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Enzyme Activities and Compounds Related to Self-Defense in UV-Challenged Leaves of Rice

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ABSTRACT : The induction of enzymes and the accumulation of their end products associated with self-defense mechanism in rice were investigated. When rice leaves were irradiated with UV light, activities of diterpene cyclase, phenylalanine ammonia-lyase (PAL), and cinnamic acid 4-hydroxylase (CA4H) were induced and rice phytoalexin, momilactone A was accumulated. The content of ρ-coumaric acid in rice leaves was closely correlated with self-defense or allelopathic potential against barnyardgrass. UV-challenged rice leaves gave rise to the inhibition of barnyardgrass growth.

Keywords : enzymes, phytoalexin, rice, self-defense

Many new chemicals of phytoalexin related to self-defense in plant have been identified in recent years. With a few exceptions, the phytoalexin agents reported from higher plants are secondary compounds that arise from either the acetate or shikimate pathway, or their chemical skeletons come from a combination of these two origins. Secondary metabolites in plants are known to contribute to the allelopathic potential and the defense mechanism against weeds, herbivores and disease organisms.

These compounds do not appear to have any central metabolic function and the more novel one has a limited occurrence throughout the plant world. Hence, the genetic capacity for their biosynthesis is important to explain their roles in a plant community. An expression of the gene related to self-defense during plant growth is controlled by different signaling mechanisms. It is crucial to understand these signaling events if the response of a crop plant to disease, weeds or other environmental stresses is to be manipulated for enhanced self-defense of crop. A comprehensive understanding of the regulation mechanisms of phytoalexin-related gene expression is required to fully understand and exploit the self-defense phenomenon for developing a highly adapted-crop cultivar to natural environment.

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Plants respond to environmental stresses through a variety of biochemical reactions, which may provide protection against the causal agents. The increase of phenolic and terpenoid compounds under environmental stresses has been well documented. For example, an irradiation of the enhanced UV-B light induces the accumulation of phenylpropanoids and flavonoids in different plant species, such as bean, parsley, potato, tomato, maize, rye, barley and rice (Hahlbrock & Scheel, 1989; Ballare *et al.*, 1995; Tevini *et al.*, 1991; Liu *et al.*, 1995).

Until recently, many studies have verified the mechanisms of a self-defense system, including allelopathy in plant, especially in phenylpropanoid and isoprenoid metabolism. All phenylpropanoids are derived from cinnamic acid which is formed from phenylalanine by the catalytic action of phenylalanine ammonia-lyase (PAL), the enzyme at a branch point between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism. It is known that many phenolic compounds show not only physiological function but also plant allelopathic potential.

The isoprenoid compounds are produced from C_5 isoprenoid units and classification of different families of isoprenoids is based on the number of C_5 isoprenoid units present in the skeleton of the compound (Banthorpe, 1991; Gershenzon & Croteau, 1993). Especially, diterpenoids are known to play an important role in the self-defense mechanism of rice, as well as, allelopathic potential. The biosynthesis of diterpenoid phytoalexines such as oryzalexin A, C and momilactone A, B induced by several stresses, is generally used as an index of stress response in rice.

The terpenoids play diverse functional roles in plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastophates), mediators of polysaccharide assembly (polyprenyl phosphates), and structural components of membranes (phytosterols). In addition to the universal physiological, metabolic, and structural functions, many specific terpenoid compounds (commonly in the C_{10}, C_{15}, C_{20} families) serve in communication and defense. For example, induced

specific terpenoids have been correlated with plant-plant, plant-insect, and plant-pathogen interaction.

Recently, Lee *et al.* (1999) isolated momilactone A and B, and oryzalexin A and C from rice straw and found that there was a strong correlation between the accumulation of these phytoalexins and allelopathic ability. It is known that the production of diterpenoid phytoalexins is induced by UV-irradiation, blast fungal infection and treatment of several elicitors such as chitin and jasmonic acid (Cartwright *et al.*, 1981). All of the rice terpenoid phytoalexins have diterpenoid structures and include momilactone A and B, oryzalexin A to F, and oryzalexin S (Kato *et al.*, 1993; Cartwright *et al.*, 1981; Kodama *et al.*, 1992). Studies on the biosynthetic mechanism of phytoalexins in rice would provide valuable information in understanding rice allelopathy or rice-weeds competition.

