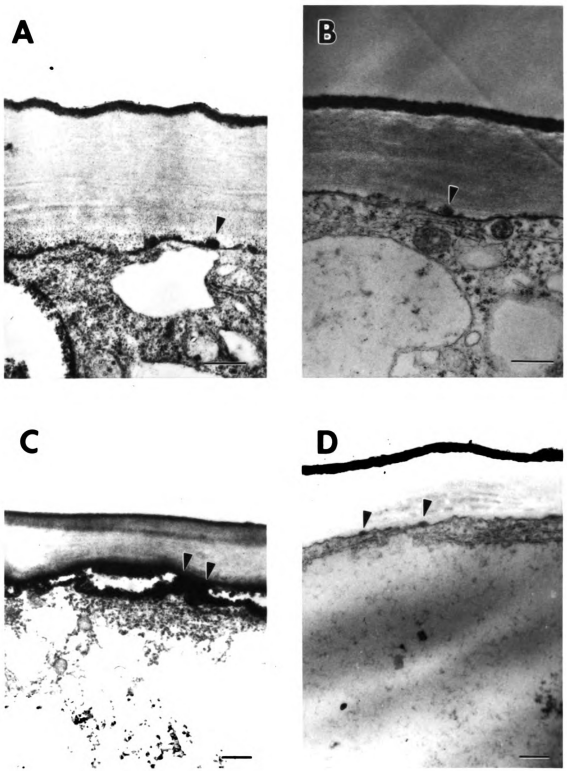
I used a technique described by Thiéry (1967) to stain glycol groups, e.g. 1,2-glycol groups, that occur in the cell wall. I could not detect any positive staining in the osmiophilic particles. However, even the staining of the cell wall was very weak. Thus, I cannot yet determine whether the osmiophilic particles contain glycol groups.

Next, I tried to stain for peroxidase since this enzyme appears to be involved in cell wall and cutin biosynthesis (Kerby and Somerville, 1992; Ferrer *et al.*, 1991). Staining for peroxidase (Sexton and Hall, 1991) yielded positive staining between the plasma membrane and outer epidermal wall (Fig. 4.9). The stain appeared to be darker in areas where osmiophilic particles usually occur. However, even though it seems possible that the osmiophilic particles contain peroxidase activity, I was unable to pinpoint the stain to the osmiophilic particles.

Table 4.4. Effect of incubation in 20 μM monensin for 12 h on the appearance of osmiophilic particles in corn and rice. Particles were counted 5 mm above the nodal septum of rice internodes grown in water (control) or 10 μM GA_3 and in the middle of 1-cm segments of corn coleoptiles incubated in water (control) or 10 μM IAA. The values represent the means $\pm$ S.E. of 20 cells.

		Number of osmiophilic particles per cell	
		- Monensin	+ Monensin
Corn	Control	2.1 $\pm$ 0.4	2.3 $\pm$ 0.6
	IAA	8.0 $\pm$ 0.5	3.8 $\pm$ 0.6
Rice	Control	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2
	GA	2.6 $\pm$ 0.4	0.5 $\pm$ 0.2

Figure 4.9. Transmission electron micrographs visualizing peroxidase activity in corn coleoptile epidermis cells. Corn coleoptiles were incubated in 10 μM IAA, chemically fixed, and stained according to Sexton and Hall (1991) for 15 min (A, B) or 1 h (C, D). A and C correspond to tissue pieces incubated in diaminobenzidine and H_2O_2 (peroxidase stain), B and D correspond to tissue pieces incubated in KCN (negative control). Bar = 500 nm.


A
B
C
D

ANTIBODY LABELING

To further analyze the nature of the osmiophilic particles, I tested antibodies against several proteins which may be secreted into the cell wall.

(1) Threonine-hydroyproline-rich glycoprotein from corn (THRGP)

(2) Lipid transfer protein (LTP)

(3) Arabinogalactan protein (AGP)

(4) Peroxidase

(5) Expansin

Threonine-hydroxyproline-rich glycoprotein from corn is an extensin-like protein that has been purified by Kieliszewski *et al.* (1991). It is localized in the cell wall and comprises less than 1% of the total cell wall. The antibody was provided by Dr. Marcia Kieliszewski (Michigan State University, E. Lansing). It bound to the outer wall of epidermal cells of corn coleoptiles but not to the osmiophilic particles, the cytoplasm or vacuole (Fig. 4.10 A). Preimmune serum (Fig. 4.12 B) or protein A gold alone (Fig. 4.10 C) showed only very few gold grains in the cell wall. Thus, the osmiophilic particles do not appear to contain THRGP.

Lipid transfer proteins transfer lipids between membranes in *in vitro* assays. They are divided into specific and non-specific LTPs. Plant LTPs are non-specific LTPs. They are soluble basic proteins with a molecular mass of about 9 kDa. They are able to transfer phospholipids and chloroplast glycolipids but not neutral lipids such as triacyl-, diacyl- or monoacylglycerols, sterols or acylsterols (for a review see Yamada, 1992). Madrid and von Wettstein (1991) suggested that LTPs

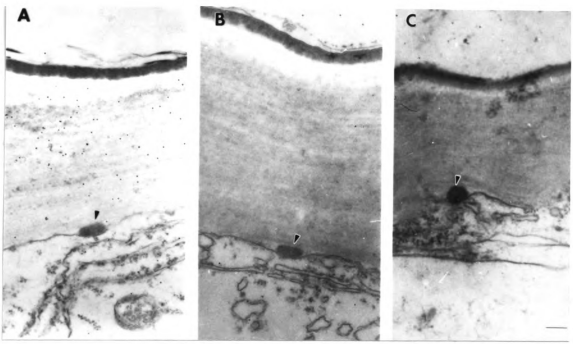


Figure 4.10. Transmission electron micrograph of cross sections of corn coleoptiles labeled with an antibody against THRGP (1:1000; A). B: preimmune; C: protein A gold alone. Bar = 200 nm

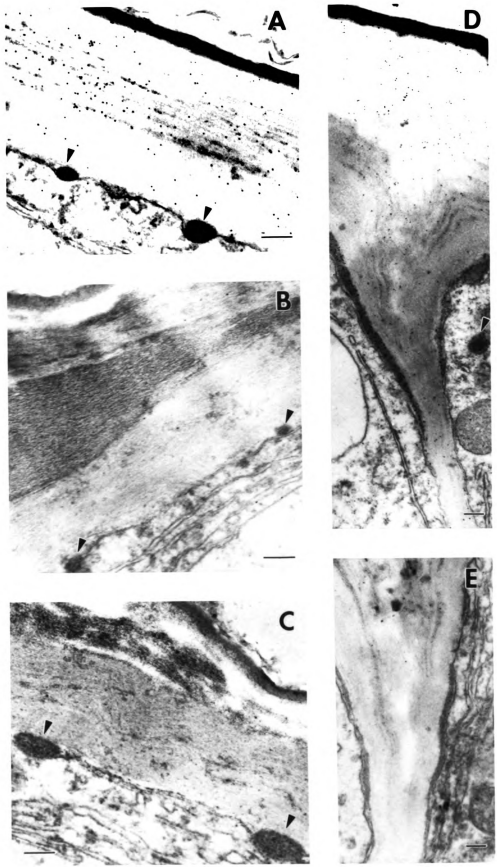
occur in two classes, one (ca. 10 kDa size) that is synthesized and translocated through the secretory pathway via the endoplasmatic reticulum and Golgi complex (e.g. in corn). The other (ca. 30 kDa size) is synthesized on free ribosomes and modified in glyoxisomes (e.g. in castor bean). A monospecific antibody against the corn LTP has been isolated by and obtained from Dr. J.-C. Kader (Université Pierre et Marie Curie, Paris, France; Sossountzov *et al.*, 1991). These authors found LTP to be localized in the epidermis of corn coleoptiles. Similarly, Thoma *et al.* (1992) also localized LTP in the epidermal cells of *Arabidopsis*. This confirmed earlier results of Sterk *et al.* (1991) who found expression of LTP mRNA in epidermal cells of carrot by *in situ* hybridization. Sterk *et al.* (1991) and Thoma *et al.* (1992) discussed the possible role of LTPs in either cutin biosynthesis or response to pathogen infection since its localization was mostly in the outer epidermal wall and mostly in tissues where the cuticle is synthesized. While the sections were not labeled with either control serum (Fig. 4.11 B, E) or protein A gold (Fig. 4.11 C), labeling of the cell walls was seen with anti-LTP antibodies as described by Thoma *et al.* (1992) for *Arabidopsis*. The osmiophilic particles showed only occasional gold labeling, but dense labeling was seen in the outer corners of some epidermal cells in an area of amorphous, electron-dense material. An anti-barley-LTP antibody of von Wettstein *et al.* (1989; obtained from Dr. P. von Wettstein-Knowles, Carlsberg Laboratory, Copenhagen, Denmark) showed the same distribution as did the antibody against corn LTP (not shown). It appears that the osmiophilic particles do not contain LTP, but that LTP may be localized in an amorphous area of the wall (Fig. 4.11 E).

