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Elicitor-Induced Cinnamyl Alcohol Dehydrogenase Activity in Lignifying Wheat (*Triticum aestivum* L.) Leaves[1]

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The substrate-specific induction of wheat (*Triticum aestivum* L. cv Fenman) leaf cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) was examined in relation to its role in regulating the composition of defensive lignin induced at wound margins. Treatment of wounds with a partially acetylated chitosan hydrolysate or spores of the nonpathogen *Botrytis cinerea* elicited lignification at wound margins and invoked significant increases in phenylalanine ammonia-lyase (EC 4.3.1.5), peroxidase (EC 1.11.1.7), and CAD activities. The substrate-specific induction of CAD with time was determined in elicitor-treated leaves and in excised lignifying wounds. In whole leaf extracts no significant increases in *p*-coumaryl and coniferyl alcohol dehydrogenase activities were detectable, but a significant 5-fold increase in sinapyl alcohol dehydrogenase activity was evident 32 h after elicitor treatment. Similarly, fungal challenge resulted in elevated levels of only sinapyl alcohol dehydrogenase in whole-leaf extracts. In excised lignifying tissues *p*-coumaryl alcohol dehydrogenase levels were similar to those observed in healthy tissue. A small yet significant increase in coniferyl alcohol dehydrogenase was apparent, but the most dramatic increase occurred in sinapyl alcohol dehydrogenase activity, which increased to values approximately 10 times higher than the untreated controls. Our results show for the first time that CAD induction in lignifying tissues of wheat is predominantly attributable to highly localized increases in sinapyl alcohol dehydrogenase activity.

Lignification occurs in many plants in response to infection or attempted infection (Vance et al., 1980). In wheat (*Triticum aestivum* L.), inoculation of wounds with nonpathogenic fungi results in rapid lignin deposition at the wound margins. The response functions primarily as a physical barrier to further invasion of the host, limiting the potential pathogen to the confines of the wound tissue (Ride, 1975). Considerable evidence supports the view that this defense response is crucial for determining the nonhost resistance of wheat (Ride, 1975). The composition of the lignin deposited at wound margins, in terms of its constituent cinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl), differs from the lignin of healthy tissues (Ride, 1975). Lignin extracted from healthy wheat leaves is essentially a guaiacyl lignin, derived principally from coniferyl alcohol. In contrast, wound lignin more closely resembles a guaiacyl-syringyl lignin, derived from both coniferyl and sinapyl alcohols. Usually, lignins contain very few *p*-hydroxyphenyl residues derived from *p*-coumaryl alcohol. However, analysis of wound lignin by nitrobenzene oxidation revealed a higher proportion of *p*-hydroxyphenyl residues, suggesting substantial *p*-coumaryl alcohol incorporation into the polymer (Ride, 1975). Potentially, the factors controlling the formation and composition of wound lignin could reside in inducible enzyme activities, which modulate the availability of lignin precursors.

General phenylpropanoid metabolism in plants leads to the formation of cinnamoyl-CoA esters, which form the precursors of numerous phenolic compounds, including lignin (Vance et al., 1980). In wheat, highly localized increases in the activities of enzymes leading to the formation of cinnamoyl-CoA esters have been reported following fungal challenge (Maule and Ride, 1976, 1983; Thorpe and Hall, 1984). However, detailed examination of the specificities of the *O*-methyltransferases (EC 2.1.1.6) and *p*-hydroxycinnamate:CoA ligases (EC 6.2.1.12) revealed that these activities could not account for the increased syringyl content of wound lignin (Maule and Ride, 1976, 1983).

The formation of cinnamyl alcohols, the immediate precursors of lignin, from their corresponding cinnamoyl-CoA esters requires two enzymic steps, catalyzed by cinnamoyl-CoA reductases (EC 1.2.1.44) and CAD (EC 1.1.1.195). CAD catalyzes the synthesis of cinnamyl alcohols from their corresponding cinnamaldehydes and is considered to be a highly specific marker for lignification. This paper is concerned with the substrate-specific induction of CAD in wheat leaves in relation to its role in regulating the composition of lignin induced at wound margins. Two lignin-inducing treatments were used, an elicitor-active, partially acetylated chitosan hydrolysate and a general nonpathogen (*Botrytis cinerea*). It is the first report in which the induction of *p*-coumaryl, coniferyl, and sinapyl alcohol dehydrogenase activities have been compared directly during the expression of a plant defense response.