In order to identify the enzymes in relation to rice self-defense mechanism, we investigated the enzyme activities, their induction, as well as the biosynthetic pathway end products.

MATERIALS AND METHODS

Plant materials and UV irradiation

Rice (*Oryza sativa* L. cv. Kouketsumochi, AUS 196, Dasanbyeo, Donginbyeo, Tang gan, Taichung Native 1 [TN 1] and Waito-C) seeds were kindly provided from the National Yeongnam Agricultural Experiment Station RDA, Korea and the International Rice Research Institute. The cultivars used were selected based on the allelopathic potential from previous study (Kim *et al.*, 1999). Rice seeds of different cultivars were sown in a plastic pot (10 cm in diameter) at one week intervals and grown in a growth chamber, 16 h light/8 h dark until appropriate stage. The leaves of rice growing in the pot were exposed to UV light for 20 min using a Hitachi germicidal lamp (15 W) at a distance of 20 cm. The irradiated plants were placed in the growth chamber until harvesting. The untreated leaves were prepared simultaneously by covering with aluminum foil during UV irradiation. The sample was harvested at 12 h interval till 60 h after UV-irradiation.

Enzyme assays

Diterpene cyclase-Rice fresh leaves (3 g) were homogenized in a mortar and pestle with 6 ml of homogenization buffer: 50 mM Hepes, 150 mM sucrose, 10 mM $Na_2S_2O_5$, 10 mM ascorbate, 10 mM DTT, 10 μM leupeptin, 10% glycerol [v/v], 1 mM EDTA, and 1 mM PMSF). The resulting suspension was squeezed through four layers of cheesecloth and then centrifuged at 39,000 g for 20 min at 4°C. The supernatant was used immediately for enzyme assay. The cyclization reaction was initiated by adding 100 μl [^{3}H]geranylgeranyl pyrophosphate (20 mCi/mMol) to a mixture of 100 μl water, 150 μl of enzyme extract, and 150 μl of incubation buffer containing 5 mM MgCl, and 10 mM KH_2PO_4, pH 7.0 (final incubation volume: 0.5 ml). The mixture was incubated in a 30°C water bath for 40 min and the reaction was stopped by the addition of 1 ml of ethanol : petroleum ether (1:3, v/v). Three successive 1.5 ml extractions with petroleum ether were employed to transfer diterpene hydrocarbons and alcohols from the aqueous reaction mixture into the organic phase. After the volume was concentrated to 0.5 ml in a stream of N_2, the organic layer was transferred to a depth of 3 cm with salicylic acid suspended in hexane. Hexane was employed as the eluting solvent; the first 7 ml of hexane eluant, which contained only the diterpene hydrocarbons, was collected. The radioactivity associated with the hydrocarbon fraction was assayed by adding the hexane eluant to cocktail and measuring the radioactivity in Beckman model LS-6100 Spectrophotometer (Ren & West, 1992).

Phenylalanine ammonia-lyase (PAL)- The fresh leaves were (0.5 g) immediately frozen in liquid nitrogen, and stored at -70°C. Homogenate was prepared by grinding leaves in a mortar using 3 ml of 50 mM Tris-HCl buffer (pH 8.8) containing EDTA (1 mM), β-mercaptoethanol (15 mM) and ascorbic acid (50 mM). All the assay were done at 4°C. The homogenate was filtered through a miracloth and then centrifuged at 39,000 g for 30 min. The supernatant was collected and adjusted to 5 ml with an extraction buffer.

