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Phenylalanine Ammonia Lyase and Cinnamic Acid 4-Hydroxylase Activities of Rice and Pepper in response to UV and Wounding

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Abstract

The metabolites related to phenylpropanoid pathway play an important role in the self-defense of plants and induced by environmental stress like wounding, pathogen attack, UV-irradiation and so on. The mRNA level of rice phenylalanine ammonia lyase (PAL) was increased at 12 h to 48 h, however it was gradually decreased 48 h to 60 h after UV irradiation. The PAL enzyme activities in rice were peaked at the time of 24 h after UV irradiation, on the other hand, it was not affected by wounding. The PAL enzyme activities in pepper were raised high at 24 h and 10 h by UV irradiation and wounding respectively. The cinnamic acid 4-hydroxylase (C4H) activities were increased by wounding treatment and were detected from 12 h to end time point of experiment, while UV-irradiation didn't affect the C4H activity in rice and pepper. These results were assumed that the action of isoflavonid has an alternative effect on the defenses which include wounding and UV irradiation and on the diverse roles in rice and hot pepper.

Key words – cinnamic acid 4-hydroxylase (C4H), pepper, phenylalanine ammonia lyase (PAL), rice

Introduction

Plants accumulate various soluble and insoluble, cell-wall associated defense compounds, including a number of phenylpropanoid derivatives as infected with a pathogen or affected by a variety of environmental stress[9,10]. For example, enhanced UV-B light induce the accumulation of phenylpropanoids and flavonoids in different plant species, such as bean, parsley, potato, tomato, maize, rye, barley and rice[10,13,15,17]. Phenylpropanoid compounds comprise a wide range of structural classes and biological functions. Biosynthesis of the compounds can be induced by different stresses such as UV and wounding and it accumulates at a different level depending on plant species or tissues of the same plant. Therefore, it is necessary to elucidate the mechanisms underlying the induction of the phenylpropanoid pathway[1,6].

The phenylpropanoid pathway gives rise to a wide array of metabolites participating in many plant defense responses[18] and absorbing potentially damaging UV-B radiation[5,11,12]. Phenylpropanoids not only serve as structural components (lignin, suberin, and other cell wall-associated phenolics) and flower pigments (flavonoids and anthocyanins) but also have important biological functions such as protectants against biotic and abiotic stresses (phytoalexins, antioxidants, and UV-absorbing compounds) and signal molecules (salicylic acid, nodulation factors).

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Due to their functional importance, the general phenylpropanoid pathway has been extensively studied in a large variety of plants to isolate genes involved in the pathway and to investigate regulation of the corresponding genes and properties of the encoded proteins [7,9,10,24]. Genes encoding some of the enzymes involved in the biosynthesis of phenylpropanoids have been cloned through purification of the soluble enzymes of the phenylpropanoid pathway. These include the genes encoding phenylalanine ammonia-lyase (PAL), ρ-coumarate Co A ligase, caffeic acid/5-hydroxyferulic acid 3/5-o-methyltransferase, chalcone synthase, and chalcone isomerase[2]. Lois *et al.* demonstrated that PAL in parsley is encoded by a small family of at least four genes and the levels of mRNA from the identified PAL genes accumulated in cultured cells by treatment of UV light or fungal elicitor, and in roots or shoots by wounding[16].

All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the catalytic action of PAL, the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism. Many phenolic compounds like ρ-coumaric acid, salicylic acid, benzoic acid and 4-hydroxybenzoic acid are produced in the phenylpropanoid pathway. It is known that those compounds have not only physiologically functional ability, but also plant defense mechanism[7].

Recently, many studies have verified the mechanisms of a self-defense system in plants, especially in phenylpropanoid and isoprenoid metabolism. These studies would provide valuable information in developing crop cultivar which has a system for resistance to a variety of environmental stress or pathogen. It will be required for developing genotypes which have a great adaptability to adverse environment such as pathogen and insect for sustainable agriculture.

This experiment focused on the PAL and C4H activities induced by UV-irradiation and wounding. The enzyme activities and their induction in the phenylpropanoid pathway were investigated in order to identify the enzymes related to higher plant self-defense mechanism.

Materials and Methods

Plant materials

Rice (*Oryza sativa* L. cv. Kouketsumochi and AUS 196) and pepper (*Capsicum annuum* L. cv. Subicho) seeds were soaked overnight in a beaker of aerated water. The germinating seeds were rinsed with water, planted in a plastic pot (10 cm in diameter) at one-week intervals and grown in a growth chamber, 14 h-light/10 h-dark until appropriate stage. For wounding treatment, rice (Kouketsumochi and AUS 196) were grown for 7 days in the dark at 25℃.

