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Specific Inhibition of Lignification Breaks Hypersensitive Resistance of Wheat to Stem Rust[1]

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ABSTRACT

When highly resistant wheat (*Triticum aestivum* L.) varieties are infected by an avirulent race of the stem rust fungus (*Puccinia graminis* Pers. f. sp. *tritici* Erics. and E. Henn.), penetrated host cells undergo rapid necrotization. This hypersensitive cell death is correlated with cellular lignification which efficiently restricts further fungal growth. Three competitive inhibitors of phenylalanine ammonia-lyase, the first enzyme of the general phenylpropanoid pathway and, thus, of lignin biosynthesis, namely α-aminooxyacetate, α-aminooxy-β-phenylpropionic acid, and (1-amino-2-phenylethyl)phosphonic acid, and two highly specific irreversible suicide inhibitors of the lignification-specific enzyme cinnamyl-alcohol dehydrogenase, namely *N*(*O*-aminophenyl)sulfinamoyl-tertiobutyl acetate and *N*(*O*-hydroxyphenyl)sulfinamoyl-tertiobutyl acetate, were applied to genetically resistant wheat plants prior to inoculation with stem rust. Treatment with any of these inhibitors decreased the frequency of lignified necrotic host cells and concomitantly led to increased fungal growth. The cinnamyl-alcohol dehydrogenase inhibitors were generally more effective than the phenylalanine ammonia-lyase inhibitors, occasionally allowing some sporulation to occur on the resistant wheat leaves. These results clearly point to a causal relationship between the formation of lignin precursors and the resistance of wheat to stem rust.

The lignin content of higher plants has long been recognized as an important factor in the resistance response against potential pathogens. Lignin is extremely resistant to microbial degradation and thus constitutes one of the most effective barriers against pathogenic invasion (25). In addition to the role of lignin as a preformed resistance factor, induced lignification has been proposed as an active resistance mechanism of plants to fungi (15). However, it is still a matter of debate whether active lignification processes are causally involved in resistance (25).

In the family Gramineae, active lignification appears to be of special importance in induced resistance mechanisms (28), possibly related to the near absence of phytoalexins in this family. In spite of intensive searches for infection-induced antimicrobial substances, no phytoalexins have been found in wheat *Triticum aestivum* L. (24). However, induced lignification has been proposed as an important mechanism of disease resistance of wheat against a variety of fungal pathogens (25). Several lines of evidence point to a role for cellular lignification in the hypersensitive resistance response of wheat to the stem rust fungus, *Puccinia gramins* Pers. f. sp. *tritici* Erics. and E. Henn.: hypersensitively reacting cells show yellow fluorescence under UV light and stain positively with phloroglucinol/HCl and chlorine/sulfite (7, 24, 30). Feeding radioactive lignin precursors to incompatible wheat-stem rust interactions leads to solvent- and alkali-insoluble incorporation of label into hypersensitively reacting host cells (7, 11, 24, 26, 30). Enzyme activities of the lignin biosynthetic pathway increase in incompatible interactions at the time of the resistance response but decrease in compatible interactions at the same time (10, 21).

The above described studies strongly suggest a correlation between lignification and resistance. However, they do not allow any conclusions concerning a causal relationship between the two phenomena. Application of AOA[3], a competitive inhibitor of PAL (3), prevents hypersensitive necrotization of penetrated wheat cells and concomitantly leads to increased fungal growth (24, 30). However, AOA is not specific for PAL, and PAL is not only involved in lignin biosynthesis. Work with the more specific PAL inhibitors, AOPP (2) and APEP (18), which differ in their modes of action may help to corroborate that the effect of all of these agents is via their inhibitory action against PAL. While such experiments may point to a role for the phenylpropanoid pathway in the resistance reaction, they do not provide conclusive evidence for the participation of lignification in these reactions.

Besides the well known PAL inhibitors, highly specific suicide inhibitors of CAD are now available (4, 9). These inhibitors have been defined by their ability to bind the cation (Zn^{2+}) involved in the enzyme active center and to act as substrate analogs (6). The combination of these properties guarantees the high specificity of these substances; besides CAD, only cinnamoyl:CoA reductase is inhibited *in vitro* (12).