The assay mixture contained 0.1 ml of extract, 1 ml of 50 mM Tris-HCl buffer (pH 8.8), 0.5 ml of 10 mM phenylalanine and 0.4 ml deionized water. The mixture was incubated for 1 h at 37°C and the reaction was terminated by addition of 0.5 ml of 6 M HCl. The blank had the same constituents except that the extract was added followed by an addition of HCl. The acidified mixture was extracted with 7.5 ml of diethylether and the organic phase was subsequently dried in vacuo at 22°C. The residue was dissolved in 3 ml of 50 mM NaOH, passed through a 0.45 μm membrane (Millipore, Bedford, MA, USA) and then the absorbance of the sample at 290 nm was determined. The amount of reaction product was calculated by relating the absorbance of the sample to the calibration curve obtained with cinnamic acid.

Cinnamic acid 4-hydroxylase (CA4H)-Extraction of CA4H from rice fresh leaves (1 g) is accomplished by homogenization of plant material in 2 ml potassium phosphate buffer (200 mM, pH 7.5) containing 2 mM 2-mercaptoethanol. After filtration and centrifugation (15 min at 10,000 g), the supernatant was passed through sephadex G-25 column equilibrated with an extraction buffer. The extract (0.2 ml)

was added to 2 ml reaction buffer (50 mM phosphate buffer containing 2 mM 2-mercaptoethanol, 2 mM trans-cinnamic acid, and 0.5 mM NADPH). Reaction mixture was incubated for 1 h at 37°C. Absorbance value was measured with 290 nm after reaction stopped with 6 M HCl and readjusted to pH 11 with 6 M NaOH (Lamb *et al.*, 1975).

Detection of UV-induced compounds

Phenolic compounds-Rice leaves (0.5 g) harvested just before heading stage were homogenized in a mortar and pestle with liquid nitrogen. Extraction was made with 10 ml of 100% HPLC grade methanol for overnight. Methanol extracts were vortexed, and centrifuged in a microcentrifuge at 10,000 rpm for 5 min. The supernatant was transferred to a new tube and the pellet was re-extracted with an additional 200 μl of methanol. The methanol fractions were combined and the methanol was removed by evaporation at 40°C under a vacuum (Smith *et al.*, 1998). Ten ml of H_2O was put in a flask including the dried methanol fraction and was shaken for 5 min. The hexane fraction (10 ml of Hexane was added to H_2O phase and shaken for 5 min.) was discarded and the water phase was collected and transferred to a new tube. Then ethyl acetate were added to the water phase and shaken for 6 h. The collected ethyl acetate fraction was dried by evaporation at 40°C under vacuum condition. Dried ethyl acetate phase was solved by 1 ml of methanol.

Twenty μL of the extracted sample was injected into a CLC-ODS column (4.6 × 250 mm) fitted with a guard column. The column was equilibrated in water containing 0.075% trifluoroacetic acid with a flow rate of 1 ml/min. After a 5 min injection of the sample, a linear gradient of 0 to 70% acetonitrile was applied to the column over 20 min. UV-absorbing compounds eluting from the column were monitored at 230 and 280 nm.

Isolation of momilactone A-UV-irradiated rice leaves were minced and soaked in 70% methanol for 24 h. The methanol extracts were homogenized in a mixer and filtered. This extract was then dried at 40°C under a vacuum and the residue dissolved in H_2O and extracted 3 times with equal volume of diethyl ether. The organic phases were combined and washed 3 times with equal volume of Pi buffer (1.4 M, pH 6.3) to remove strong acids. The diethyl ether was removed at 25°C under a vacuum and the residue were taken into 90% ethanol and applied to a Sephadex LH-20 column (ϕ2 cm× 45 cm). The fraction was analyzed by GC-MS (HP 6890 GC/HP 5973 MS).

Allelopathic effects of UV-induced compounds

Ground powder of UV-challenged rice leaves was put in distilled water to a final concentration of 10% [v/v] and shaken overnight. The extract was filtered through a funnel and miracloth. Barnyardgrass was sown in a plastic pot filled with air-dried soil and the soil was wet to saturation with the water extract. At every 12 h, the water level was readjusted with distilled water and the shoot length of barnyardgrass was measured after one week.