UV irradiation and wounding

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 from the top of the plants. The irradiated plants were placed in the growth chamber until harvesting. The untreated leaves were simultaneously prepared by covering with aluminum foil during UV irradiation. The sample was harvested at 12 h intervals till 60 h after UV-irradiation.

For wounding treatment, etiolated seedlings of rice were harvested and the stems were cut into 1 cm sections with a blade. Control (no wounding) tissues were immediately frozen in liquid nitrogen and stored at -70℃. The remainder of the wounded sections was incubated in the dark until collecting for analysis.

Pepper (Subicho) leaves of 8th or 10th leaf stage were detached and exposed with UV light for 15 min using a Hitachi germicidal lamp (15 W) at a distance of 15 cm. The irradiated leaves were placed at 26℃ in a petri-dish with distilled water in a growth chamber, 14 h-light/10 h-dark until harvesting at various times.

For wounding, detached pepper leaves were scratched with iron brush and incubated in the dark until sampling at various times.

RNA blot analysis

Total RNA was isolated from UV-exposed and wounded leaves of rice at various time courses using the phenol/chloroform method[20]. Total RNA (20 μg) was fractionated on 1.0% agarose gel containing formaldehyde and transferred onto nylon membrane (Hybond N+, Amersham). The RNA blot hybridized with the random primed ^{32}P-labeled rice PAL cloned by PCR fragments using a Megaprime Labeling Kit (Amersham) in a QuikHyb solution (Stratagene) at 68℃ for 1 h and then after hybridization the membrane was washed twice at room temperature for 15 min in 2×SSC, 0.1% SDS (w/v) and then once at 60℃ with 0.2×SSC, 0.1% SDS (w/v) for 30 min by the manufacture's protocol (Stratagene).

Enzyme assays

PAL (phenylalanine ammonia lyase) - The fresh leaves (0.5 g) were immediately frozen in liquid nitrogen after harvesting, and stored at -70℃ until use. Homogenate was prepared by grinding leaves in a mortar using 1-2 (v/w) of 50 mM Tris-HCl buffer (pH 8.5) containing β-mercaptoethanol (14 mM) and 5% (w/v) PVP. All the operations were done at 4℃. The homogenate was filtered through miracloth and then centrifuged at 10,000 g for 10 min. Small aliquots (0.1 mL) of the supernatant were incubated with 0.9 mL of 12.1 mM L-phenylalanine in 50 mM Tris-HCl (pH 8.5) at 40℃. A parallel of sample with 0.9 mL of 12.1 mM D-phenylalanine was incubated as control. The formation of cinnamic acid was monitored by taking absorbance readings at 30 min intervals in quartz cuvettes at 290 nm. The amount of reaction product was calculated by relating the absorbance of the sample to the calibration curve obtained with cinnamic acid[8]. PAL activity in μgkats/kg protein as: 27780×($\triangle A_{290}$ L-Phe/60 min - $\triangle A_{290}$ D-Phe/60 min)/μg protein per incubation.

C4H (cinnamic acid 4-hydroxylase) - The fresh leaves (1 g) were immediately frozen in liquid nitrogen after harvesting, and stored at -70℃ until use. Extraction from rice fresh leaves (0.5 g) was accomplished by homogenization of plant material in 1 mL Tricine buffer (50 mM, pH 7.5) containing 1 mM EDTA, 0.3 M mannitol, 0.1% BSA and 10% PVP. After filtration and then centrifuged at 10,000 g for 5 min. Then the supernatant was centrifuged at 21,000 g for 15 min. For precipitation of microsomes, the supernatant was centrifuged at 106,000 g for 1 h. The precipitate was resuspended with potassium phosphate buffer (10 mM, pH 7.5) containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, and 15% glycerol. Protein amounts adjusted 15 mg/mL by Bradford protein quantification method. Fifty $\mu\ell$ microsomal extract was added to 480 $\mu\ell$ Tricine buffer (50 mM, pH 7.5) containing 1 mM EDTA, 10 mM KCl, 0.1 mM ascorbic acid, 2 mM trans-cinnamic acid, and 0.5 mM NADPH. Reaction mixture was incubated for 30 min at 30℃. Absorbance value was measured at 340 nm after reaction stopped with 6 N HCl and readjusted to pH 11 with 6 N NaOH.

