The Formation of Phenolic Acids in the Root of Downy Mildew-Infected Japanese Radish*

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Abstract

Quinic, prephenic, phenylpyruvic, trans-cinnamic, p-coumaric, and caffeic acids were identified by gas-liquid chromatography in the root extract of Japanese radish infected by Peronospora parasitica. Ferulic and sinapic acids were not detected. These phenolic acids detected did not originate from the infecting hyphae but from the host cells. These phenolic acids were not detected in the healthy root. Since the phenolic acids are considered to be precursors of lignin, the relationship between the phenolic acids detected and lignin formation in the infected plants was discussed.

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Introduction

The biosynthesis of lignin in plants, or the lignification process, has attracted the attention of many lignin chemists during recent years. In the field of plant pathology, the elucidation of host-parasite interaction is one of the most important problems, and lignification of the cell walls in infected plants is considered to be a result of interaction between the fungal hyphae and hosts. Although knowledge on the role of phenolic compounds in plants is comprehensive, little has been known in connection with lignin formation and phenolic metabolism in fungus-infected plants. In a previous paper, we reported the formation of lignin in the Japanese radish root infected by Peronospora parasitica. In order to understand the presence of lignin precursors in the infected root, we examined the constituents of phenolic acids.

Materials and methods

Inoculation. Well-developed roots of about 3-month-old Japanese radish (Raphanus sativus L. var. hortensis Backer f. minowase Kitam.) cultivated in our experimental farm were used. The roots (ca. 5 cm in diam.) were aseptically cut into slices about 1 cm thick. The slices were inoculated with conidium suspensions of Peronospora parasitica Pers. ex Fr., which had been cultured on root slices. Inoculated slices were incubated in moistened Petri dishes for 4 days at 20°C under 110 lux illumination from white fluorescent lamps. Subsequently the fungus-infected tissues (ca. 3 mm thick) were removed with a knife. Non-inoculated roots without incubation served as controls.

Isolation of phenolic acids. Uritani and Muramatsu's method was slightly modified. Five

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kg of slices were boiled at 100°C for 1 hr and homogenized with a Waring blender. The homogenate was extracted twice with 10 l of acetone for 48 hr and filtered with a Büchner funnel. The filtrate was concentrated with a rotary evaporator to ca. 2 l. Kieselgur was added to 2% and the mixture was stirred for 2 hr and filtered. Five hundred ml of 10% lead acetate was added and the mixture centrifuged at 2,500 × g for 5 min. The precipitate was then extracted twice with 500 ml of 5% acetic acid under reflux for 1 hr and filtered. The filtrate was adjusted to pH 8.5 with 28% NH₄OH and centrifuged at 2,500 × g for 5 min. The precipitate was washed with 200 ml of water and centrifuged at 2,500 × g for 5 min. The precipitate was suspended with 200 ml of water and slowly mixed with 100 ml of 10% H₂SO₄ and centrifuged at 2,500 × g for 5 min. Then KH₂PO₄ was added in the supernatant to make 2 Mol and the pH was adjusted to 2.2 with 20% NaOH. The solution was extracted with 500 ml of ethyl acetate under shaking for 2 hr and centrifuged at 2,500 × g for 5 min and crude phenolic acids were obtained.

Identification of phenolic acids. Trimethylsilylation. A mixture of hexamethyldisilazane and trimethylchlorosilane in dioxane (1:1:8) was added to 15 mg of the extract. After occasional stirring for 5 min, the liquid was centrifuged at 2,500 × g for 5 min. An aliquot (5 μl) of the supernatant was injected into the gas chromatograph. Gas-liquid chromatography. Analyses were carried out by dual column operation with a Shimadzu GC-5A equipped with a hydrogen flame ionization detector using glass columns of 2 m × 3 mm inside diameter, containing Chromosorb W, 60-80 mesh, coated with 1.5% SE-30. The column temperature was programmed to provide a 1 min interval at 150°C, after injection, then a 10°C/min rise to 250°C followed by 5 min at the upper limit. Injection port temperature was 250°C. Nitrogen was used as carrier gas, and gas flow was adjusted around 60 ml/min, and the flow rate of hydrogen gas was 50 ml/min. The auxiliary gas was air, with flow rate of 700 ml/min.

Results and discussion

Fig. 1 shows the gas-liquid chromatograms of phenolic acids obtained from the healthy (control) and diseased roots. The trimethylsilylated extract of the diseased roots showed 20 separated peaks with retention times at 3.7, 4.3, 4.6, 4.9, 5.1, 5.7, 5.9, 6.3, 6.7, 7.2, 7.4, 7.8, 8.1, 8.3, 8.7, 9.3, 10.5, 11.8, 12.9 and 14.6 mins. Among these retention times, 3.7, 4.9, 5.7, 5.9, 6.3, 8.1, 8.3, 9.3, 10.5 corresponded with those for trans-cinnamic acid, p-hydroxyphenylacetic acid, phenylpyruvic acid, vanillic acid, prephenic acid, quinic acid, p-coumaric acid, and caffeic acid, respectively. The others were not identified. Further gas-liquid chromatography of the extract and a mixture of authentic compounds gave identical traces. No shikimic, chorismic, p-hydroxyphenylpyruvic, ferulic, sinapic and chlorogenic acids were detected. Also, no phenolic acids except p-hydroxyphenylacetic acid were detected in extracts from healthy roots. Therefore, it is apparent that the metabolism of aromatic compounds in the diseased roots differs from that occurring in the healthy roots.