Electron microscopic observation of barnyardgrass leaves

Sampling was done from the barnyardgrass leaves with and without a treatment of the water extract for a week and fixed in a modified Karnovsky's fixation for 2 h in vacuum. Samples were rinsed with 0.5 M cacodylate buffer solution, postfixed with 1% osmium tetroxide in 0.5 M cacodylate buffer and contained overnight with 0.5% aqueous uranyl acetate. Samples were then dehydrated in graded uranyl acetate and dehydrated in a graded ethanol series, and processed through propylene oxide and embedded in Spurr's medium. Embedded tissue was trimmed and sectioned with a Diatome diamond knife using a Sorvall MT-2B ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate, then viewed on a Zeiss 109 electron microscope.

RESULTS AND DISCUSSION

Activity of enzymes associated with allelopathic activity

There is a lot of evidence for the role of secondary metabolites such as phenolic and terpenoid compounds on plant allelopathic activity. These compounds accumulate in different plant species after being challenged by various environmental stresses. This experiment focused on the effects of UV irradiation on the enzyme activities associated with biosyntheses of terpenoid and phenolic compounds in rice.

Diterpene cyclase activities of Kouketsumochi and Dasanbyeo were rapidly induced from 12 h after UV-challenging and showed to be 2 to 3 times higher than that of AUS 196, as shown in Fig. 1. However, in AUS 196, the activity increased slowly and sustained at a low level. It has been shown that diterpene cyclase has been detected in the UV-treated rice (Wickham & West, 1992). The high level of activity seemed to remain constant from the 24 h to the 48 h and declined rapidly thereafter. The diterpene cyclase activity was induced by not only UV, but also wounding, chitin, fungal invasion and heavy metal (Komada *et al.*, 1988).

PAL activities of Kouketsumochi and AUS 196 were measured at various times after UV irradiation. The PAL

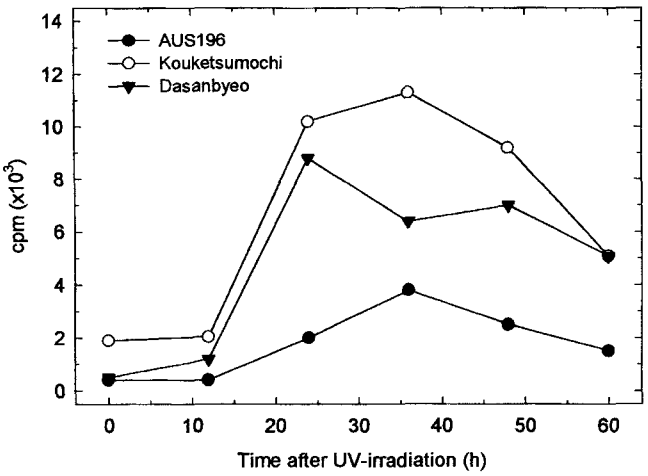

Fig. 1. Diterpene cyclase activity of rice cultivars as affected by UV-irradiation. The rice plant were harvested at the indicated time after UV-irradiation for 20 min.

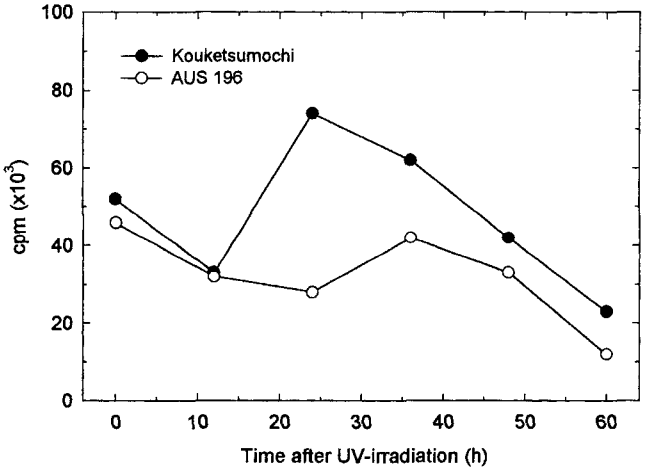

Fig. 2. Time course of PAL activity in different rice cultivars irradiated by UV. The rice plants were harvested at the indicated time after UV-irradiation for 20 min.