Results and Discussion

PAL expression in rice cultivar Kouketsumochi and AUS 196

In order to investigate regulation of PAL gene expression by UV irradiation, patterns of mRNA accumulation of rice were analyzed. The amount of mRNA of PAL of rice cultivar Kouhetusmochi was increased at 12 h to 48 h after UV irradiation however decreased at 48 h to 60 h. The PAL expression patterns of rice cultivar AUS 196 were much the same of Kouketsumochi, on one side, the amount of mRNA smaller than Kouketsumochi (Fig. 1). Lois *et al.* (1989) demonstrated that the mRNA levels of three PAL genes were considerably higher in UV- or elicitor-stimulated

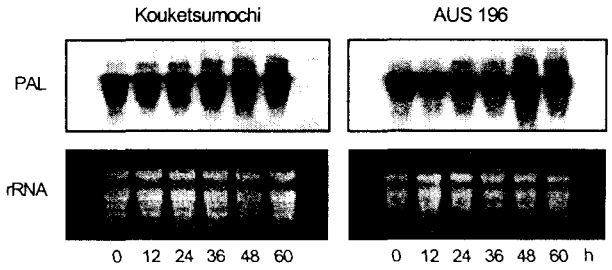

Fig. 1. Total RNA gel blot analysis of PAL in UV-exposed leaves of rice. Total RNA was extracted from the rice leaves collected at 12, 24, 36, 48, and 60 h after UV-exposed and untreated leaves as a control. Twenty μg of total RNA were separated per lane on a denatured formaldehyde gel. After transferring RNA into nylon membrane, the blot was sequentially hybridized with ^{32}P-labeled PAL fragment.

cells, as well as in wounded leaves or roots, than in untreated controls in parsley[14]. The PAL gene was induced even in the control plant which is not irradiated. This suggests that the gene was constitutively expressed under normal condition[16].

PAL enzyme activities in rice cultivar Kouketsumochi and AUS 196

PAL activities of Kouketsumochi and AUS 196 were measured at various times after UV-irradiation and wounding (Fig. 2). The PAL activity of Kouketsumochi increased from 12 h after UV-irradiation, peaked at 24 h and thereafter gradually decreased till 60 h. However, the activity of AUS 196 was not induced by UV. It is hard to correlate the PAL mRNA transcripts with PAL enzyme activity because the PAL activity was greatly induced at 24 h after UV-irradiation in Kouketsumochi but the PAL mRNA accumulation was not changed upon treatment of plant with UV light. There are many studies showing that PAL activity in various plants was increased by UV-irradiation and various elicitors[21]. By wounding, the PAL activities were not stimulated in two cultivars that was different responses to the respective stresses.

PAL enzyme activities in pepper cultivar Subicho

It was apparent that PAL of pepper was induced by UV-irradiation and wounding in pepper. The PAL activity was not only stimulated by UV-irradiation with a maximum at 24 h after exposure but also induced by wounding with a maximum at 8 h after treatment. However, the activity declined to the level of untreated control in 60 h after UV-irradiation and in 36 h after wounding (Fig. 3).

Comparing to rice PAL activity, different response on the wounding was observed in pepper. This indicates that there are some differences in the mechanism of response to stress including signal reception, transduction, and gene regulation between plant species.

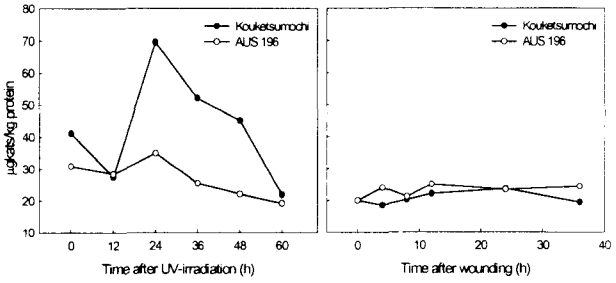

Fig. 2. Time course of PAL activities in different rice cultivars in response to UV exposure and wounding. At the indicated times after UV-irradiation and wounding the shoots were harvested and their PAL activity was determined.

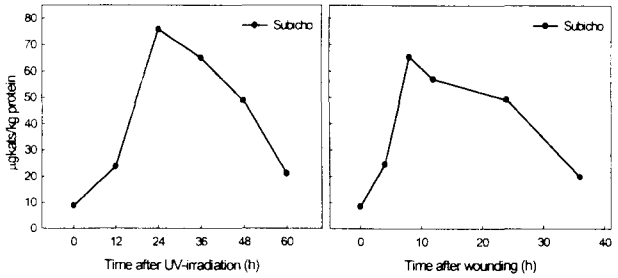

Fig. 3. Time course of PAL activities in pepper in response to UV exposure and wounding. At the indicated times after UV-irradiation and wounding the shoots were harvested and their PAL activity was determined.