Extensive studies carried out principally by Freudenberg and his colleagues in the early 1940's suggested that coniferyl alcohol was involved in the polymerization reactions of lignification. Brown and Neish extended their studies with radiotracers to a number of phenylpropanoid acids which they considered as potential intermediates in lignification, and p-coumaric and caffeic acids were found to be comparable to phenylalanine, which was previously shown to be efficiently utilized as lignin precursors. In the meantime, Koukol and Conn demonstrated the presence of phenylalanine ammonia-lyase (EC 4.3.1.5), an enzyme catalyzing the conversion of L-phenylalanine to trans-cinnamic acid and ammonia. The enzyme was also found only in higher plants and some fungi,
Fig. 1. Gas-liquid chromatograms of trimethylsilylated phenolics detected in the healthy (H) and diseased (D) roots.

1: trans-Cinnamic acid, 4: p-Hydroxyphenylacetic acid, 6: Phenylpyruvic acid, 7: Vanillic acid, 8: Prephenic acid, 13: Quinic acid, 14: p-Coumaric acid, 17: Caffeic acid. 2, 3, 5, 9, 10, 11, 12, 15, 16, 18, 19, 20: Unidentified.

but is not generally present in animals or lower plants. Minamikawa and Uritani11) reported the existence of phenylalanine ammonia-lyase in sliced sweet potato roots infected by the black-rot fungus. We also found the enzyme in the root of Japanese radish infected by Peronospora parasitica23). It seems, therefore, that phenylpropanoid acids in the downy mildew-infected root are probably derived from phenylalanine by the action of this enzyme during the process of the infection. Moreover, since the material used did not contain the infecting hyphae, it is likely that the phenolic acids obtained did not originate from the fungus but from the diseased host cells.

Fig. 2 shows the relationship between the phenolic acids detected and the suggested pathway of lignin biosynthesis. Generally, the hexose monophosphate shunt seems to be the predominant pathway for glucose catabolism in diseased plants. For such activation of the shikimic acid pathway in the root, enzyme proteins which catalyze each of the changes should partially be activated. Therefore,
subsequent work should be focused to the enzyme activation. It remains to be elucidated whether
the phenolic acids produced are of significance only in lignification, or whether they have a wider
significance in the host-parasite interaction. It would be of interest to study the fungitoxicity of
the phenolic acids formed in the infected tissues. Recently, Seevers and Daly\textsuperscript{14} reported that no
significant differences in the content of total phenolic compounds were found among healthy or
inoculated, resistant and susceptible plants at any stage of disease development in wheat stem rust.
In our work, the fungus grew in the roots until they were severely damaged, so the idea of toxicity
appears to be in doubt.

![Diagram](Fig. 2. The relationship between the phenolic acids detected (flames)
and not detected (dotted flames) in the diseased roots and the
suggested pathway of lignin biosynthesis.

G-1-P: Glucose-1-phosphate, G-6-P: Glucose-6-phosphate, EMP: Embden-Meyerhoff
pathway, HMP: Hexose monophosphate pathway, DOPA: 3, 4-dihydroxyphenylalanine.
Arrows with broken lines indicate probable pathway of more than 2 steps. Thick
lines show clearly indentified pathway in the root.

Although ferulic and sinapic acids have been suggested as lignin precursors, we did not detect
these free acids in the material examined. Shimada \textit{et al.}\textsuperscript{15} reported the metabolism of \textit{p}-coumaric
acid during lignification of bamboo. They mentioned that the content of \textit{p}-coumaric acid increased
drastically from the top toward the lower parts of bamboo shoots, whereas ferulic acid did not
increase as markedly. Bland and Logan\textsuperscript{3} mentioned that no free ferulic acid was found during
lignification in \textit{Eucalyptus} and suggested that esterification of 3-methoxy-4-hydroxyphenylalanine was
followed by deamination to ferulic acid. Rohringer \textit{et al.}\textsuperscript{8,12} studied the metabolism of aromatic
compounds in healthy and rust-infected wheat leaves. They mentioned that when either quinate-U-
\textsuperscript{14}C or shikimate-U-\textsuperscript{14}C was metabolized by healthy wheat leaves, more activity was recovered in
insoluble esters of ferulic and \textit{p}-coumaric acids than in soluble esters; resistant-reacting leaves
accumulated still more activity in the insoluble esters. However, susceptible-reacting leaves contained
more activity in soluble esters than in insoluble esters. Since at least part of the insoluble esters are
intermediates in the synthesis of wheat leaf lignin, the relatively high activity of these compounds
in resistant-reacting leaves may reflect a greater lignification in these leaves. This is consistent with
our data which showed the absence of free ferulic acid in the radish root.

*p*-Coumaric acid can act as a cofactor in the oxidation of both indoleacetic acid and reduced nicotinamide adenine dinucleotide, while other cinnamic acid derivatives, such as ferulic acid, inhibit these reaction at least in vitro\(^{13}\). In this paper, the role of phenolic acids in the infected tissues was discussed from the standpoint of the lignin formation. It seems profitable to study the relationship between the phenolic compounds and indoleacetic acid and/or phytoalexins, because indoleacetic acid and pisatin, one of the phytoalexins, originate from a common biosynthetic pathway with the phenolic compounds.

Literature cited


和文要旨

ベト病菌感染ダイコン根でのフェノール化合物生成

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ベト病罹病ダイコン根での生成フェノール化合物をガスクロマトグラフ法でしらべた結果、キナ酸、プレフェノニン酸、フェニールピルビン酸、トランス桂皮酸、p-クマール酸、コーヒー酸が同定された。同重量の健全組織からは、これらのフェノール化合物は見出されない。供試病態材料には菌糸が含まれていないので、生成フェノール化合物は宿主起源である。罹病状態でフェルラ酸やシナビン酸は見出されなかった。以上の結果に基づき、罹病組織におけるリグニン前駆物質としてのフェノール化合物について論述した。