activity of Kouketsumochi increased from 12 h after UV-irradiation, peaked at 24 h and thereafter gradually decreased till 60 h, the end of the experimental time course (Fig. 2). However, PAL activity of AUS 196 seemed not to be induced by UV, showing that there was some difference in induction of PAL activities between the two cultivars. At 24 h after UV irradiation, PAL activity in Kouketsumochi was at least two times higher than that of AUS 196, indicating different response to UV between rice cultivars. There are many studies showing that PAL activity in various plants have increased by UV irradiation and various elicitors (Lois *et al.*, 1989). PAL enzyme has been studied extensively in parsley plants and cell cultures in order to investigate the relationship between the activity and plant responses to UV and pathogens (Chappell & Hahlbrock,

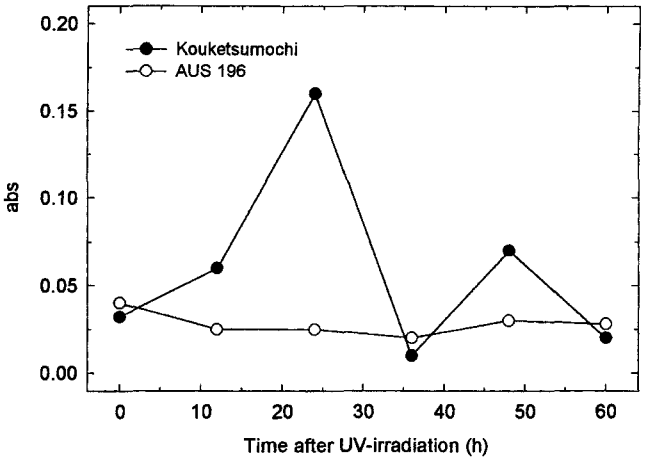

Fig. 3. Change in the CA4H activity of different rice cultivars affected by UV irradiation. The rice plants were harvested at the indicated time after UV-irradiation for 20 min.

1984, Kuhn *et al.*, 1984; Schmelzer *et al.*, 1985; Hahlbrock & Scheel, 1989).

CA4H is the enzyme catalyzing cinnamic acid to ρ-coumaric acid which is a key reaction in the biosynthesis of a large number of phenolic compounds in higher plants. CA4H activity was measured to elucidate how the activity is influenced by UV irradiation in rice leaves. The activity of CA4H in Kouketsumochi was induced by UV and showed peak activity at 24 h after UV irradiation. In contrast, there was no change in the CA4H activity in AUS 196 (Fig. 3). This may indicate that differenial response to UV or other environmental stresses exists in among rice cultivars.

Detection of rice diterpenes in UV-challenged leaves

Several classes of phytoalexins have been found and investigated in connection with allelopathy in rice. Recently, a great deal of attention has been paid on diterpenoid phytoalexins such as momilactones and oryzalexins. In has been reported that momilactone A was detected in UV-challenged leaves (Hahlbrock & Scheel, 1989). As shown in Fig. 4, a certain compound detected in the retention time of 42.54 min by GC/MS analysis was determined as momilactone A based on comparison with standard momilactone. However, it was not detected in the control plant.

In isoprenoid pathway, UV-irradiation increased diterpene cyclase activity in rice and it can be assumed that diterpene compounds can be synthesized in response to UV-irradiation. Many diterpenoids have been detected in rice, for example momilactone A and B, oryzalexins A to F, oryzalexin S and (Kato *et al.*, 1993; Cartwright *et al.*, 1981;