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[3] Abbreviations: AOA, α-aminooxyacetate; AOPP, α-aminooxy-β-phenylpropionic acid; APEP (1-amino-2-phenylethyl)phosphonic acid; CAD, cinnamyl-alcohol dehydrogenase; HMC, haustorial mother cell; NH_2-PAS, *N*(*O*-aminophenyl)sulfinamoyl-tertiobutyl acetate; OH-PAS, *N*(*O*-hydroxyphenyl)sulfinamoyl-tertiobutyl acetate; PAL, phenylalanine ammonia-lyase.

Both enzymes are involved only in lignin biosynthesis. The mechanism of inhibition is thought to be a pseudo-irreversible inactivation of the suicide type (5, 6). Such inhibitors are ideally suited for *in vivo* assays, as they are highly specific and generally metabolized only slowly (1).

We have now investigated the effects of the above mentioned PAL inhibitors, AOPP and APEP, and of two of the CAD inhibitors, OH-PAS and NH_2-PAS, on the development of lignified necrotic host cells and fungal growth in highly incompatible interactions between wheat and the stem rust fungus.

MATERIALS AND METHODS

Plants and Fungus

The experiments were carried out with the wheat (*Triticum aestivum* L.) cultivars Feldkrone, Mannitou, and Prelude-Sr5 (Reliance/6* Prelude). These varieties are highly resistant to attack by race 32 of the stem rust fungus; inoculation of untreated leaves always yielded an infection type 0. Control experiments were performed with the fully susceptible wheat varieties Little Club and Prelude (infection type 4).

Uredospores of race 32 of the stem rust fungus (*Puccinia graminis* Pers. f. sp. *tritici* Erics. & E. Henn.) were cultivated on six-week-old wheat plants (cv Little Club).

Plant Growth Conditions

Wheat seedlings were raised in prefertilized soil at 20°C and 50% RH under a 16 h photoperiod in automatically regulated growth chambers. Illumination was provided by high pressure mercury vapor lamps producing approximately 15 klux at soil level.

Inhibitors

The PAL inhibitor AOA was purchased from Sigma. AOPP and APEP were kindly provided by Dr. N. Amrhein, ETH Zürich, Switzerland, and Dr. F. J. Schwinn, Ciba Geigy, Basel, Switzerland, respectively.

The CAD inhibitors, OH-PAS and NH_2-PAS were synthesized according to the protocol outlined by DeBlic *et al.* (9) and Baltas *et al.* (4).

Treatment with Inhibitors

Two different methods were used to apply inhibitors to healthy wheat leaves prior to inoculation:

1. Six-d-old seedlings were cut approximately 1 cm above soil level and placed in dark glass vials containing inhibitor solutions (10 mL, 0.1 mM) in 0.1% of Polycrescal (Philips-Duphor). These plants dessicated within a few days when cultivated under the usual conditions of our growth chambers. Thus, they were placed in a spot in the laboratory receiving indirect sunlight. One day later, these plants (3–5 per vial) were inoculated with uredospores of the stem rust fungus as described earlier (21).

2. Seven-d-old primary leaves were infiltrated with distilled water or 0.1 mM solutions of the inhibitors using a hypodermic syringe mounted on forceps with silicon gaskets (22). The soaked area extended approximately 3 cm to each side of the injection site, corresponding to 3 to 10 μL of injected solution per cm^2 leaf area. Two h later, the injected water had evaporated completely and plants were inoculated as described.

Application via the cut end of seedlings had the advantage of a continuous supply of inhibitor. On the other hand, application by hypodermic injection minimized wounding and allowed plant cultivation under standard growth conditions. However, injection of water alone sometimes shifted incompatibility slightly toward compatibility. Both methods led to essentially identical results concerning the influence of inhibitors on host cell necrosis and fungal growth.

Microscopic Evaluation

Primary leaves were harvested at the indicated times after inoculation, cleared and fixed in lactophenol-ethanol, and stained with diethanol (Uvitex 2B, Ciba Geigy, Basel) (17). Evaluation was with a Zeiss epifluorescent photomicroscope under UV-light (light source HBO 50W; heat protection filter KG 1; red quenching filter BG 38; filter combination BP 390-440, FT 460, LP 470). Fungal mycelium exhibited blue fluorescence, while necrotic host cells showed yellow autofluorescence under these conditions (30).

Statistical treatment of the results was by standard procedures, namely Behrens-Fischer-test, paired sample *t*-test, and Kolmogoroff-Smirnoff-test.