MATERIALS AND METHODS

Production and Inoculation of Plants

All experiments were performed on 8-d-old seedlings of wheat (*Triticum aestivum* L. cv Fenman) grown and wounded following the method of Ride (1975). Enzyme activities were

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Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); CAD, cinnamyl alcohol dehydrogenase; ϵ, molar absorption coefficient; PAL, phenylalanine ammonia-lyase; PO, peroxidase.

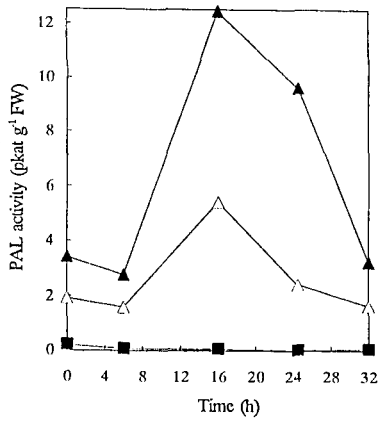

Figure 1. Time course of elicitor-induced PAL activity in wheat leaves. Untreated leaves (■), wounded leaves treated with water (△), and wounded leaves treated with elicitor (▲). FW, Fresh weight.

determined in untreated leaves, and wounded leaves were treated with 10 μL per wound of either water, elicitor, or fungal inoculum. Experiments were performed on replicates of three leaves for each treatment, with each leaf receiving 10 compression wounds. All experiments were performed in duplicate, and the results from a representative experiment are presented. Lignin deposition was localized by chlorine sulfite staining (Wardrop, 1971) following the procedure of Ride (1975).

Preparation of Elicitor and Fungal Inoculum

Partially acetylated chitosan was prepared by partial reacetylation of crab shell chitosan (degree of acetylation 8%; Miya et al., 1980). Chitosan (100 mg) was dissolved in 4 mL of 10% (v/v) acetic acid at 80°C for 2 h and cooled to room temperature. Methanol (9 mL), containing 100 μL of acetic anhydride, was added dropwise with vigorous stirring. After 3 h the partially acetylated chitosan was dialyzed against excess 10 mM acetic acid, followed by excess distilled water. Dry weight analysis and estimation of GlcN residues (Tsuji et al., 1969) were used to determine the degree of acetylation of the polysaccharide. The gelatinous chitosan was partially hydrolyzed by incubation with 100 μg of wheat-leaf chitinase, isoenzyme 6 (Ride and Barber, 1990), at 30°C for 24 h. The hydrolysis was terminated by heat treatment (80°C for 1 h), and the sample was centrifuged (500*g* for 5 min) to remove insoluble material. The chitosan hydrolysate was diluted to a final concentration of 1.0 mg mL^{-1} prior to inoculation of wounds.

Spores of *Botrytis cinerea* were obtained from 8-d-old cultures grown on 2% (w/v) malt agar and a suspension prepared (5×10^5 spores mL^{-1}) as described previously (Ride, 1975).

Preparation of Enzyme Extracts

Individual leaf sections (6 cm) were homogenized in 300 μL of appropriate ice-cold extraction buffer: PAL, 100 mM Tris-HCl buffer (pH 8.8) containing 0.05% (w/v) EDTA and 0.05% (v/v) mercaptoethanol; PO, 100 mM phosphate buffer (pH 7.0); CAD, 100 mM phosphate buffer (pH 7.3) containing 40 mM mercaptoethanol. Particulate material was removed by centrifugation (11,600*g* for 5 min), and the supernatants were used directly in the enzyme assays.