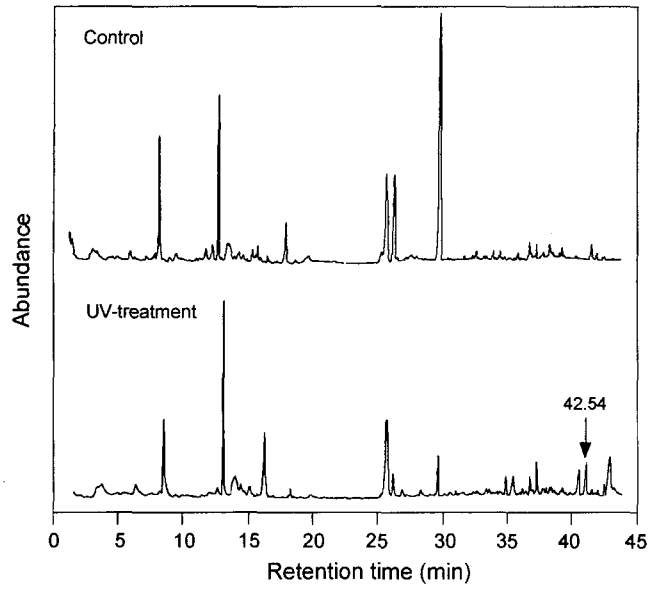

Fig. 4. Gas chromatogram of compounds from Dassanbyeo leaves.

Kodama *et al.*, 1992). However, most of the researches were focused on induction of diterpenoids by environmental stresses and little on cultivar differences. UV-irradiation was employed to induce synthesis of the phytoalexins by many researchers (Back *et al.*, 1998; Kodama *et al.*, 1998; Kato *et al.*, 1994).

Phenolic compounds determined in rice plants

In the enzyme activity of the general phenylpropanoid pathway, the levels of PAL and CA4H activity were changed in the UV-challenged rice leaves, showing rice varietal difference. The biosynthesis of phenolic compounds is regulated by many enzymes in the phenylpropanoid pathway. Therefore, it was necessary to see any variation in the amounts of the phenolic compounds related to the enzymes. Fig. 5 showed that various phenolic compounds detected in different rice cultivars. Especially, the contents of ρ-coumaric acid was about 3 to 5 times higher in Tang gan, Kouketsumochi and TN 1 than any other cultivars when it was determined from rice leaves harvested just before heading stage, the end of vegetative growth. From this stage, secondary metabolites including phenolic compounds will be accumulated, as a similar effect found by UV-irradiation, environmental stresses or pathogen invasion in young plants. In the phenylpropanoid pathway, ρ-coumalic acid is an important intermediate to produce other phenolic compounds and great cultivar difference in the amount of ρ-coumalic acid was observed at the end of vegetative growth stage. But, we are not sure that how the contents of ρ-coumalic acid are influenced according to growing stages in this experiment.

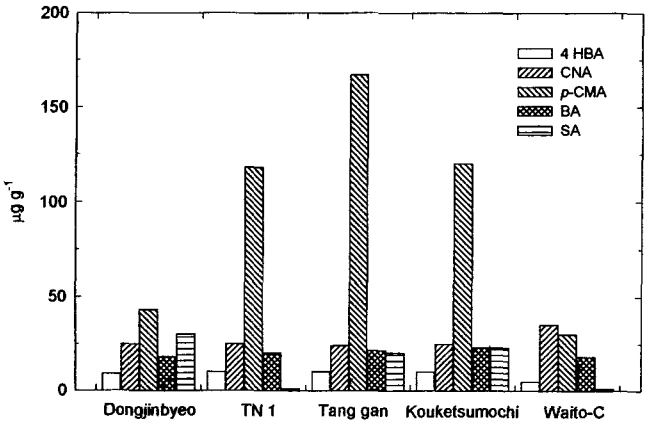

Fig. 5. Intermediate compounds in the phenylpropanoid pathway in various cultivars dentified by HPLC. The rice leaves were extracted with 10 ml methanol and 20 μl of treated sample was injected into a CLC-ODS column. 4HBA : 4-hydroxybenzoic acid, CNA : cinamic acid, ρ-CMA : ρ-coumaric acid, BA : benzoic acid, SA : Salicylic acid.

Allelopathic effects of UV-induced compounds

Differences in the enzyme activity and the amount of phenolic compounds observed in the phenylpropanoid pathway among rice cultivars might result in differential cultivar responses to environmental conditions. The shoot growth of barnyardgrass was completely inhibited by treatment of 10% water extract from Tang gan which contained the highest amount of ρ-coumaric acid (Fig. 6). It is necessary to correlate the amount of ρ-coumaric acid and phytotoxicity to clarify the plant-plant interaction.