Figure

abeled

A gold

Figure 4.11. Transmission electron micrograph of corn coleoptile epidermal cells labeled with an antibody against LTP (1: 50; A, D). B, E: control serum; C: protein A gold alone. Bar = 200 nm


A
B
C
D
E

As could be seen from the cytochemical stain, some peroxidase activity is localized between the plasma membrane and the outer epidermal wall. An anti-peroxidase antibody from Kirby and Somerville (1992; Michigan State University, E. Lansing) raised against a barley cell wall peroxidase was used to further study the localization of this enzyme. This antibody cross-reacts with a doublet band from rice on a Western blot (not shown). It bound specifically to corn coleoptile epidermis cells (Fig. 4.12). The antibody bound to the cell wall but not to the osmiopilic particles (Fig. 4.12 A). Preimmune serum showed no labeling (Fig. 4.12 B). It appears that the osmiophilic particles do not contain the peroxidase which is recognized by this antibody. However, since there are several peroxidase isozymes in plants, I cannot exclude the possibility that the osmiophilic particles contain a peroxidase.

Based solely on light microscopy staining, Schopfer (1990) proposed that the osmiophilic particles contain arabinogalactan proteins (AGPs). AGPs are cell wall components that are thought to anchor the cell wall to the plasma membrane. I used a rat monoclonal antibody (MAC 207) against an epitope containing arabinose and glucuronic acid to test whether the osmiophilic particles contain AGPs. The antibody was obtained from Dr. Keith Roberts (John Innes Institute, Norwich, UK; Pennel *et al.*, 1989). MAC 207 bound as described by Pennell *et al.* (1987). The gold grains were localized along the plasma membrane but not in the osmiophilic particles, indicating that they are not AGPs containing these specific carbohydrate residues (Fig. 4.13 A). A negative control, JIM4, an antibody recognizing AGPs specific of certain cells of *Daucus carota* (Knox *et al.*, 1989), showed no specific binding to the sections (Fig. 4.13 B).

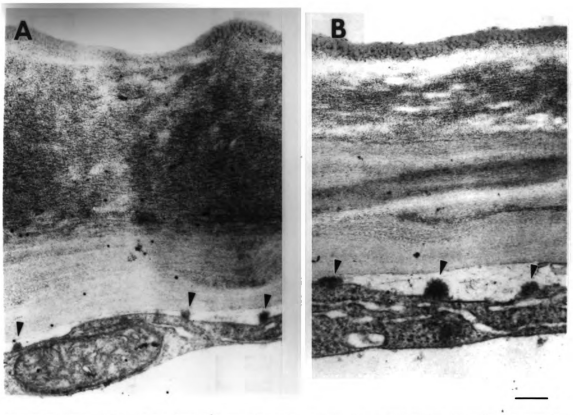


Figure 4.12. Transmission electron micrograph of corn coleoptile epidermal cells labeled with an antibody against a barley cell wall peroxidase (1:100; A). B: preimmune serum. Bar = 200 nm

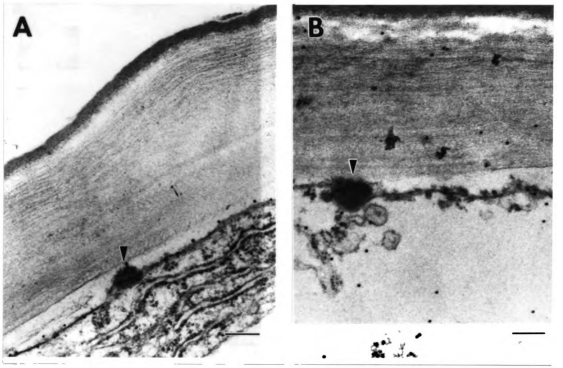


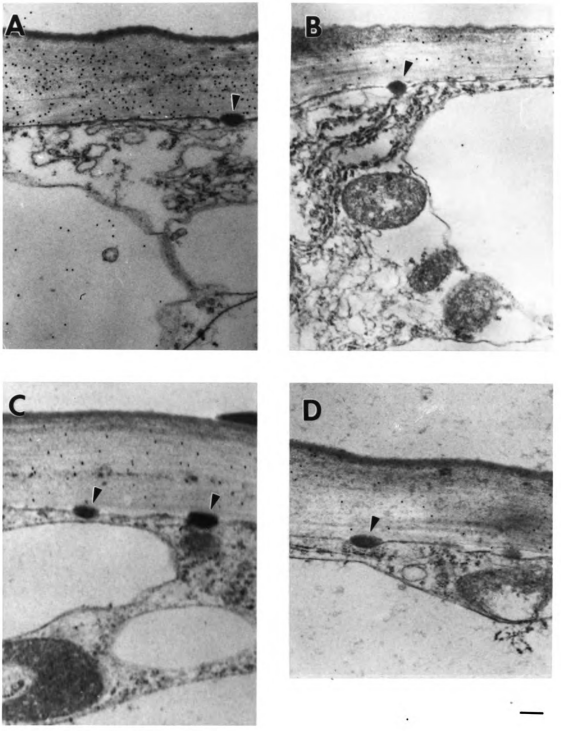
Figure 4.13. Transmission electron micrographs of corn coleoptile epidermal cells labeled with monoclonal antibodies against AGPs. The antibodies used were MAC 207 (A) and JIM4 (B). Bar = 200 nm

Expansins are two proteins that mediate cell wall extension without detectable hydrolytic breakdown of the wall. They were isolated from the walls of the growing region of cucumber hypocotyls and mediate wall extension in other dicots as well and, to a lesser extent, in monocots (McQueen-Mason *et al.*, 1992). The two proteins, S1 and S2 (29 and 30 kD, respectively), do not have endoglycanase activity. Both, total serum and monospecific antibodies against them, were obtained from Dr. Daniel Cosgrove (Pennsylvania State University, USA; Zhen-Chang Li *et al.*, 1993). The monospecific antibodies against both proteins bound to the cell wall, predominantly of the epidermal cells, but not to the osmiophilic particles in either corn (Fig. 4.14) or cucumber (not shown). The level of labeling was higher in rapidly growing corn coleoptiles than in slowly growing ones and could sometimes also be seen over Golgi vesicles (not shown).

CONCLUSIONS

The osmiophilic particles in rice and corn were predominantly localized between the plasma membrane and the outer epidermal wall in rapidly growing internodes and coleoptiles, respectively (Fig. 4.2, 4.3). In rice, they appeared during rapid internodal elongation (Fig. 4.4) and were localized in regions of increased cell elongation (Table 4.2).

Figure 4.14. Transmission electron micrographs of corn coleoptile epidermis cells labeled with antibodies against expansin S2 (A: 1:400 dilution) and S1 (C: 1:25 dilution). Binding of the corresponding control sera is given in B (for S2) and D (for S1). Bar = 200 nm


A
B
C
D

Enzyme-gold labeling showed that the osmiophilic particles are, at least in part, proteinaceous (Figs. 4.6, 4.7, Table 4.3). This result was confirmed by digestion experiments performed either between primary and secondary fixation (Table 4.4 B) or after embedding (Fig. 4.4 A).

Treatment of rice or corn plants with monensin, an inhibitor of Golgi transport, led to an almost complete disappearance of the osmiophilic particles. This indicates that they were translocated through the secretory pathway of the Golgi complex (Fig. 4.9, Table 4.4).

Immunogold labeling showed that the osmiophilic particles did not contain extensin-like THPRG (Fig. 4.10), LTP (Fig. 4.11), arabinogalactan proteins recognized by JIM4 and MAC 207 (Fig. 4.13), a peroxidase recognized by an antibody obtained from Kirby and Somerville (1992; Fig. 4.12), or expansins (Fig. 4.14). However, a peroxidase stain (Fig. 4.9) was localized in the area where osmiophilic particles appeared, indicating that they may contain peroxidase, but an isozyme that is different from the one of Kirby and Somerville (1992). The osmiophilic particles could contain other cell wall components, e.g., phenolics, acetyl CoA, or acetyl CoA-cutin transacylase. The latter two may be involved in cutin biosynthesis, but no antibodies exist against them. The osmiophilic particles could also consist of several components similar to multi-enzyme complexes.

REFERENCES

Barckhausen, R., Rosenstock, G. (1973) Feinstrukturelle Beobachtungen zur traumatogenen Suberinisierung beim Knollenparenchym von *Solanum tuberosum* L. *Z. Pflanzenphysiol.* **69,** 193-203.

Bendayan, M. (1984a) Protein A gold electron microscopic immunocytochemistry: methods, applications, and limitations. *J. Electron Microsc. Tech.* **1,** 243-270.

Bendayan, M. (1984b) Enzyme-gold electron microscopic cytochemistry: a new affinity approach for the ultrastructural localization of macromolecules. *J. Electron. Microsc. Tech.* **1,** 349-372.