Enzyme Assays

PAL (EC 4.3.1.5) was assayed radiochemically using L-Phe as the substrate at 35°C, based on the method of Thorpe and Hall (1984). The reaction mixture (500 μL) consisted of 100 mM Tris-HCl buffer (pH 8.8), 100 μM L-[U-^{14}C]Phe (2 μCi μmol^{-1}), and 100 μL of wheat-leaf extract. The reaction was terminated by the addition of 50 μL of 0.1% (w/v) *trans*-cinnamic acid in 50 mM NaOH, followed by 50 μL of 50% (w/v) TCA. Precipitated protein was removed by centrifugation (2900*g* for 5 min), and the reaction product was partitioned into 200 μL of toluene. After the sample was centrifuged (2900*g* for 5 min) 100 μL of the upper toluene phase was mixed with 5 mL of scintillation fluid and counted in a Beckman scintillation counter.

PO (EC 1.11.1.7) was assayed colorimetrically using ABTS as the electron donor at 20°C, based on the method of Shindler et al. (1976). The assay mixture (1.6 mL) comprised 500 μM ABTS, 25 mM sodium acetate buffer (pH 4.4), 250 μM H_2O_2, and suitably diluted wheat leaf extract. The formation of the radical cation was monitored at 412 nm ($\epsilon = 32.4 \times 10^3\ M^{-1}\ cm^{-1}$; Shindler et al., 1976).

CAD (EC 1.1.1.195) was measured following the oxidation of the appropriate hydroxycinnamyl alcohol at 30°C, based on the method of Wyrambik and Grisebach (1975). The assay mixture (1 mL) contained 100 μM of appropriate cinnamyl alcohol, 100 mM Tris-HCl buffer (pH 9.3), 100 μM $NADP^+$, and 200 μL of enzyme extract. Assays using coniferyl alcohol as the substrate were monitored by following the formation of coniferaldehyde at 400 nm ($\epsilon = 2.10 \times 10^4\ M^{-1}\ cm^{-1}$; Wyrambik and Grisebach, 1975). Assays using *p*-coumaryl or sinapyl alcohols were monitored at 340 nm following the formation of NADPH ($\epsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

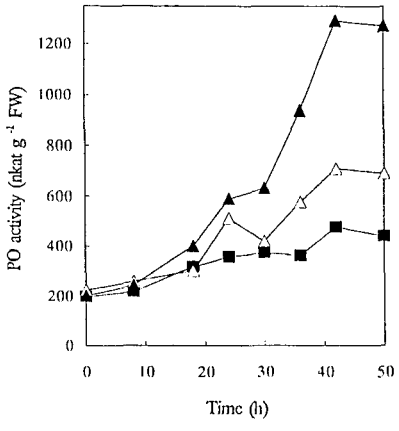

Figure 2. Time course of elicitor-induced PO activity in wheat leaves. Untreated (■), wounded leaves treated with water (△), and wounded leaves treated with elicitor (▲). FW, Fresh weight.

Table I. *Enzyme activities in wheat leaves following inoculation with B. cinerea*

Enzyme levels were determined in untreated leaves, wounded leaves treated with water, and wounded leaves inoculated with spores of *B. cinerea*. Significant increases above the untreated controls determined using a *t* test ($P \leq 0.05$, $n = 3$) are indicated with an asterisk.

Enzyme	Substrate	Enzyme Activity			
		Untreated	Water	*B. cinerea*	Fold increase[c]
		pkat g⁻¹ fresh wt			
PAL[a]	L-Phe	13	18*	44*	3.4
PO[b]	ABTS	3.99×10^5	6.16×10^5	$10.73 \times 10^{5*}$	2.7
CAD[b]	*p*-Coumaryl alcohol	1383	1555	1771	1.3
CAD[b]	Coniferyl alcohol	401	461	567	1.4
CAD[b]	Sinapyl alcohol	199	298	562*	2.8

[a] Activity determined 16 h after treatment. [b] Activity determined 42 h after treatment. [c] Increase in activity of *B. cinerea*-treated wounds compared to untreated leaves.