According to earlier studies, ρ-coumaric acid was found

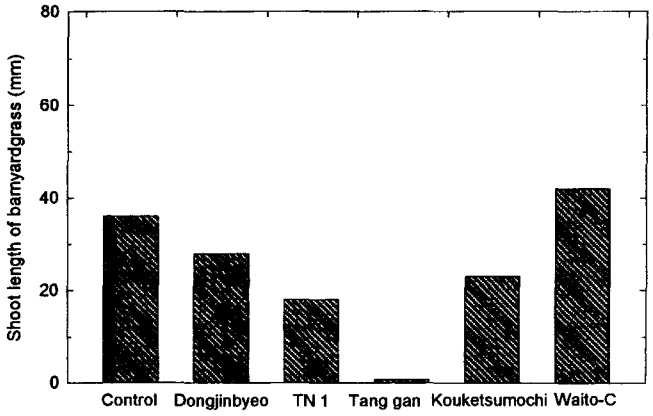

Fig. 6. Shoot growth of barnyardgrass as affected by water extracts from various rice cultivars. Ten percent of water extracts were used for the bioassay.

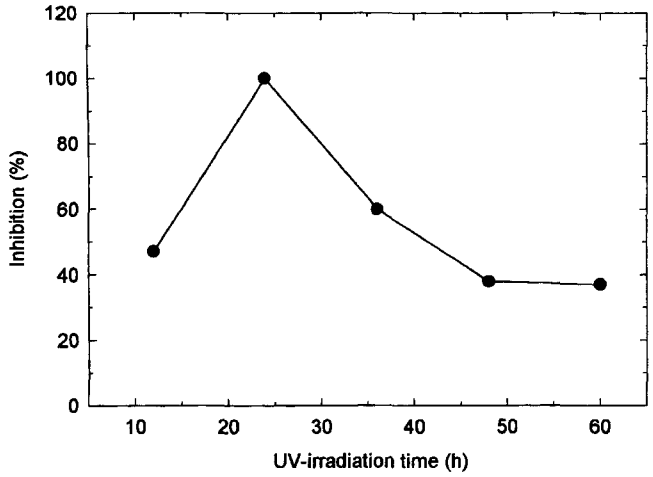

Fig. 7. Shoot growth of barnyardgrass as affected by water extracts from Kouketsumochi irradiated with UV. Ten percent of water extracts were used for the bioassay.

to be an allelopathic substance and existed in rice plants and rice fields. Based on this result, the level of ρ-coumaric acid in plants will be a parameter for determining plant-plant interaction in rice.

In order to determine the effect of UV irradiation on allelochemicals accumulation in plants, water extracts from Kouketsumochi leaves irradiated with UV in different time periods were prepared and used to treat to barnyardgrass (Fig. 7). Shoot growth of barnyardgrass was highly inhibited by UV-irradiated water extracts and showed complete inhibition at 24 h after UV irradiation. Particularly, root growth was completely inhibited by the extracts. This indicates that some metabolites having allelopathic potential might be newly synthesized or highly elevated in rice plants by UV irradiation.

Physical changes in leaf cells of barnyardgrass affected by water extract from rice

The barnyardgrass treated by water extract from rice leaves exhibited several phytotoxic symptoms. First, the most severe symptom was turning the leaf color to yellow or brown, drying and ultimately withering to death. Second, among the inhibitory effect, there was no further growth of the shoot and the root, and they remained very little and weak.

Compared to the cell of untreated barnyardgrass, the mesophyll cell affected by rice leaf extract was damaged. The chloroplast membrane and cell wall were destroyed and the inner materials within the cell were effused (Fig. 8). This means that a compound(s) in rice plants can be served as a toxic chemical to destroy cells of other plants, suggesting a possibility of developing a natural plant-inhibiting chemical.

Fig. 8. Electron microscopy of barnyardgrass leaves as affected by rice leaf extracts. Lower: Electron micrograph of mesophyll cell of untreated control. Intact chloroplast was shown (approx.× 55,000). Upper: Electron micrograph of mesophyll cell affected by the extract. Completely damaged chloroplast was shown (approx. × 55,000).

ACKNOWLEDGMENTS

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