Ferrer, M.A., Muñoz, R., Ros Barceló, A. (1991) A biochemical and cytochemical study of the cuticle-associated peroxidases in *Lupinus*. *Ann. Bot.* **67,** 561-568.

Hoffmann-Benning, S., Kende, H. (1992a) On the role abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol.* **99,** 1156-1161.

Hoffmann-Benning, S., Kende, H. (1992b) Characterization of osmiophilic particles associated with rapid growth in corn and rice. *Plant Physiol.* **99,** Abstract # 177.

Kende, H. (1987) Studies on internodal growth in deepwater rice. In: D.J. Cosgrove, D.P. Knievel eds., *Physiology of Cell Expansion during Plant Growth.* American Society of Plant Physiologists, Rockville, MD, pp. 227-238.

Kieliszewski, M., Lamport, D.T.A. (1987) Purification and partial characterization of a hydroxyproline-rich glycoprotein in a graminaceous monocot, *Zea mays*. *Plant Physiol.* **85,** 823-827.

Kirby, K., Somerville S.C. (1992) Purification of an infection-related, extracellular peroxidase from barley. *Plant Physiol.* **100,** 397-402.

Knox, J.P., Day, S., Roberts, K. (1989) A set of cell surface glycoproteins forms an early marker of cell position, but not cell type, in the root apical meristem of *Daucus carota* L. *Development* **106,** 47-56.

Kutschera, U., Bergfeld, R., Schopfer, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of rice coleoptiles. *Planta* **170,** 168-180.

Kutschera, U., Kende, H. (1988) The biophysical basis of elongation growth in internodes of deepwater rice. *Plant Physiol.* **88,** 361-366.

Kutschera, U., Kende, H. (1989) Particles associated with the outer epidermal wall in internodes of deepwater rice. *Ann. Bot.* **63**, 385-388.

Kutschera, U. (1989) Tissue stresses in growing plants. *Physiol Plant.* **77**, 157-163.

McQueen-Mason, S., Durachko, D.M., Cosgrove, D.J. (1992) Two endogenous proteins that induce cell wall extension in plants. *Plant Cell* **4**, 1425-2433.

Métreaux, J.-P., Kende, H. (1983) The role of ethylene in the growth response of submerged deepwater rice. *Plant Physiol.* **72**, 441-446.

Madrid, S., von Wettstein, D. (1991) Reconciling contradictory notions on lipid transfer proteins in higher plants. *Plant Physiol. Biochem.* **29**, 705-711.

Olesen, P. (1980) The visualization of wall-associated granules in thin sections of higher plant cells: occurrence, distribution, morphology and possible role in cell wall biogenesis. *Z. Pflanzenphysiol.* **96**, 35-48.

Pennell, R.I., Knox, J.P., Scofield, G.N., Selvendran, R.R., Roberts, K. (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. *J.Cell Biol.* **108**, 1967-1977.

Raskin, I, Kende, H. (1984) Regulation of growth in stem sections of deepwater rice. *Planta* **160**, 66-72.

Robards, A.W. (1969) Particles associated with developing plant cell walls. *Planta* **88**, 376-379.

Roland, J.C., Vian, B. (1991) General preparation and staining of thin sections. In: *Electron Microscopy of Plant Cells*, J.L. Hall and C.Hawes eds. Academic Press Limited, 1991.

Sexton, R., Hall, J.L. (1991) Enzyme cytochemistry. In: *Electron Microscopy of Plant Cells*, J.L. Hall and C.Hawes eds. Academic Press Limited, 1991.

Sossountzov, L., Ruiz-Avila, L., Vignols, F., Joillot, A., Arondel, V., Tschang, F., Grosbois, M., Guerbette, F., Miginiac, E., Delseny, M., Puigdomenech, P., Kader, J-C. (1991) Spacial and temporal expression of a maize lipid transfer protein gene. *Plant Cell* **3**, 923-933.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., De Vries, S.C. (1991) Cell specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **3**, 907-921.

Thoma, S., Yasuko, K., Somerville, C. (1992) The non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein.

Thiéry, J.-P. (1967) Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *J. Microsc.* **6,** 987-1018.

Yamada, M. (1992) Lipid transfer proteins in plants and microorganisms. ***Plant Cell Physiol.*** **33,** 1-6.

CHAPTER 5

CUTICLE BIOSYNTHESIS AND COMPOSITION IN GIBBERELLIC ACID-TREATED DEEPWATER RICE INTERNODES

ABSTRACT

Submergence induces rapid elongation of rice coleoptiles (*Oryza sativa* L.) and of deepwater rice internodes. This adaptive feature helps rice to grow out of the water and to survive flooding. Earlier, I found that three plant hormones, ethylene, gibberellin (GA), and abscisic acid (ABA) participate in regulating the growth response of submerged deepwater rice plants. Here I show that in GA-treated, rapidly growing rice stem sections the cuticle is a growth-limiting structure. In addition, I show that GA treatment leads to an increase in the incorporation of [^{14}C]palmitic acid and [^{14}C]oleic acid into the cuticle of growing internodes and to increased levels of at least one cuticular component, a dihydroxyhexadecanoic acid. From the changes in the chromatographic patterns of cutin monomers I conclude that the activities of enzymes that lead to hydroxylation of fatty acids may be enhanced.

INTRODUCTION

Deepwater rice (*Oryza sativa* L.) is a rice type that elongates rapidly when the plants become submerged. This feature helps the plants to emerge from the water and to avoid drowning. In seedlings, submergence promotes coleoptile growth (Kordan *et al.*, 1977; Turner *et al.*, 1981; Yamada, 1959) and in adult deepwater rice plants, elongation of the internode (Métreaux and Kende, 1983; Vergara *et al.*, 1976). In both instances, the plants respond to the altered gas composition of their submerged organs, namely to reduced partial pressure of O_2, to increased partial pressure of CO_2, and to the accumulation of ethylene (Ku et al., 1970; Ohwaki, 1967; Ranson and Parija, 1955; Raskin and Kende, 1984 a,b; for a review, see Jackson and Pearce, 1991). In adult deepwater rice plants, the reduced O_2 concentration leads to an increase in ethylene biosynthesis and, subsequently, to an increase in responsiveness of the internodal tissue to GA. Growth of deepwater rice can also be induced by application of GA (Raskin and Kende, 1984 a,b).

How does GA promote internodal growth? Hofmeister (1859) found that separated outer and inner tissues of plants changed their dimensions upon separation: the isolated epidermal layer shrinks while the inner tissue expands. He concluded that, in intact shoots, the epidermis is under tension and the inner tissues under compression. This can be shown by splitting isolated stem segments longitudinally. They will bend outwards showing that the epidermis contracts and the inner tissue expands. Sachs (1865) called this phenomenon tissue tension. It appears that the plastic extensibility of the inner tissues is much larger than that of the outer epidermal

wall (Kutschera and Briggs, 1987; Kutschera *et al.*, 1987; Cosgrove, 1989) which makes the outer epidermal wall the more rigid structure of the growing organ. Kutschera (1989) proposed that "the relatively inextensible, thick outer epidermal wall maintains the cells with extensible, thin walls in a stage of compression." Consequently, the longitudinal tensile stress of inner walls is low and that of the outer epidermal wall is high, creating tissue tension. Since the epidermis is the cell layer responding to growth-inducing hormones (e.g. auxin, Went and Thimann, 1937), the appearance of tissue tension was seen as an indication that the epidermis is the growth-limiting structure. In rice internodes, where GA promotes growth, the epidermis and, in particular, the outer epidermal wall also limits growth (e.g. Kutschera and Kende, 1988). Therefore, it has to be assumed that GA acts, at least in part, on the outer epidermis to enhance growth. While most research focuses on the effect of plant hormones on the cell wall, little attention has been given to the role of the cuticle in restricting growth and to the effect of plant hormones on the biosynthesis of the cuticle. An exception to this is the work of Bowen and Walton (1988) who found an up to 2.5-fold increase of [^{14}C]palmitic acid incorporation into cutin monomers of GA-treated pea internodes.

The plant cuticle is a lipid layer on the outside of the outer wall of epidermis cells. Its structural component is cutin, a biopolyester, which is composed of a family of C_{16} and C_{18} monomers (Kolattukudy, 1980a). These monomers often contain one or more hydroxy or epoxy groups. The major C_{16} component of cutin is dihydroxypalmitic acid; the major C_{18} components are 18-hydroxyoctadecanoic acid, 9,10-epoxy-hydroxyoctadecanoic acid, 9,10,18-trihydroxy-octadecanoic acid, and their

Δ^{12} unsaturated counterparts (Kolattukudy, 1980b). It appears that half the monomers are cross-linked through hydroxy groups (Deas and Holloway, 1977; Kolattukudy, 1977; Kolattukudy, 1984). Small amounts of phenolic acids are covalently linked to the polymer (Riley and Kolattukudy, 1975). Croteaux and Kolattukudy (1974) found that a particulate fraction sedimenting at 1000 *g* from *Vicia faba* leaves incorporated labeled hydroxy acids into cutin when ATP and coenzyme A were present. This hydroxyacyl transferase activity seems to be responsible for the polymerization of cutin from monomers and is thought to occur outside the epidermal cells.