Enzyme Localization

Enzyme localization experiments were performed by excising wounds with an average of 1.0 mm of healthy surrounding tissue and assaying these separately from the leaf remains after removal of the wounds. Protein content of the crude enzyme extracts was determined by the Bradford (1976) dye-binding assay using BSA as a standard.

Chemicals

Unless otherwise stated all chemicals were obtained from Sigma (Poole, UK). L-[U-^{14}C]Phe was obtained from NEN Research Products (Stevenage, UK). Scintillation fluid (OptiPhase "Safe") was obtained from FSA Laboratory Supplies (Loughborough, UK). Sinapyl and *p*-coumaryl alcohols were the generous gift of Stéphane Quideau (U.S. Dairy Forage Research Center, University of Wisconsin).

RESULTS

PAL, PO, and Lignin Induction

Treatment of wounds with 80% acetylated chitosan elicited an intense chlorine sulfite reaction at wound margins diagnostic of lignin deposition. Prolonged enzymic hydrolysis of this chitosan, with purified wheat leaf chitinase, dramatically reduced the viscosity of the preparation without diminishing its elicitor activity. The levels of enzyme activity induced by the chitosan hydrolysate at wound margins were measured and statistically analyzed using a *t* test at a 5% level of significance. The chitosan hydrolysate was capable of inducing significant increases in both PAL (Fig. 1) and PO activity (Fig. 2). PAL induction was temporal, reaching a maximum 16 h after elicitor treatment and declining to basal levels thereafter. The increase in PO was apparent after 24 h, reaching a maximum at 42 h. Similarly, fungal inoculation of the wounds with spores of the nonpathogen *B. cinerea* resulted in extensive lignification and significantly elevated levels of both PAL and PO (Table I).

Elicitor-Induced CAD

The substrate-specific induction of CAD with time was determined in untreated, wounded, and elicitor-treated leaves. No significant increase in *p*-coumaryl alcohol dehydrogenase activity was observed in elicitor-treated leaves above the untreated controls (Fig. 3a). Coniferyl alcohol dehydrogenase activity in elicitor-treated leaves increased slightly in comparison to the control levels but not significantly (Fig. 3b). A significant and sustained increase in sinapyl alcohol dehydrogenase activity was evident as early as 8 h after elicitor treatment, and it continued to increase, reaching a maximum of approximately 5 times higher than that of the untreated controls after 32 h (Fig. 3c).

Fungal-Induced CAD

The substrate-specific induction of CAD was determined in untreated, wounded, and *B. cinerea*-inoculated leaves. No significant increase in either *p*-coumaryl or coniferyl alcohol dehydrogenase activity was observed in fungal-treated leaves. In contrast, fungal challenge resulted in a significant, 3-fold increase in sinapyl alcohol dehydrogenase activity above the untreated control (Table I).

Localization of Enzyme Activities

The specific activities of PAL, PO, and CAD were determined in untreated leaves, elicitor-treated excised wounds with an average of 1.0 mm of healthy surrounding tissue, and the leaf remains after removal of the wounds. Significant increases in PAL and PO activities were observed in both the excised wounds and the leaf remains as compared to the untreated leaves. In contrast, CAD was highly localized to the excised tissues, where significant increases in coniferyl and sinapyl alcohol dehydrogenase activities were found. The most dramatic increase occurred in sinapyl alcohol dehydrogenase activity, which reached values approximately 10 times higher than in the untreated leaves (Table II).

DISCUSSION

The mechanisms controlling the induction, composition, and location of lignin deposition at wound margins in wheat are largely unexplored but are presumably closely regulated. Obvious candidates for regulatory control of the response are the biosynthetic enzymes leading to the formation of lignin.

