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CINNAMATE 4-HYDROXYLASE AND HYDROXYCINNAMATE:CoA LIGASE IN WHEAT LEAVES INFECTED WITH *BOTRYTIS CINEREA*

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Key Word Index—*Triticum aestivum*, Gramineae, wheat, *Botrytis cinerea*, fungi, lignification, control, cinnamate 4-hydroxylase, hydroxycinnamate CoA ligase

Abstract—Increases in cinnamate 4-hydroxylase and hydroxycinnamate:CoA ligase activities preceded the deposition of lignin around wounds in wheat leaves infected with a non-pathogenic isolate of *Botrytis cinerea*. The increases were localized in the lignifying tissues Little or no increase in activity was observed in uninfected, non-lignifying wounded leaves. The CoA ligase showed a preference for hydroxycinnamate substrates, particularly *p*-coumaric acid, but had no activity with sinapic acid. The substrate specificity did not change significantly on inoculation with *B. cinerea* and, hence, it is unlikely that the increased proportion of syringyl groups found in infection-induced lignin is controlled by hydroxycinnamate.CoA ligase activity.

INTRODUCTION

Lignification occurs rapidly around wounds in primary wheat leaves in response to non-pathogenic fungi and may be important in restricting the spread of such fungi from the wounds [1]. The response appears to be relatively specific for filamentous fungi and wounding alone has no effect unless the wounds become contaminated with fungi [2]. The induced lignin contains a much higher proportion of syringyl groups than the predominantly guaiacyl lignin present in healthy leaves [1]. Studies of the metabolic changes associated with this phenomenon could yield information concerning factors controlling the lignin biosynthetic pathway as well as providing further insight into lignification as a defence mechanism against fungal infection

Ammonia-lyase and *O*-methyltransferase activities have been shown to increase following the inoculation of wounds with the non-pathogenic fungus *Botrytis cinerea*, the increases being localized in the lignifying tissues [3]. However, the substrate specificity of the *O*-methyltransferase activity could not explain the increased proportion of syringyl groups found in infection-induced lignin This paper reports on the activities of two more enzymes likely to be important in the process of induced lignification: cinnamate 4-hydroxylase and hydroxycinnamate CoA ligase. Although both enzymes may be involved in the biosynthesis of other phenylpropanoid compounds, apart from lignin, increases in their activities could nevertheless be vital for induced lignification. The possible localization of the activities in the lignifying tissues has thus been investigated. The substrate specificity of the CoA ligase activity in healthy and infected leaves has also been examined, as changes in the ability to activate sinapic acid could explain the increased syringyl content of induced lignin

RESULTS AND DISCUSSION

Cinnamate 4-hydroxylase activity

Using a modified radiochemical assay from Russell [4], cinnamate 4-hydroxylase activity was detected in a microsomal fraction prepared from cell-free extracts of wheat leaves Attempts to demonstrate activity by the method of Nair and Vining [5], using tetrahydrofolic acid as a cosubstrate, were unsuccessful The enzyme from wheat tissue appeared very similar to that isolated from peas [4] in that it was highly unstable, activity being lost within 12 hr at 4°C It had a pH optimum of 7.5 and a specific requirement for NADPH as an electron donor and 2-mercaptoethanol for maximum activity The activity was also localized exclusively in the 105 000 *g* particulate fraction and presumably, as in other plants [6], the reaction is catalysed by a microsomal system containing cytochrome P-450. Attempts at a more rapid isolation of a wheat microsomal fraction by Mg^{2+} precipitation [7] were unsuccessful, no activity being detected with microsomes precipitated between 0 and 100 mM Mg^{2+}

A time course study of changes in the hydroxylase activity showed that inoculating wounded wheat leaves with *B. cinerea* caused a rapid and significant ($P < 0.01$) increase in activity between 12 and 18 hr after inoculation (Fig. 1) This increase closely parallels those observed with phenylalanine ammonia-lyase and tyrosine ammonia-lyase [3] and immediately precedes the period of rapid lignification between 18 and 24 hr [1]. No increase in activity was observed in uninfected, non-lignifying leaves, whether wounded or not (Fig. 1). Excision of infected wounds together with immediately adjacent tissue revealed that more that 99% of the increase in hydroxylase activity was confined to an area extending not more than

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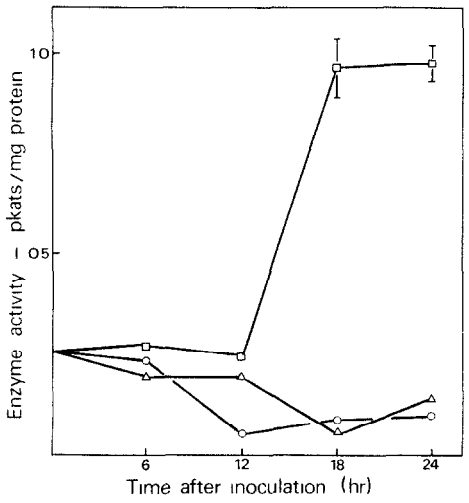