The cuticle is a major diffusion barrier in water and gas exchange (Schönherr, 1976) and also impedes the entry of pathogenic microorganisms into the plant (van den Ende and Linskens, 1974). Kolattukudy (1965, 1970) found that cutin biosynthesis occurs more rapidly in expanding *Vicia faba* and *Brassica oleracea* leaves than in slowly expanding tissues. The C_{16} family of monomers predominates in rapidly expanding plant organs whereas a mixture of the C_{16} and C_{18} families of monomers is found in slower growing organs with thicker cuticle. In addition, the relative composition of the cuticle appears to change under various environmental conditions and at different ages of the plant (Kolattukudy *et al.*, 1974).

In this chapter, I report on cuticle biosynthesis in rapidly growing, GA-treated deepwater rice internodes and on the potential role of the cuticle as a growth-limiting structure.

MATERIALS AND METHODS

Growth of plants. Rice plants (*Oryza sativa* L., cv. Habiganj Aman II) 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 as described by Métraux and Kende (1983). These treatments were performed in the same growth chamber in which the plants had been grown. After two days, 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 water (control) or in 10 μM GA_3 solution and incubated in plastic cylinders through which humidified air was passed at a rate of 80 ml/min.

Treatment with cutinase. Cutinase, which had been purified as described in Sebastian and Kolattukudy (1988), was obtained from Dr. P.E. Kolattukudy (Ohio State University). Stem sections were grown in either water or GA_3 for a total of 24 h as described above. After 20 or 22 h, they were injected between the leaf and the

youngest internode with 50 μl of either buffer (0.05 M NaHCO/NaCO, pH 9.0, 0.05% Tween 20) or 40 μg/ml cutinase in the above buffer and incubated for the last 2 or 4 h in water or GA_3. One set of water- and GA-treated stem sections were not injected as further controls. Twentyfour h after start of the GA treatment, the leaf sheaths were peeled off, the lowest 3-cm zone of the internode was excised and cut longitudinally into quarters. These segments were incubated in distilled water for ca. 15 min, photocopied, and the angle of curvature was determined as shown below in Figure 5.1.

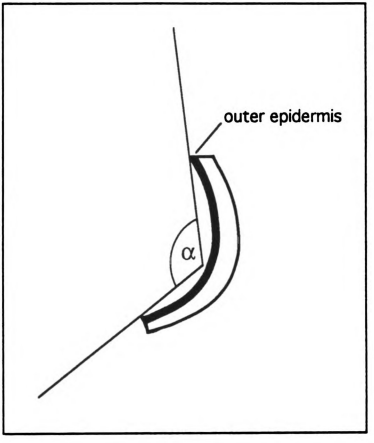


Figure 5.1. Angle of curvature in tissue segments displaying tissue tension.

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, Irvine, CA) in 25 μl aquaeous solution were injected between the leaf and the youngest internode at various times. Two h later, the leaf sheath was removed, the lowest two centimeters of the internode were excised, rinsed with water, and frozen in liquid N_2. For large-scale cuticle preparations to be used for thin layer chromatography, the cuticle was labeled for 20 h prior to excision.

Preparation of rice cuticle. Cuticle extraction was modified from Kolattukudy (1970). Internodal tissue was ground in liquid N_2. Ten to 20 ml of distilled water were added to the ground tissue, mixed, and subsequently centrifuged at 27000 *g* for 10 min at 4° C. The pellet was extracted several times in chloroform/methanol (2:1, v/v) until the tissue was totally white and all membrane lipids extracted. The remaining tissue contained only cell wall polysaccharides and cuticle. It was dried, and the radioactivity determined by liquid scintillation counting. For cutin analysis, the dried sample was refluxed under N_2 for 24 h in distilled tetrahydrofuran containing LiAlH. The amount of LiAlH was three times the sample dry weight. After 24 h, some drops of water were carefully added to react with the remaining LiAlH, and the pH of the solution was lowered to $\approx$ 3. At this point, the cuticle was depolimerized and its fatty acid components reduced to fatty alcohols. These products were in solution while the cell wall formed a precipitate. The solution was filtered through Whatman No. 4 filter paper and extracted three times with ethyl acetate. The ethyl acetate phase was collected, evaporated, and resuspended in a small volume of

chloroform/methanol (2:1).

Thin layer chromatography (TLC) was performed according to Kolattukudy (1970). For TLC separation of fatty alcohol derivatives of the cuticle, a silica TCL plate was activated at 110° C for ca. 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 in the bands determined using a phosphor imager (Molecular Dynamics).

Electron microscopy. For measurements of cell wall- and cuticle thickness plants were treated and fixed as described in chapter 4. Ultrathin sections were viewed in a Phillips 201 transmission electron microscope, and measurements were taken from the center of the cells on photographic prints.

RESULTS AND DISCUSSION

Since the outer epidermis of deepwater rice internodes is considered a growth-limiting structure, I examined the ultrastructure of epidermis cells of rice plants that had been submerged or treated with GA_3. Kutschera and Kende (1988) have shown that submergence leads to the appearance of osmiophilic particles between the plasma membrane and the outer epidermal wall. These particles are associated with rapidly growing tissue and are most likely involved in cell wall or cuticle biosynthesis. I measured the dimensions of the epidermal walls and the cuticle of rice plants that

Table 5.1. Thickness of the epidermal cell walls and the cuticle. Tissue was taken 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 values represent the means of 13 measurements $\pm$ S.E.

	Thickness (nm)		
	Control	Submerged	GA_3
Cell Wall			
Outer Epidermal Wall	112.5 $\pm$ 7.6	122.8 $\pm$ 4.2	116.6 $\pm$ 8.5
Lateral Wall	20.4 $\pm$ 4.8	24.3 $\pm$ 2.7	20.0 $\pm$ 2.7
Inner Epidermal Wall	51.3 $\pm$ 4.2	55.2 $\pm$ 3.0	65.6 $\pm$ 2.8
Cuticle	37.7 $\pm$ 4.1	42.5 $\pm$ 3.9	52.2 $\pm$ 4.9

had either been air-grown, submerged or treated GA_3 (Table 5.1). There was no change in the thickness of the outer epidermal wall. The cuticle and inner wall of GA-treated rice stems appeared thicker than that of air-grown stems. The value for the submerged plants was intermediate. Since neither the cell wall nor the cuticle became thinner, their biosynthesis had to be maintained during rapid growth. The observation that GA_3 leads to an increase in cuticle thickness prompts two questions:

1) Is the cuticle not only a physical barrier but also a growth-limiting structure?

2) Does cuticle biosynthesis change during GA-induced growth in deepwater rice?

To examine the first question, I treated air-grown or GA_3-treated rice internodes for various times with cutinase or buffer, sliced them longitudinally and determined their curvature after incubation in water (Figure 5.2, Tables 5.2, 5.3). The left half of Figure 5.2 shows differences in tissue tension between slowly versus rapidly growing sections. Slowly growing tissue did not show any outward bending, while segments of rapidly growing tissue displayed a rapid outward bending upon transfer of the stem segments into water. This is interpreted as showing that the inner tissue can expand freely while expansion of the epidermis or, more specifically the outer epidermal wall is limited. Measurements of the internal angle of curvature (Tables 5.2, 5.3; visualized in Figure 5.2) showed that application of cutinase had no effect on the bending of internodal segments from air-grown plants. However short-term application of cutinase to rapidly growing internodes led to a significant decrease in tissue tension as indicated by a reduction in outward bending of the internode segments. Treatment with buffer had no effect on tissue tension. I conclude that enzymatic digestion of the cuticle leads to a reduction in tissue tension and that the

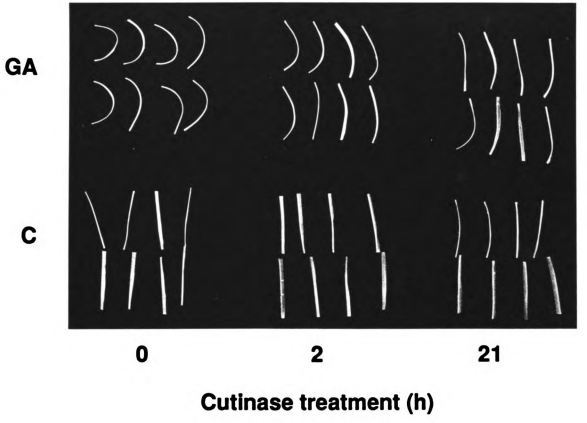


Figure 5.2. Effect of cutinase on tissue tension in the lowest 3 cm portion of the youngest internode of deepwater rice. Stem sections had been grown in water (C) or in 10 μM GA_3 for a total of 24 h. Treatment with cutinase took place during the last two or 21 h. The outer epidermis is on the concave side of each section.