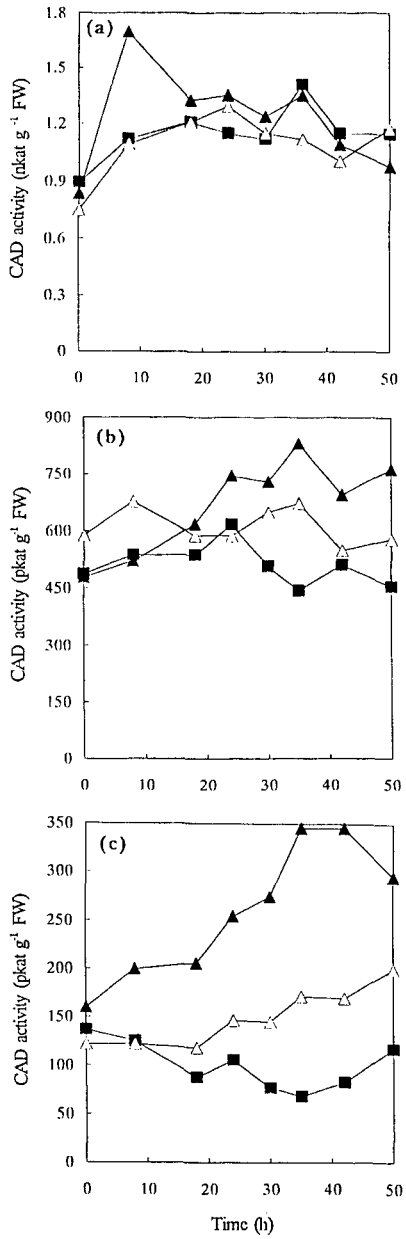

Figure 3. Time course of elicitor-induced CAD activity in wheat leaves. a, *p*-Coumaryl alcohol dehydrogenase activity; b, coniferyl alcohol dehydrogenase activity; c, sinapyl alcohol dehydrogenase activity. ■, Untreated leaves; △, wounded leaves treated with water; ▲, wounded leaves treated with elicitor. FW, Fresh weight.

Fungal challenge and elicitor treatment are known to elevate the levels of PO and general phenylpropanoid enzymes, such as PAL (Maule and Ride, 1976, 1983; Thorpe and Hall, 1984), and our data are in agreement with these observations. However, these activities are relatively nonspecific markers for lignification in comparison to the lesser studied cinnamoyl-CoA reductases and CADs (Moerschbacher, 1989). CAD induction has been studied in relatively few plant defense responses, and in all of these cases only coniferyl alcohol dehydrogenase activity was measured (Moerschbacher et al., 1986, 1988; Grand et al., 1987; Walter et al., 1988; De Sa et al., 1992).

We have examined the substrate-specific induction of CAD in lignifying wheat leaves following elicitor treatment and fungal challenge. This is the first report in which *p*-coumaryl, coniferyl, and sinapyl alcohol dehydrogenase activities have been compared during the expression of a plant defense response involving lignin deposition. Our results show that CAD induction in lignifying tissues of wheat is predominantly attributable to highly localized increases in sinapyl alcohol dehydrogenase activity.

Analysis of wound lignin by nitrobenzene oxidation has revealed that the polymer contains higher proportions of syringyl and *p*-hydroxyphenyl residues, derived from sinapyl and *p*-coumaryl alcohols, respectively (Ride, 1975). Our finding that elicitor treatment and fungal challenge induce highly localized sinapyl alcohol dehydrogenase activity is consistent with the increased syringyl content of wound lignin (Ride, 1975) and with its intense chlorine sulfite staining (Wardrop, 1971). This suggests that the regulatory mechanisms controlling the formation and composition of wound lignin could reside in inducible activities, favoring the accumulation of sinapyl and *p*-coumaryl alcohols.

Corresponding, sustained increases in *p*-coumaryl alcohol dehydrogenase activity were not detected in whole-leaf extracts or in the excised lignifying tissues. Thus, it seems likely that the apparent increased *p*-hydroxyphenyl content of wound lignin arises from an artifact of the nitrobenzene oxidation assay, used to analyze the composition of wound lignin, rather than from increased *p*-coumaryl alcohol dehydrogenase activity in the lignifying tissues. The product derived from the nitrobenzene oxidation of *p*-hydroxyphenyl residues of lignin, *p*-hydroxybenzaldehyde, can also arise from protein (Stone et al., 1951). We suspect, therefore, that a significant proportion of the elevated *p*-hydroxyphenyl content of wound lignin previously reported (Ride, 1975) may have arisen from mycoprotein in the wounds inoculated with fungal spores.