Fig 1 Changes in cinnamate 4-hydroxylase activity in healthy (wounded untreated) (△), control (wounded treated with water) (○) and inoculated leaves (□) with time Each point is the mean of three tissue samples Bars represent s e

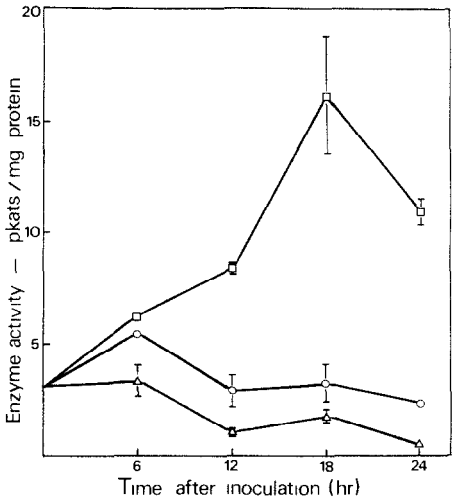

Fig 2 Changes in hydroxycinnamate CoA ligase activity in healthy (unwounded untreated) (△), control (wounded treated with water) (○) and inoculated leaves (□) with time Each point is the mean of three tissue samples Bars represent s e

0.5 mm from the wound (Table 1) Hence, the increase is localized in the tissues undergoing lignification. A similar correlation between lignin formation and cinnamate 4-hydroxylase activity has been observed during ethylene stimulated lignification of swede roots [8]

Hydroxycinnamate CoA ligase activity

Hydroxycinnamate.CoA ligase activity was detected in crude extracts and ammonium sulphate precipitates of extracts of wheat leaves using a modified spectrophotometric assay from Gross and Zenk [9] Treatment with Dowex 1 failed to further increase the overall activity, as had been reported for the CoA-ligase from *Forsythia* [9], and redissolved ammonium sulphate precipitates were used for routine assays. As with other plants [10], the enzyme showed a requirement for ATP, Mg^{2+} and a thiol compound Dithiothreitol used in the assays could not be replaced by 2-mercaptoethanol. Activity was enhanced slightly by the addition of NaF, indicating that ATPase was a source of interference, as reported for the CoA ligase from potato tubers [11]

A time course of CoA ligase activity with *p*-coumaric as substrate is shown in Fig. 2 As with cinnamate 4-hydroxylase a rapid increase in activity was observed in the infected tissues compared to the uninfected controls. The increase was again localized in the lignifying tissue immediately surrounding the infected area (Table 1) The rise in hydroxycinnamate CoA ligase activity due to infection (Fig 2) apparently occurred a little earlier than the rises in cinnamate-4-hydroxylase (Fig 1) and ammonia-lyase [3] activities, in that a significant ($P < 0.001$) and reproducible increase was apparent as early as 12 hr after inoculation This may imply the activation of pre-existing phenolic pools prior to the input of carbon from phenylalanine

An examination of the substrate specificity of the CoA ligase from healthy tissue revealed that, as in most other plants tested [10, 12], the enzyme will activate hydroxylated cinnamic acids whereas the unsubstituted parent cinnamic acid is not accepted as a substrate (Table 2) The preferred substrate is *p*-coumarate and increasing the substitution on the benzene ring causes a decrease in activity, with no activity at all being detected with

Table 1 Localization of enzyme activities in tissues surrounding wheat leaf wounds infected with *B cinerea*

	Cinnamate 4-hydroxylase activity (pkat/mg protein)	Hydroxycinnamate CoA ligase activity† (pkat/mg protein)
Untreated leaves	0 00	2 57
Leaf remains after removal of infected wounds	0 18	8 18
Isolated infected wounds*	23 2	44 7

*Wounded, infected tissue plus an average of 0 5 mm 'healthy' tissue surrounding it, taken 24 hr after inoculation

†Determined with *p*-coumarate as substrate

Table 2. Substrate specificity of the hydroxycinnamate:CoA ligase activity from healthy and *Botrytis* infected wheat leaves

Substrate	Relative activity (%)*		
	Healthy tissue†	Control tissue‡	*Botrytis*-inoculated tissue§
Cinnamate	0	n d	n d.
p-Coumarate	100	100	100
Caffeate	82	n d.	n d.
Ferulate	77	82	101
5-Hydroxyferulate	34	n.d	n d.
Sinapate	0	0	0

*Activity with *p*-coumarate = 100%.
†Tissue harvested at time 0
‡Tissue harvested 24 hr after wounding and inoculation with water
§Tissue harvested 24 hr after wounding and inoculation with *B. cinerea*
n.d., Not determined

sinapate. The activation of *p*-coumarate, ferulate and sinapate is supposed to be important in the biosynthesis of lignin and, hence, the relative activity with these three substrates was determined for both wounded wheat leaves and *B. cinerea* inoculated leaves. As with healthy leaves, no significant activity could be detected using sinapate as substrate despite the high syringyl content of the lignin induced in *B. cinerea* inoculated leaves: a slight increase in the relative activity with ferulate was the only change (Table 2). The substrate specificity of the CoA ligase activity cannot, thus, explain the modified structure of the induced lignin. The lack of activity with sinapate as substrate, despite the high syringyl content of the induced lignin, also suggests that the hydroxycinnamic acids may be activated by the CoA ligase mainly at the level of *p*-coumarate, which is the first, and preferred, substrate. Changes in the substitution pattern on the benzene ring may then occur whilst the acid is in its CoA ester form. This is the reverse of the normal proposed scheme where the substituted cinnamic acids are first formed and then individually activated by the CoA ligase [10].