Table 5.2. Effect of cutinase on the tissue tension in the lowest 3 cm of the youngest internode of deepwater rice. Stem sections had been grown for 24 h in water (control) or in 10 μM GA_3. Treatment with cutinase took place during the last 2 h of this time period. The values represent the means ± S.E. of the internal angle determined from 44 stem segments.

	Duration of cutinase treatment (h)	
Treatment	0	2
Water	166 ± 2	169 ± 1
GA_3	98 ± 4	149 ± 3

Table 5.3. Effect of cutinase or buffer on the tissue tension of the lowest 3 cm in the youngest internode of rice. Stem sections were grown in 10 μM GA_3 for 24 h. The values represent the mean ± S.E. internal angle of 32 stem segments (16 at 4 h).

	Duration of treatment (h)		
Injected with	0	2	4
Buffer	97 ± 5	83 ± 4	89 ± 4
Cutinase	97 ± 5	123 ± 4	131 ± 5

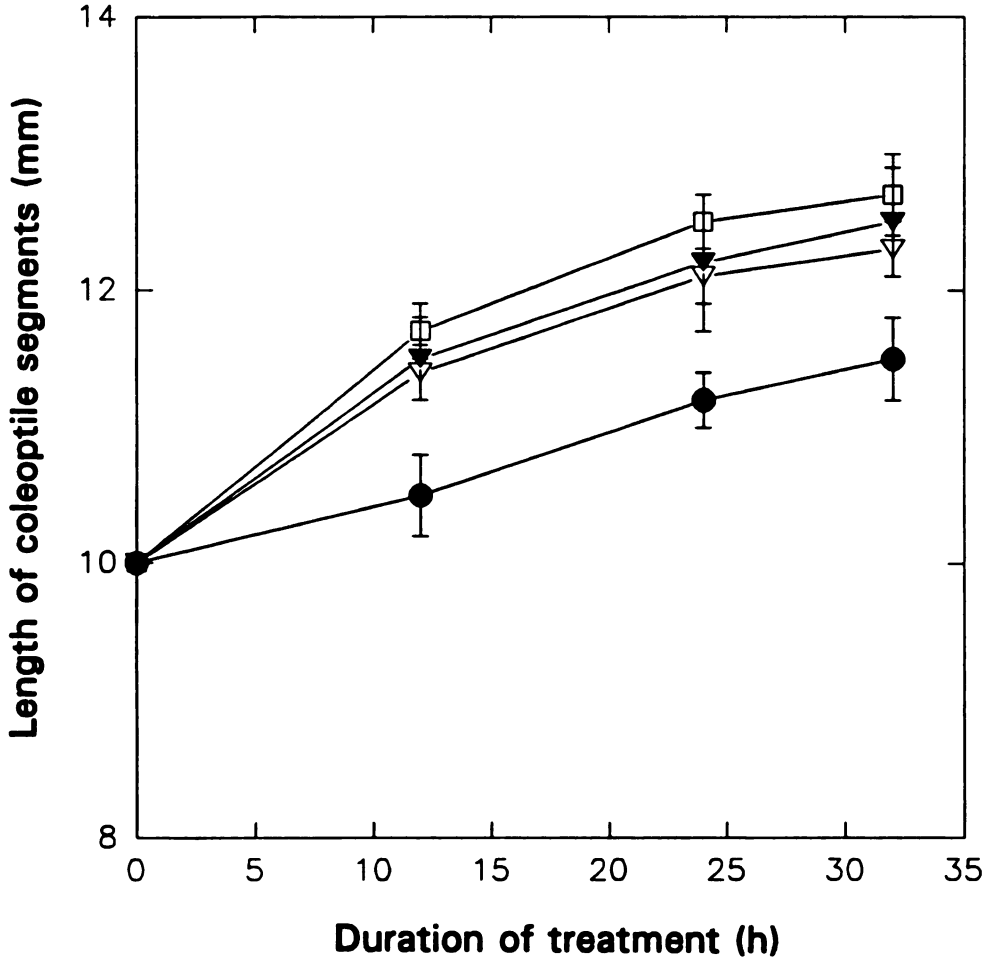


Figure 5.3. Length of coleoptile segments incubated in water or 10 μM IAA with or without cutinase, respectively, for various times (+ IAA, + cutinase: ▼; + IAA, - cutinase: □; - IAA, + cutinase: ▽; - IAA, - cutinase: ●. The values correspond to the means ± S.E. three independent experiments with 4 coleoptile segments each.

cuticle may - at least in part - play a role in limiting growth. A further indication that the cuticle may play a role in limiting growth is my finding that application of cutinase leads to an increase of corn coleoptile growth (Fig. 5.3).

Since the cuticle does not become thinner during increased growth and may contribute to the limitation of growth by the epidermis, there must be increased cuticle synthesis. GA_3 could, thus, affect cuticle biosynthesis either indirectly by inducing growth or, less likely, by acting on enzymes of cuticle biosynthesis directly. I compared the incorporation of [^{14}C]palmitic acid into the cuticle of control and GA_3-treated stem sections. Figures 5.4 and 5.5 show the labeling of the rice cuticle in the lowest and second lowest centimeter of the youngest internode. In both zones, treatment with GA_3 led to an increase in the incorporation of [^{14}C]palmitate when compared to the control plants. This was already visible after the first 2 h of treatment with GA_3 and had increased to a level 20- to 30- fold above that of control plants after 48 h. It was slightly reduced after 72 h, presumably because the internode stopped growing. This result shows that cuticle biosynthesis increases in internodes induced to grow rapidly with GA_3.

Cuticle from control internodes and internodes treated with GA_3 for 48 h was labeled with palmitate and depolymerized using reductive hydrolysis with LiAlH (Kolattukudy, 1970). Its monomeric compounds were separated by TLC, and the presence and amount of radioactivity associated with individual bands was determined using a phosphor imager (Figure 5.6). Ten radioactive bands were evident in the cutin hydrolysate of internodes treated with GA_3. The most prominent band at the running front corresponds to palmitic acid. The amount of radioactivity incorporated into this

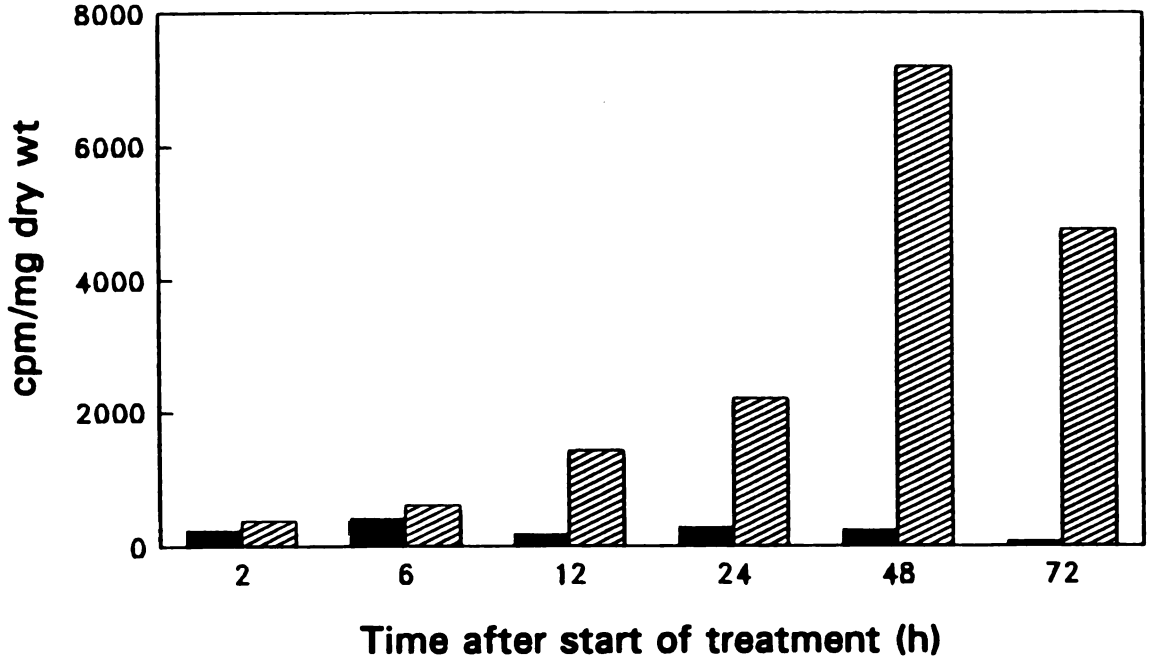


Figure 5.4. Incorporation of [^{14}C]palmitic acid into the lowest 1-cm zone of the youngest internode of deepwater rice. Plants were grown in water (closed bars) or in 10 μM GA_3 (hatched bars) for the indicated times. Labeling was performed during the last 2 h of each period. Each value represents the means of four experiments.