RESULTS

PAL Inhibitors

The PAL inhibitors AOA, AOPP, and APEP, were shown to inhibit in a dose-dependent manner the *in vitro* activity of PAL in wheat leaf extracts, AOPP being the most active compound (data not shown). Inhibitors were used at 0.1 mM in the *in vivo* experiments. Lower concentrations had no apparent effect on the host/parasite interaction, while treatments with a higher concentration (1 mM) yielded similar results as the ones shown here.

The effect of AOA on fungal growth and host cell necrosis was described earlier (24, 30). We used AOA as a positive control in our experiments, while water-treated plants served as a negative control.

In AOA-treated leaves, roughly twice as many colonies were free of host cell necrosis compared to water-treated controls (Table I). The effect was especially conspicuous regarding epidermal cell necrosis. Treatments with AOPP or APEP yielded very similar results (Table I). In small colonies, (<10 HMC) where individual cells can be distinguished, about 60% of HMC-associated host cells were necrotic and lignified in the water controls compared to about 40% in leaves treated with any of the PAL inhibitors.

This general decrease in host cell lignification was accompanied by an increase in fungal growth (Table I and Fig. 1). In water-treated plants, more than 70% of the colonies had developed only one or two HMC, and only 10% had grown to more than 10 HMC. In contrast, in AOA-treated leaves, this percentage increased to about 30. Similarly, in AOPP-

Table I. *Influence of the PAL Inhibitors, AOA, AOPP, and APEP, and the CAD Inhibitors, NH_2-PAS and OH-PAS, on Fungal Development and Host Cell Necrosis in Highly Resistant Wheat Leaves Inoculated with Stem Rust*

Data are from the experiments described in Figures 1 and 3.

Treatment	PAL Inhibitors				CAD Inhibitors		
	H_2O	AOPP	APEP	AOA	OH-PAS	NH_2PAS	H_2O
Number of colonies (*n*)	488	323	635	510	1257	951	1094
Percent necrosis-free colonies	14	22	18	29	31	33	8
Percent colonies with epidermal necrosis	45	25	22	21	31	21	69
Percent necrotized HMC-associated cells	59	42	42	37	26	28	58
Percent colonies with 1-2 HMC	71	44	47	47	32	31	57
Percent colonies with >10 HMC	10	24	24	30	20	18	4
Mean diameter of colonies (μm)	96	123	132	165	137	132	110

and APEP-treated leaves, 24% of the colonies had differentiated more than 10 HMC. Accordingly, the mean diameter of fungal colonies increased from 96 μm in water controls to 165/123/132 μm in AOA/AOPP/APEP-treated leaves. Fungal colonies in inhibitor-treated leaves, thus, resembled those in less highly resistant wheat cultivars which exhibit infection type 0;.

The differences between water-treated controls and PAL inhibitor-treated leaves were shown to be significant ($P < 0.001$), while those between the PAL inhibitors were not ($P \gg 0.05$).

CAD Inhibitors

Similar results were obtained with the two inhibitors of the lignification-specific enzyme CAD, OH-PAS and NH_2-PAS. Figure 2 shows a typical colony in a highly resistant wheat leaf 4 d after inoculation (water-treated control); penetrated epidermal and mesophyll cells of the host were necrotic and exhibited yellow autofluorescence under UV-light, indicative of cellular lignification, and fungal colonies were small (mean diameter 110 μm). Figure 2 also shows an inhibitor-treated leaf at the same time after inoculation; necrotic lignified host cells were rare, and most fungal colonies were significantly ($P < 0.001$) larger than in the control (mean diameter 137/132 μm, OH-PAS/NH_2-PAS; not significantly different from each other, $P \gg 0.05$).

Figure 3 represents the quantitative evaluation of the influence of CAD inhibitors on host cell lignification and fungal development (see also Table I). Generally, the effects of the irreversible CAD inhibitors were more pronounced than those of the competitive PAL inhibitors. In controls, 92% of the colonies were associated with lignified host cells, compared with 32% necrosis-free colonies in inhibitor-treated leaves. In small colonies where individual cells can be distinguished, about 60% of HMC-associated host cells were necrotic and lignified in the controls, compared with less than 30% in CAD inhibitor-treated leaves. In water-treated control plants, almost 60% of fungal colonies had developed not more than two HMC 7 d after inoculation. In contrast, in inhibitor-treated plants, this percentage decreased to approximately 30, while a considerable portion of colonies (approximately 20%) had developed more than 10 HMC (compared with 4% in the control). Occasionally, limited sporulation was observed in the inhibitor-treated area of the resistant wheat leaves.