The good correlation that exists, both spatially and temporally, between lignification and cinnamate 4-hydroxylase and hydroxycinnamate:CoA ligase activity suggests that increased levels of the enzymes are an important part of the defence mechanisms of the plant, although the way in which the levels are stimulated by the invading fungus is not yet clear.

EXPERIMENTAL

Materials *Trans*-cinnamic and [3(α)-^{14}C]*trans*-cinnamic acid (58 mCi/mmol) were obtained from CEA, Gif-sur-Yvette, France

Plant and fungal material The maintenance of cultures, production of spore suspensions of *Botrytis cinerea* and production and inoculation of 7-day-old seedlings of *Triticum aestivum* cv Joss Cambier were as described previously [1]

Enzyme preparations All procedures were carried out of 0–4° unless otherwise stated For the measurement of cinnamate 4-hydroxylase activity, 20 sections (6 cm, 10 wounds each) of primary leaves were ground to a cell-free extract with 10% by wt Polyclar AT and a small quantity of ground glass in *ca* 8 ml 0 1 M Pi buffer, pH 7.5. After centrifugation at 2000 *g* for 10 min, followed by 12 000 *g* for 15 min and 105 000 *g* for 1 hr, the final pellet was resuspended in 2.5 ml extraction buffer containing 3 mM 2-mercaptoethanol and assayed immediately for enzyme activity. For the hydroxycinnamate·CoA ligase assays, 10 leaf sections were ground with 10% by wt Polyclar AT and a small quantity of ground glass in *ca* 4 ml 0 05 M Pi buffer, pH 7.3 containing 1 mM dithiothreitol After centrifugation at 2000 *g* for 10 min and 35 000 *g* for 1 hr, $(NH_4)_2SO_4$ was added to the supernatant to 75% satn and the resulting ppt dissolved in a minimum vol of the extraction buffer This concd preparation was diluted as necessary to obtain maximum activity within the linear portion of the activity vs enzyme concn curve

Enzyme assays. Cinnamate 4-hydroxylase activity was assayed by a modification of the method of ref [4]. [3-^{14}C]*trans*-cinnamic acid (1 ml, 1 29 μCi), 0 4 ml 2.5 mM *trans*-cinnamic acid in 0.1 M Pi buffer, pH 7 5, and 0.6 ml of NADPH-regenerating system (G-6-P dehydrogenase 0 66 units/ml, NADP 3 3 mM, G-6-P 16.7 mM) were equilibrated together at 30° for 5 min Enzyme preparation (1 ml) was added and the whole incubated in unstoppered tubes with shaking for 1 hr at 30° The reaction was stopped by the addition of 0.2 ml 5 M HCl and the digest extracted twice with 1 vol of Et_2O Et_2O was removed by evaporation *in vacuo* at room temp The residue was dissolved in a small vol (30–50 μl) of Me_2CO, applied to a TLC plate (Si gel) and co-chromatographed with cold *p*-coumaric acid in C_6H_6–HOAc–H_2O (10 7:3, upper phase). Extracts from boiled enzyme controls or mixtures lacking NADP were always run on the same plate Areas corresponding to *p*-coumaric acid and cinnamic acid were scraped from the plates and eluted twice with Me_2CO (3 ml), the gel being centrifuged between washings The supernatants were pooled in scintillation vials, the Me_2CO removed under a stream of air at room temp. and 10 ml toluene-based scintillant added prior to counting in a scintillation counter. Corrections were made for the efficiency of isolation of the product prior to calculating the enzyme activity Hydroxycinnamate CoA ligase was assayed by a modification of the optical assay of ref. [9]. Substrate (0.67 mM in 0 05 M Pi buffer, pH 7 3, 0.3 ml), ATP (10 mM in 0 05 M Pi buffer, pH 7 3, 0 2 ml), CoA (1 mM, 0 2 ml), dithiothreitol (9 mM) containing $MgCl_2$ (0 1 M) (0 1 ml), and NaF (0 2 M) in 0.05 M Pi buffer (pH 7 3, 0.1 ml) were equilibrated together at 30° Enzyme (0.1 ml) was added, and after thorough mixing the *A* at the max of the product

was recorded for at least 5 min A changes in control samples (minus CoA) were recorded similarly The initial slope obtained after correction for the control was used to calculate the enzyme activity using the extinction coefficients cited in ref [13] Protein concn in the enzyme preparations was determined by the method of ref [14]

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