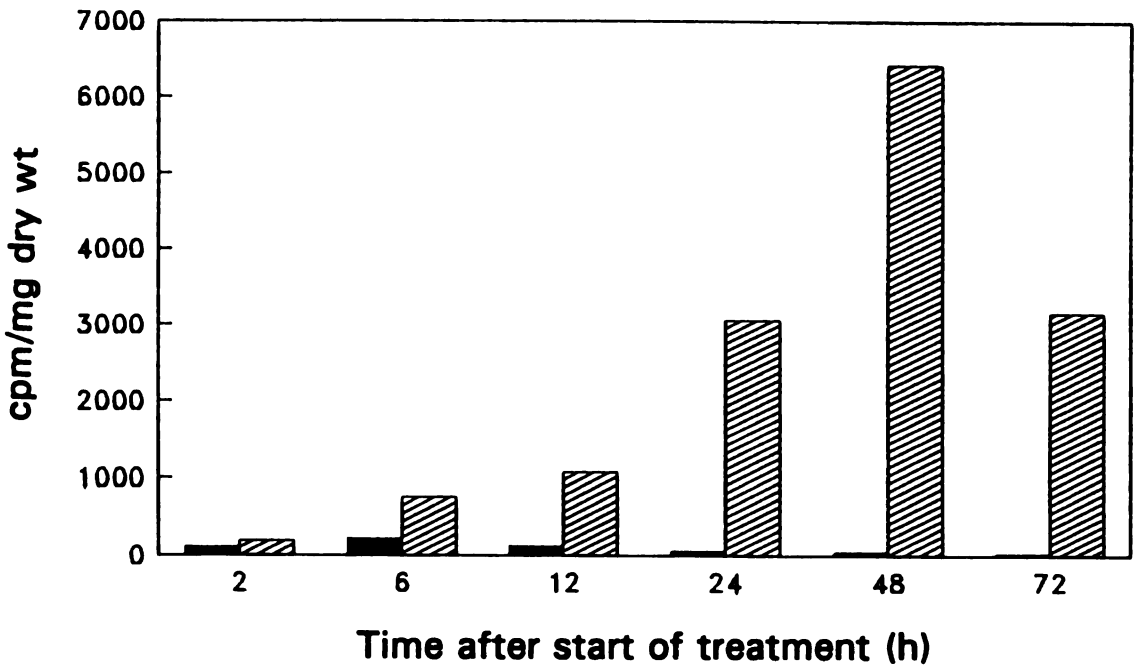
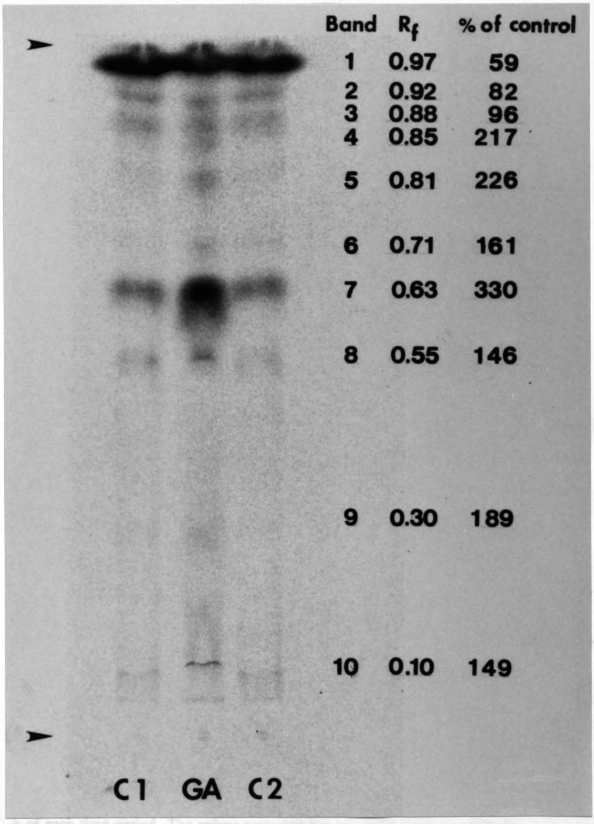


Figure 5.5. Incorporation of [^{14}C]palmitic acid into the second lowest 1-cm zone of the youngest internode of deepwater rice. Plants were grown in water (closed bars) or in 10 μM GA_3 (hatched bars) for the indicated times. Labeling was performed during the final 2 h of each time period. Each value represents the means of four experiments.

band was calculated from a phosphor imager printout measuring the radioactivity in every band and was found to be 59% of the control level in GA_3-treated plants. The second most prominent band (R_f = 0.63) appeared in both, control and GA_3-treated plants. However, it contained over three times more radioactivity in the cuticle hydrolysate of plants treated with GA_3 than in that of control plants. In addition, incorporation of radioactivity into six more bands (R_f = 0.85, 0.81, 0.71, 0.55, 0.30, 0.10) was increased upon GA-treatment. These changes were not evident in plants that had been treated with GA_3 for only 4 h and labeled for the entire time period as compared to plants treated for 48 h (not shown). From the TLC data of Walton and Kolattukudy (1972), it appears that the top three bands correspond to unmodified fatty acids that were incorporated into the cuticle, while the bands below them correspond to mono-, di-, or tri-hydroxy fatty acids (or their epoxy derivatives). This indicates that the activity of enzyme(s) responsible for hydroxylation (Walton and Kolattukudy, 1972) is enhanced in GA_3-treated plants.

The level of [^{14}C]oleic acid incorporation into the rice cuticle was much lower than that of palmitic acid but it also showed an increase in sections treated with GA_3 (Figure 5.7). In this case, GA_3 lead to a two-fold increase in incorporation within 6 h. At 72 h, there was a five-fold increase above the control. The control plants showed no change in the level of oleic acid incorporation. The results of Figure 5.4, 5.5, and 5.7 show that cuticle biosynthesis increased in GA-treated, rapidly growing deepwater rice internodes. However, far more palmitic (C_{16} fatty acid) than oleic acid (C_{18} fatty acid) was incorporated. This confirms the findings of Kolattukudy *et al.* (1974) that the C_{16} family of monomers predominates in rapidly growing plants.

Figure 5.6. Phosphor-imager printout indicating the radioactivity in cutin monomers produced by reductive hydrolysis and separated by TLC. The cutin was obtained from stem sections that were grown in water (C1 and C2) or 10 μM GA_3 (GA) for 48 h and that had been labeled with [^{14}C]palmitic acid for the last 20 h of that time period. Lane C1 contains the same amount of radioactivity as lane GA, lane C2 contains the same amount of extract as lane GA. Percent of control stands for the amount of radioactivity in lane GA as compared to the C1. The values were taken from a phosphor-imager printout scan of each band. The arrowheads at the bottom and top indicate the origin and the solvent front, respectively.


Band
R_f
% of control
1 0.97 59
2 0.92 82
3 0.88 96
4 0.85 217
5 0.81 226
6 0.71 161
7 0.63 330
8 0.55 146
9 0.30 189
10 0.10 149
C1
GA
C2

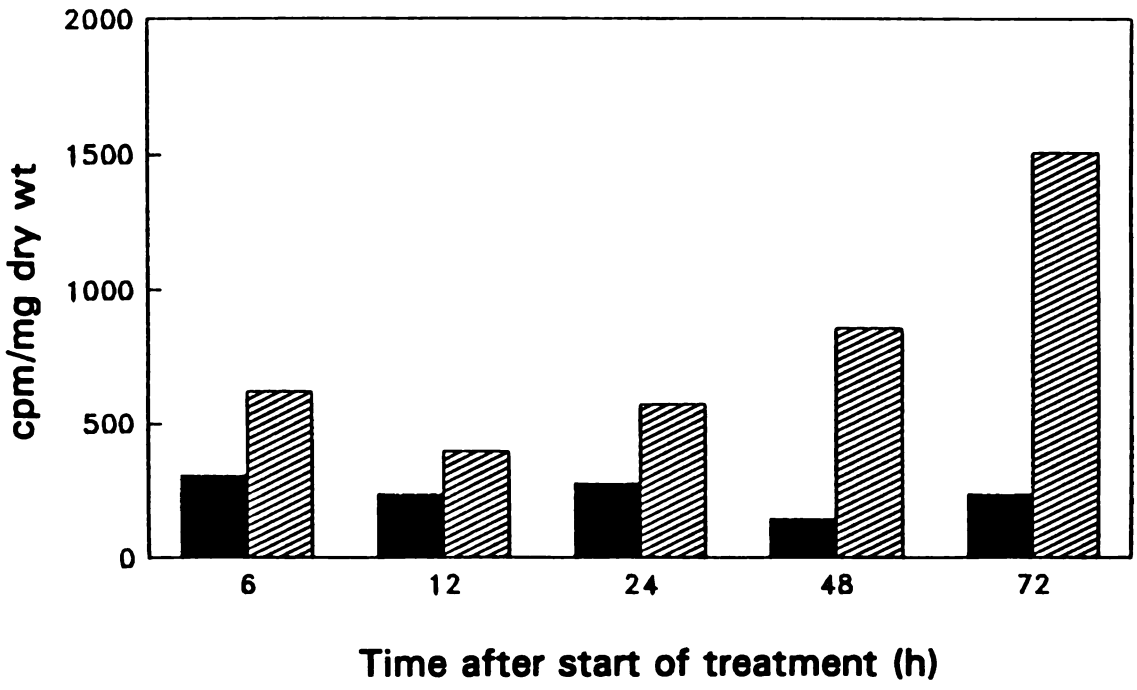
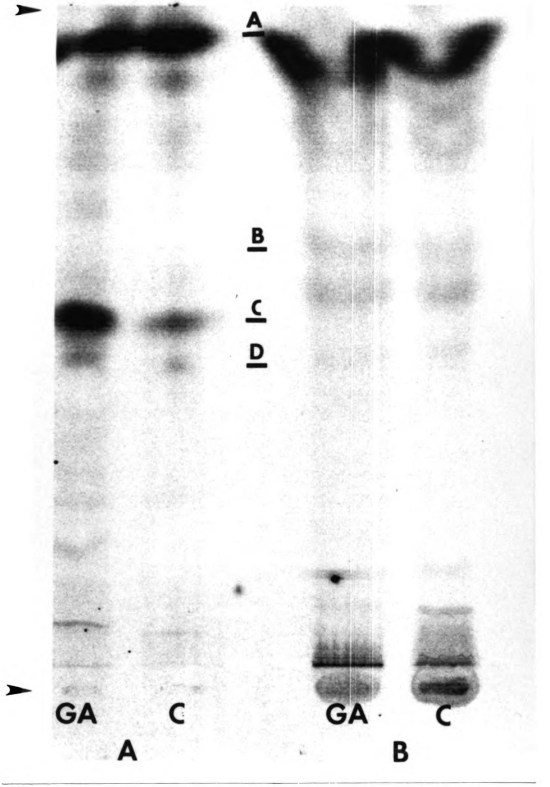


