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Mechanism of Avenanthramide Induction*

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Oat leaves produce phytoalexins, avenanthramides, in response to pathogen infection and elicitor treatment. Feeding experiments with labeled precursors and measuring enzyme activities revealed the biosynthetic pathway for avenanthramides. In addition, the enzyme that catalyzes the final biosynthetic reaction was identified. An accumulation of similar hydroxycinnamic acid amides was found in maize and barley under stress, suggesting the generality of the defense reaction that involves hydroxycinnamic acid amides. The metabolism of avenanthramides was analyzed using labeled compounds. Avenanthramides were metabolized by the oat leaf itself and were incorporated into cell walls. The elicitor treatment induced peroxidase activity that accepts avenanthramides as substrates. These findings suggested that avenanthramides serve as substrates for the reinforcement of cell walls.

Keywords: phytoalexin, avenanthramide, biosynthesis, metabolism, *Avena sativa*.

1. INTRODUCTION

Plants express various biochemical reactions in response to biological stress including pathogen infection. Among these reactions, the accumulation of low-molecular-weight antimicrobial compounds, phytoalexins, plays an important role. In oats, avenanthramides, a series of substituted cinnamic acid amides with anthranilates, have been identified as phytoalexins. The significance of the accumulation of avenanthramides has been shown in the interactions between oat cultivars and crown rust fungus races. In addition to the pathogen infection, the treatments with elicitors, such as *N*-acetylchitooligosaccharides, HV-toxin C, and heavy metal ions, cause avenanthramides to accumulate.

In the present study, the biosynthesis of avenanthramides was investigated to fill the gaps in knowledge regarding the biochemical changes induced by elicitors in oats. In addition, since avenanthramides can be classified as hydroxycinnamic acid amides, the generality of the defense reaction involving these amides was examined in other gramineous plants, maize and barley. The results suggested the biological significance of the metabolism of avenanthramides in the defense reaction. Thus, the metabolism of avenanthramides was further analyzed in elicited oat leaves.

* See Part II for the full Japanese article.

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2. BIOSYNTHESIS OF AVENANTHRAMIDES

To elucidate the biosynthetic pathway producing avenanthramides, feeding experiments with putative precursors labeled with isotopes were carried out. Oat leaf segments were treated with elicitor in the presence of [ring-U-^{14}C]anthranilate, [2,3,4,5,6-$^{2}H_5$]L-phenylalanine or [8,9-$^{13}C_2$]*p*-coumaric acid. These compounds were effectively incorporated into avenanthramides. Thus, the hydroxyanthranilate part of avenanthramides is derived from anthranilate, while the hydroxycinnamic acid part is synthesized from L-phenylalanine *via* the phenylpropanoid pathway. Sodium [$^{13}C_2$]acetate was also incorporated into avenanthramide L which has an avenalumoyl (5-(4-hydroxyphenyl)-2,4-pentadienoyl) moiety, indicating that the avenalumoyl moiety is synthesized from *p*-coumaric acid through elongation of the side chain with an acetic acid unit.

The amounts of avenanthramides and the activities of putative biosynthetic enzymes were measured in elicited leaves. The amounts of avenanthramides reached a maximum 24-48 hr after elicitation. The accumulation was preceded by a marked increase in the activities of anthranilate synthase (AS), chorismate mutase (CM), and enzymes of the phenylpropanoid pathway. Thus, the accumulation of avenanthramides is the result of a concerted activation of the enzymes in the biosynthetic pathway.

In plants, it has been demonstrated that several isozymes are present in AS and CM and that the isozymes may have distinct biological roles. The expression of the isozyme genes

of the anthranilate synthase α subunit was investigated in elicited oat leaves. A probe specific to each isozyme was obtained by RT-PCR with poly $(A)^+$ RNA from elicited oat leaves using primers designed from ASα gene sequences in rice. RNA gel blot analysis with the probes revealed that at least two isozyme genes are present in oats and that one is responsive to elicitation. In the case of CM, the activity separated into two peaks on anion-exchange chromatography with MonoQ. A comparison of the chromatograms for extracts from elicited and intact leaves indicated that one isozyme is elictor-inducible. Accordingly, specific isozymes of AS and CM are responsible for the biosynthesis of avenanthramides.

The final step in the biosynthesis was thought to be the condensation reaction between hydroxyanthranilic acids and hydroxycinnamic acids. The author assumed that CoA esters of hydroxycinnamic acids are the substrates for the reaction, and examined the enzyme (hydroxyanthranilate *N*-hydroxycinnamoyl transferase, HHT) activity in the crude extracts from oat leaves. A marked activity was detected in elicited leaves, while no activity was found in the intact leaves. All of the putative precursors of avenanthramides served as substrates for HHT, indicating that the enzyme is directly involved in the formation of avenanthramides.