Similarly, no significant increases in coniferyl alcohol dehydrogenase activity were apparent in whole-leaf extracts, but small increases were detectable in the excised lignifying tissues. In contrast, dramatic increases in coniferyl alcohol dehydrogenase activity in whole-leaf extracts have been reported during the hypersensitive lignification response of wheat to the stem rust pathogen *Puccinia graminis* f. sp. *tritici* (Moerschbacher et al., 1986, 1988). This apparent anomaly may perhaps be explained in terms of the composition of the lignin deposited in the two responses. The guaiacyl-syringyl lignin induced at wound margins (Ride, 1975) is preceded by a large increase in sinapyl alcohol dehydrogenase activity. Conversely, the induction of coniferyl alcohol dehydrogenase activity associated with the hypersensitive response of wheat suggests that this lignin may more closely resemble the guaiacyl lignin of healthy tissue.

Lignin deposition at wound margins in wheat leaves is first detectable 24 h after elicitor treatment (Ride and Barber, 1987) and continues to be deposited in the following 48 h (Pearce and Ride, 1982). The timing of this response is consistent with the sequential induction of PAL, CAD, and PO following elicitor treatment. In contrast to CAD, both

Table II. *Localization of enzyme activities in tissues surrounding wheat leaf wounds treated with elicitor*

Enzyme levels were determined in untreated leaves, elicitor-treated excised wounds with an average of 1.0 mm of healthy surrounding tissue, and leaf remains after removal of the wounds. Significant increases above the untreated controls determined using a *t* test ($P \leq 0.05$, $n = 3$) are indicated with an asterisk.

Enzyme	Substrate	Specific Activity			
		Untreated leaves	Excised wounds	Leaf remains	Fold increase[c]
		pkat mg^{-1} of protein			
PAL[a]	L-Phe	1.2	4.9*	4.1*	4.1
PO[b]	ABTS	8.0×10^3	$40.6 \times 10^{3*}$	$35.4 \times 10^{3*}$	5.1
CAD[b]	*p*-Coumaryl alcohol	118.9	174.8	177.4	1.5
CAD[b]	Coniferyl alcohol	28.1	81.6*	30.9	2.9
CAD[b]	Sinapyl alcohol	10.2	99.6*	21.6	9.8

[a] Activity determined 16 h after treatment. [b] Activity determined 42 h after treatment. [c] Increase in specific activity of elicitor-treated excised wounds compared to untreated leaves.

PAL and PO were elevated in the leaf remains surrounding the lignifying tissues. The systemic induction of PAL and PO was not investigated further, although this phenomenon has been reported previously (Thorpe and Hall, 1984).

In suspension cultures of *Phaseolus vulgaris* L., coniferyl alcohol dehydrogenase activity appears in elicited tissues before the induction of more general phenylpropanoid enzymes such as PAL (Walter et al., 1988). It has been suggested that early induction of CAD reduces the levels of hydroxycinnamyl acids known to repress the expression of phenylpropanoid enzymes earlier in the pathway (Walter et al., 1988). Corresponding early increases in CAD activities were not apparent in our investigation, suggesting that a similar regulatory mechanism is unlikely to operate in lignifying wheat leaves.

The identification of a highly localized increase in sinapyl alcohol dehydrogenase activity in lignifying tissues suggests that specific sinapyl alcohol dehydrogenases may be induced in these tissues. Multiple forms of CAD from healthy wheat have been partially purified, but none of these exhibit a preferential activity for sinapyl alcohol (Pillonel et al., 1992). The isolation of the sinapyl alcohol dehydrogenase from lignifying wheat leaves is currently in progress. Characterization of this activity should yield important information regarding the control mechanisms regulating lignin deposition at wound margins in wheat.

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