Figure 5.7. Incorporation of [^{14}C]oleic acid into the lowest 1-cm zone of the youngest internode of deepwater rice. Plants were grown in water (closed bars) or in 10 μM GA_3 (hatched bars) for the indicated times. Labeling was performed during the last 2 h of each time period. The values represent the means of three experiments.

To compare the incorporation pattern of oleic and palmitic acid and to identify at least some of the cuticular components on the TLC plate, I separated the products of reductive hydrolysis of the cuticle from stem sections that had been treated with GA_3 or were incubated in water and that had been labeled with [^{14}C]palmitic acid or with [^{14}C]oleic acid. The radioactivity of individual bands was determined using a phosphor imager (Figure 5.8). There were no differences in the pattern of oleic acid incorporation between cuticles of contol and GA_3-treated stem sections (Fig. 5.8 B). However, the differences in the incorporation pattern of [^{14}C]palmitic acid into of stem sections treated with GA_3 or water were confirmed (Figure 5.8 A). The bars between the two sets of radioactive cutin monomers represent the positions of the monomers of apple cutin which had been obtained from Dr. Kolattukudy (Walton and Kolattukudy, 1972) and were run as standards. From a comparison of the R_f values from the second-most prominent band visible in the cuticle of GA_3-treated, palmitic acid-labeled stem sections ($R_f \approx 0.63$) and the standards I conclude that this band corresponds to a hexadecatriol (C). Reductive hydrolysis converts fatty acid components of the cuticle to their corresponding alcohols. Thus, the band from rice cutin with an $R_f \approx 0.63$ was derived from a dihydroxy-hexadecanoic acid in the cuticle. Since it ran parallel with the band in the apple standard that corresponds to 10,16-dihydroxyhexadecanoic acid, I assume that it is the same compound. The above results indicate that treatment with GA_3 leads to a change in the composition of only the C_{16} family of fatty acids while the relative amounts of the fatty acids from the C_{18} family to each other remained the same. In addition, the increase in [^{14}C]palmitic acid incorporation occurred only into cutin components below R_f 0.85.

Figure 5.8. Phosphor-imager printout indicating 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. The arrowheads at the bottom and top indicate the origin and the solvent front, respectively. The bars indicate the positions of standards (A: monool, B: diols, C: triols, D: tetraols).


A
B
C
D
GA
C
GA
C
A
B

According to the literature (Walton and Kolattukudy, 1972) these lower bands contain derivatives of hydroxylated fatty acids. This indicates that the enzyme activity for fatty acid hydroxylation (Walton and Kolattukudy, 1972) is enhanced in GA_3-treated plants. Because total cutin biosynthesis was increased as well, activity of the enzyme responsible for polymerization (cutin transacylase, Croteau and Kolattukudy, 1974) may be enhanced as well. However, I could not find a significant increase in *in vitro* cutin-transacylase activity in GA_3-treated plants as compared to air-grown ones (not shown).

CONCLUSIONS

Gibberellin induces rapid growth in deepwater rice internodes. This growth and the accompanying tissue tension show that the epidermis is the growth-limiting tissue. In the case of rice stem sections there is no thinning out of either the cuticle or the cell wall. In order to maintain the thickness of the cuticle, there has to be an increase in cutin biosynthesis. This indicates that GA may, at least indirectly, influence cuticle biosynthesis and that the cuticle may play a role in influencing growth. Treatment with cutinase shows that this is indeed the case (Figures 5.2, 5.3; Tables 5.1, 5.2). Application of cutinase leads to a reduction of the tissue tension in rice stems and leads to an increase in the growth of corn coleoptiles. I conclude that the cuticle may be a growth-limiting factor.

I also found that GA_3-induced rapid growth leads to an increase in cuticle biosynthesis (Figures 5.4, 5.5, 5.7) in deepwater rice stems, with at least one

quantitative change in cuticle composition (Figures 5.6, 5.8). The cutin monomer whose level increases is probably a dihydroxyhexadecanoic acid. In addition, all other bands with increased incorporation of [^{14}C]palmitic acid appear more hydrophilic on the TLC plate than the free fatty acid derivative and may thus be hydroxylated or otherwise modified fatty acids. Bowen and Walton (1988) had investigated the possibility that GA-induced growth influences cuticle biosynthesis and composition by comparing the incorporation of [^{14}C]palmitic acid into three selected cutin monomers of control and GA-treated stems of *Pisum sativum*. However, in contrast to my results, they found a similar increase of palmitate incorporation into all monomers and concluded that the enhancement of cutin production occurred at the level of monomer biosynthesis. Our experiments indicate that, in GA-treated deepwater rice internodes overall cutin synthesis as well as hydroxylation of cutin monomers are promoted.

REFERENCES

Bowen, D.J., Walton, T.J. (1988) Cutin composition and biosynthesis during gibberellic acid-induced stem extension of *Pisum sativum* var. Meteor. *Plant Sci.* **55**, 115-127.

Croteau, R., Kolattukudy, P.E. (1974) Biosynthesis of hydroxyfatty acid polymers. Enzymatic synthesis of cutin from monomer acids by a cell-free preparation from the epidermis of *Vicia faba* leaves. *Biochemistry* **13**, 3193-3202.

Cosgrove, D. (1989) Characterization of long-term extension of isolated cell walls from growing cucmber hypocotyls. *Planta* **177**, 121-130.

Deas, A.H.B., Holloway, P.J. (1977) The intermediate structure of some plant cutins. In: Tevini, M., Lichtenthaler, H.K. eds., *Lipids and Lipid Polymers in Higher Plants*. Springer Verlag, Berlin, pp. 293-300.

Hoffmann-Benning, S., Kende, H. (1992) On the role of abscisic acid and gibberellin in the regulation of growth in deepwater rice. *Plant Physiol.* **99**: 1156-1161.

Hofmeister, W. (1959) Über die Beugung saftreicher Pflanzen nach Erschütterung. *Ber. Verh. Königl.-Sächs. Ges. Wiss. Leipzig* **11**, 175-204.

Jackson, M.B., Pearce, D.M.E. (1991) Hormones and morphological adaptation to aeration stress in rice. In: M.B. Jackson, D.D. Davies, H. Lambers, eds., *Plant Life under Oxygen Deprivation.* SPB Academic Publishing, The Hague, Netherlands, pp. 47-67.

Kolattukudy, P.E. (1970) Cutin biosynthesis in *Vicia faba* leaves. *Plant Physiol.* **46**, 759-760.

Kolattukudy, P.E. (1977) Lipid polymers and associated phenols, their chemistry, biosynthesis, and the role in pathogenesis. In: F.A. Loewus, V.C. Runeckles, eds., *The Structure, Biosynthesis, and Degradation of Wood.* Plenum Publishing Corp., New York, pp. 185-246.

Kolattukudy, P.E. (1980a) Biopolyester membranes of plants: Cutin and suberin. *Science* **208**, 990-1000.

Kolattukudy, P.E. (1980b) Cutin, suberin, and waxes. In: P.K. Stumpf, ed., *The Biochemistry of Plants. Vol. 4 Lipids: Structure and Function.* Academic Press, New York, pp. 571-645.