C4H enzyme activities in rice cultivar Kouketsumochi and AUS 196

C4H (cinnamic acid 4-hydroxylase) is the enzyme catalyzing hydroxylation of trans-cinnamic acid into trans-ρ-coumaric acid which is a key reaction in the biosynthesis of a variety of phenolic compounds in higher plants. The enzyme activity was determined to elucidate how the activity is affected by environmental stresses and is regulated in different plant species. The C4H activities were not induced by UV-irradiation, regardless of plant species or cultivar and UV-irradiation times (Fig. 4 and Fig. 5). However, there was increase in the activity in rice cultivar, Kouketsumochi and pepper by wounding. The C4H activities in Kouketsumochi and pepper peaked at about 10 h after wounding.

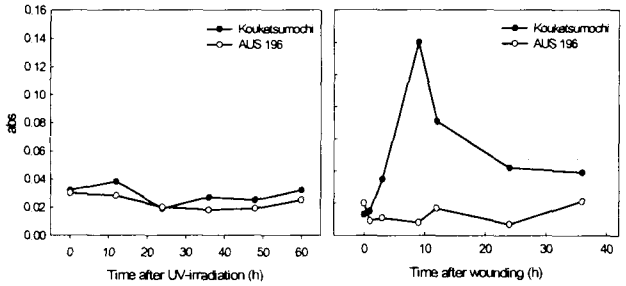

Fig. 4. Time course of C4H activities in different rice cultivars in response to UV exposure and wounding. At the indicated times after UV-irradiation and wounding the shoots were harvested and their C4H activity was determined.

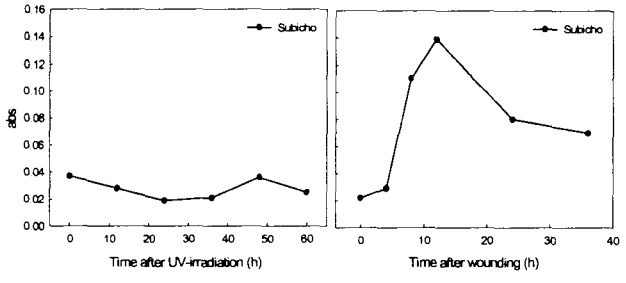

Fig. 5. Time course of C4H activities in pepper in response to UV exposure and wounding. At the indicated times after UV-irradiation and wounding the shoots were harvested and their C4H activity was determined.

Based on the results, it can be concluded that each genotype of rice possesses its own system for responding to environmental stresses. It will make possible to screen the germplasm which has a potential to resist various stresses.

Many phenylpropanoid compounds are induced in response to wounding or to feeding by herbivores[22]. Teutsch *et al.* reported that C4H is highly inducible by light and involved in the synthesis of plant defense compounds by wounding and pathogen infection. A number of stimuli including light, elicitors, and wounding stimulated the C4H activity[3,4,19,23].

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초록 : 벼와 고추에서 UV와 상처가 PAL 및 C4H 효소 활성에 미치는 영향

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Phenylpropanoid pathway 생성물질은 식물의 self-defense에 관계하며 이러한 물질들은 UV뿐 아니라 wounding, pathogen과 같은 environmental stress에 의해 생성되는 것으로 알려져 있다. 벼에서 PAL mRNA 는 UV 조사 후 12시간에서 48시간까지는 증가하였으나 48시간부터 60시간까지는 점점 줄어드는 경향을 보였다. 한편 PAL의 활성은 UV 조사 후 24시간에서 가장 높았지만 상처에 의해서는 PAL의 활성이 벼에서는 증가하지 않았다. 그러나 고추에서는 UV조사와 상처를 준 후 24시간과 10시간에서 각각 높은 활성을 나타내었다. 벼와 고추 모두 cinnamic acid 4-hydroxylase의 활성은 상처를 준 후 12시간에서 증가하였지만 UV 조사는 C4H 활성에 영향을 주지 않았다. 이러한 결과로 볼 때 벼와 고추에서는 UV 조사와 상처가 모두 PAL, C4H 효소활성에 영향을 주는 것으로 나타났다.