3. INVOLVEMENT OF HYDROXYCINNAMIC ACID AMIDES IN DEFENSE MECHANISMS IN MAIZE AND BARLEY

The phenylpropanoid pathway has been demonstrated to be activated in response to biological stress in numerous plants. However, the compounds produced *via* the pathway under the stressed conditions have been rarely identified. The finding that the pathway is activated for the production of avenanthramides in oats suggested that an accumulation of similar hydroxycinnamic acid amides may be induced in other gramineous plants as a common response to stress. Thus, the generality of the defense reaction involving hydroxycinnamic acid amides was examined in maize and barley.

The first leaves of maize seedlings were damaged mechanically, and incubated. An HPLC analysis of the leaf extracts revealed that *p*-coumaroyl and feruloytyramines accumulated in response to wounding. The accumulation of hydroxycinnamoyltyramines was accompanied by an increase in tyramine *N*-hydroxycinnamoyltransferase (THT) activity. The accumulation of *p*-coumaroyl and feruloyltyramines was also induced by infection with the causal agent of southern corn leaf blight, *Bipolaris maydis*. The accumulation of amides may thus be a part of the defense reaction of maize against pathogenic fungi.

The effects of jasmonic acid (JA) on the production of hydroxycinnamic acid amides were investigated in barley. JA has been indicated to mediate the signals from various types of stress to evoke defensive reactions. Treatment of barley leaves with 100 μM JA induced the accumulation of *p*-coumaroylagmatine. The enzyme activity that catalyzes the condensation reaction between agmatine and *p*-coumaroyl-CoA (agmatine *N*-*p*-coumaroyltransferase, ACT) was also enhanced by JA treatment.

In gramineous plants, three different species namely oats, maize and barley were demonstrated to produce hydroxycinnamic acid amides in response to stress. Avenanthramides in oats have been indicated to possess antimicrobial activity, and shown to act as phytoalexins. However, antimicrobial activity has not been detected in the amides in maize and barley, suggesting a different function for these compounds. One plausible function of the amides is in the reinforcement of cell walls under stress conditions. In potato and tobacco, hydroxycinnamic acid amides have been indicated to be incorporated into cell walls in wounded tissues. Similarly, the incorporation of amides into cell walls may also be important in the gramineous plants.

4. METABOLISM OF AVENANTHRAMIDES

Some evidence to support the metabolism of avenanthramides has been obtained from studies on the biosynthesis of avenanthramides. For instance, the amounts of avenanthramides reached a maximum 24–48 hr after elicitation, and thereafter, gradually decreased. Feruloyl-CoA was the best substrate for HHT, but avenanthramide B which has a feruloyl moiety was not necessarily the major component among avenanthramides. This can be explained by a rapid metabolism of avenanthramide B in comparison with other avenanthramides. On the basis of this circumstantial evidence, the metabolism of avenanthramides was analyzed.

First, the author attempted to detect the metabolism using isotope-labeled avenanthramides. The transfer of elicited oat leaves to solutions containing avenanthramides labeled with ^{13}C atoms resulted in a rapid decrease in the labeled avenanthramides, indicating the metabolism of avenanthramides. Next, the rates of biosynthesis and metabolism of avenanthramides A and B were analyzed in detail based on a model of isotope-labeling dynamics. Avenanthramide B was found to be more actively biosynthesized and metabolized than avenanthramide A. Radiolabeled avenanthramide B was fed to elicited oat leaves to elucidate its metabolic fate. The analysis revealed that elicitor treatment enhanced the incorporation of avenanthramide B into the cell walls. The conversion of avenanthramide B to its dehydrodimers in elicitor solution was also activated by elicitation.

The formation of dehydrodimers of phenolic compounds is catalyzed by peroxidase. Thus, the effects of peroxidase inhibitors on the avenanthramide metabolism were investigated. The decrease in labeled avenanthramides was suppressed by catalase, salicylhydroxamic acid, and sodium ascorbate. In addition, peroxidase activity that accepts avenanthramide B as a substrate was induced in apoplastic fractions by elicitor treatment. Furthermore, multiple basic isoperoxidases were detected by activity staining with 3-amino-9-ethylcarbazole coupled with the isoelectric focusing of proteins from elicitor-

treated leaves. These findings indicated that avenanthramides are metabolized in apoplasts by inducible isoperoxidases.

In addition to the production of avenanthramides in oats, the involvement of hydroxycinnamic acid amides in the defense reaction has been found in maize and barley. The hydroxycinnamic acid amides may have originally been the substrates for the reinforcement of cell walls because the amides in maize and barley have no antimicrobial activity. Avenanthramides may have been recruited as phytoalexins from those compounds due to their toxicity against pathogens, and may still have dual roles, i.e. as phytoalexins in chemical defense and as substrates for the reinforcement of cell walls in physical defense. It seems interesting that the degree of contribution to these roles is dependent on the species of avenanthramides because this suggests a functional differentiation among avenanthramide species.