Kolattukudy, P.E. (1984) Biochemistry and function of cutin and suberin. *Can. J. Bot.* **62**, 2918-2933.

Kolattukudy, P.E., Croteau, R., Brown, L. (1974) Structure and biosynthesis of cuticular lipids. *Plant Physiol.* **54**, 670-677.

Kolattukudy, P.E., Espelie, K.E., Soliday, C.L. (1981) Hydrophobic layers attached to cell walls, cutin, suberin, and associated waxes. *Encycl. Plant Physiol. New Ser.* **13B**, 225-254.

Kordan, H.A. (1977) Coleoptile emergence in rice seedlings in different oxygen environments. *Ann. Bot.* **41**, 1205-1209.

Ku, H.S., Suge, H., Rappaport, L., Pratt, H.K. (1970) Stimulation of rice coleoptile growth by ethylene. *Planta* **90**, 333-339.

Kutschera, U., Briggs, W.R. (1987) Differential effect of auxin on *in vivo* extensibility of cortical cylinder and epidermis in pea internodes. *Plant Physiol.* **84**, 1361-1366.

Kutschera, U., Bergfeld, R., Schopfer, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. *Planta* **170,** 168-180.

Kutschera, U., Kende, H. (1988) The biophysical basis of elongation growth in internodes of deepwater rice. *Plant Physiol.* **88,** 361-366.

Kutschera, U. (1989) Tissue stresses in growing plants. *Physiol. Plant.* **77,** 157-163.

Métreaux, J.-P., Kende, H. (1983) The role of ethylene in the growth response of submerged deepwater rice. *Plant Physiol.* **72,** 441-446.

Ohwaki, Y. (1967) Growth of rice coleoptiles in relation to oxygen concentrations. *Sci. Rep. Tôhoku Univ.* **33,** 1-5.

Ranson, S.L., Parija, B. (1955) Experiments on growth in length of plant organs. II. Some effects of depressed oxygen concentrations. *J. Exp. Bot.* **6,** 80-93.

Raskin, I., Kende, H. (1984a) Regulation of growth in stem sections of deepwater rice. *Planta* **160,** 66-72.

Raskin, I., Kende, H. (1984b) Role of gibberellin in the growth response of submerged deep water rice. *Plant Physiol.* **76,** 947-950.

Riley, R.G., Kolattukudy, P.E. (1975) Evidence for covalently attached *p*-coumaric and ferulic acid in cutins and suberins. *Plant Physiol.* **56,** 650-654.

Sachs, J. (1865) In: *Handbuch der Experimentalphysiologie der Pflanzen.* Engelmann, Leipzig.

Schönherr, J., Ziegler, H. (1980) Water permeability of *Betula* periderm. *Planta* **147,** 345-354.

Sebastian, J., Kolattukudy, P.E. (1988) Purification and characterization of cutinase from a fluorescent *Pseudomonas putida* bacterial strain isolated from phyllosphere. *Arch. Biochem. Biophys.* **263,** 77-85.

Stünzi, J.T., Kende, H. (1989) Gas composition in the internal air spaces of deepwater rice in relation to growth induced by submergence. *Plant Cell Physiol.* **30,** 49-56.

Turner, F.T., Chen, C.-C., McCauley, G.N. (1981) Morphological development of rice seedlings in water of controlled oxygen levels. *Am. J. Bot.* **29,** 721-738.

Van den Ende, G., Linskens, H.F. (1974) Cutinolytic enzymes in relation to pathogenesis. *Annu. Rev. Phytopathol.* **12,** 247-258.

Vergara, B.S., Jackson, B., DeDatta, S.K. (1976) Deep-water rice and its response to deep-water stress. In: *Climate and Rice.* International Rice Research Institute, Los Baños, Philippines, pp. 310-319.

Walton, T.J., Kolattukudy, P.E. (1972) Enzymatic conversion of 16-hydroxypalmitic acid into 10,16-dihydroxypalmitic acid in *Vicia faba* epidermal extracts. *Biochem. Biophys. Res. Commun.* **46,** 16-21.

Went, F.W., Thimann, K.V. (1937) In: *Phytohormones*, Macmillan Co., New York, NY, pp. 54-55.

Yamada, N. (1954) Auxin relationships of the rice coleoptile. *Plant Physiol.* **29,** 92-96.

CHAPTER 6

GENERAL CONCLUSIONS

Deepwater rice (*Oryza sativa* L.) has a number of physiological and metabolic adaptations that enhance its chances for survival under conditions of temporary flooding. One of these is the capacity of plants to elongate rapidly when they become submerged. This feature helps rice to emerge from the water and to avoid drowning. In seedlings, submergence promotes coleoptile growth (Kordan *et al.*, 1977; Turner *et al.*, 1981; Yamada, 1959) and in adult deepwater rice plants, elongation of the internode (Métraux and Kende, 1983; Vergara *et al.*, 1976). In both instances, the plants respond to the altered gas composition of their submerged organs. In adult rice plants, the reduced O_2 tension promotes ethylene biosynthesis which, in turn, increases the responsiveness of the internodal tissue to GA, which is the ultimate growth-promoting hormone. It causes an increase in cell division and elongation and several ultrastructural changes (for a review see Kende, 1987).

The first question in this thesis was how ethylene causes the responsiveness of the internodal tissue to GA. Through application of inhibitors and measurements of endogenous hormones concentrations, we found that ethylene may change the responsiveness to GA by altering the ratio of a growth inhibitor (ABA) to a growth promoter (GA). Upon ethylene-treatment and upon submergence, the level of endogenous ABA is reduced and the level of GA is increased prior to the onset of growth (Hoffmann-Benning and Kende, 1992a; Chapter 3). Similarly, a reduction of endogenous ABA content by fluridone, an inhibitor of carotenoid biosynthesis, is also

correlated with increased growth of rice coleoptiles (Chapter 2). However, in coleoptiles the growth-promoting hormone is not GA but probably IAA.

In response to submergence, osmiophilic particles appear between plasma membrane and the outer epidermal wall of deepwater rice internodes. These particles were found in the rapidly but not in slowly growing corn coleoptiles (Kutschera *et al.*, 1987), deepwater rice internodes (Kutschera and Kende, 1989; Hoffmann-Benning and Kende, 1992b), and in cucumber hypocotyls (Chapter 4). Experiments using monensin indicate that the osmiophilic particles are secreted via the Golgi pathway. Binding of proteinase K gold to the osmiophilic particles is evidence that they are, at least in part, proteinaceous. They do not appear to be lipid transfer proteins, threonine hydroxyproline-rich glycoproteins, arabinogalactan proteins, or expansin; however, they may contain some peroxidase activity (Chapter 4). My hypothesis is that they are involved in either cell wall or cutin biosynthesis.

To keep up with rapid internodal elongation, there would have to be changes in the rate of cutin biosynthesis or in cutin composition. Both appear to be the case. In deepwater rice that was induced to grow rapidly with GA_3, the rate of incorporation of [^{14}C]palmitic acid into the cuticle was more than 20-fold higher than in control plants. In addition, some cutin monomers were incorporated preferentially in GA_3-treated plants, one being a dihydroxypalmitic acid. This indicates that hydroxylation of cutin monomers and, possibly, their incorporation into cutin is increased in GA_3-treated deepwater rice plants (Chapter 5).

REFERENCES

Hoffmann-Benning, S., Kende, H. (1992a) On the role of abscisic acid and gibberellin in the regulation of growth in rice. *Plant Physiol.* **99,** 1156-1161.

Hoffmann-Benning, S., Kende, H. (1992b) Characterization of osmiophilic particles associated with rapid growth in corn and rice. *Plant Physiol.* **99,** Abstract # 177.

Kende, H. (1987) Studies on internodal growth using deepwater rice. In: Cosgrove, D.J., Knievel, D.P., eds. *Physiology of Cell Expansion during Plant Growth.* The American Society of Plant Physiologists. pp 227-238.

Kordan, H.A. (1977) Coleoptile emergence in rice seedlings in different oxygen environments. *Ann. Bot.* **41,** 1205-1209.

Kutschera, U., Bergfeld, R., Schopfer, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of rice coleoptiles. *Planta* **170,** 168-180.

Kutschera, U., Kende, H. (1989) Particles associated with the outer epidermal wall in internodes of deepwater rice. *Ann. Bot.* **63,** 385-388.

Métreaux, J.-P., Kende, H. (1983) The role of ethylene in the growth response of submerged deepwater rice. *Plant Physiol.* **72,** 441-446.

Turner, F.T., Chen, C.-C., McCauley, G.N. (1981) Morphological development of rice seedlings in water of controlled oxygen levels. *Am. J. Bot.* **29,** 721-738.

Vergara, B.S., Jackson, B., DeDatta, S.K. (1976) Deep-water rice and its response to deep-water stress. In: *Climate and Rice.* International Rice Research Institute, Los Baños, Philippines, pp. 310-319.

Yamada, N. (1954) Auxin relationships of the rice coleoptile. *Plant Physiol.* **29,** 92-96.