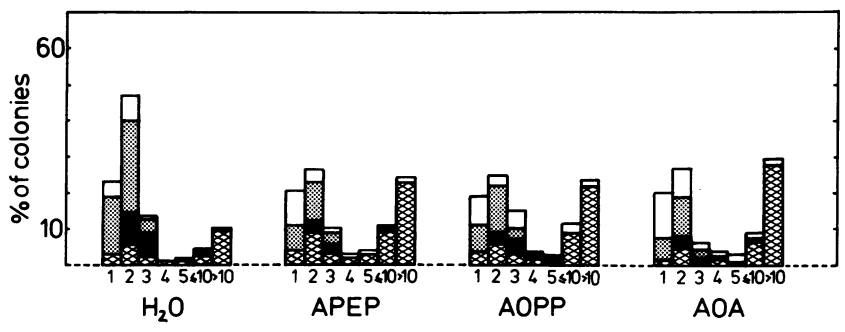

Figure 1. Influence of the PAL inhibitors, AOA, AOPP, and APEP, on fungal growth and host cell necrosis. Percentages of fungal colonies with n haustorial mother cells in highly incompatible interactions between wheat (cvs Feldkrone and Prelude-Sr5) and stem rust 7 d after inoculation. Inhibitors were injected into the intercellular spaces of the leaves. Colonies associated with no □, epidermal □, epidermal and mesophyll ■, or mesophyll ▨ necrosis. The differences between water- and inhibitor-treated leaves were shown to be significant ($P < 0.001$); the differences between the inhibitors were not P ($\gg 0.05$).

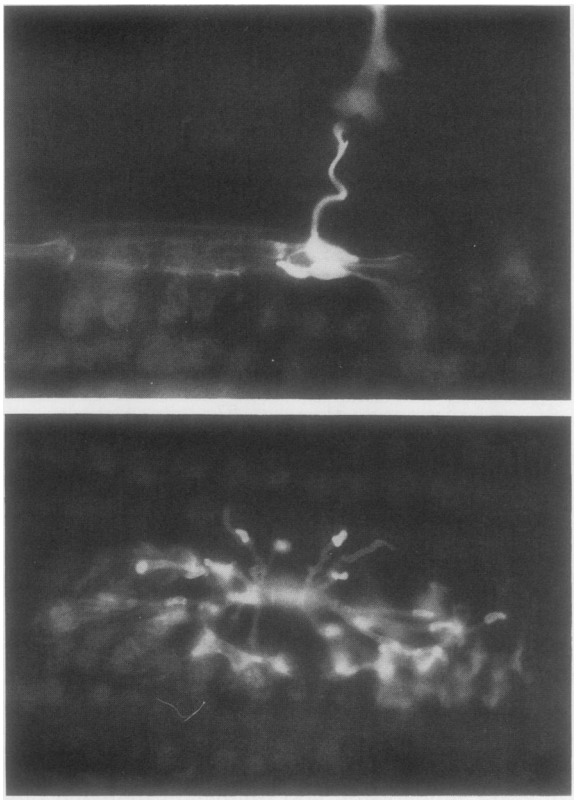

Figure 2. Fluorescence micrograph showing, in surface view, fungal development and host cell reaction in a highly incompatible interaction of wheat leaf and stem rust 4 d after inoculation. (Top panel) Water-injected controls. The majority of colonies has produced only two haustorial mother cells; penetrated wheat cells have reacted hypersensitively exhibiting yellow autofluorescence indicative of lignin or lignin-like compounds (21, 24, 30). (Bottom panel) CAD inhibitor (NH_2-PAS)-injected leaves. Colonies are significantly larger than in the controls and most of the haustorial mother cells are not associated with hypersensitive host cells. Similar results were obtained with OH-PAS and the PAL inhibitors.

Fungal growth, thus, resembled that in wheat cultivars exhibiting infection types 0; to 1.

Both PAL and CAD inhibitors exhibited similar effects in three different highly resistant wheat lines. No influence of these inhibitors on fungal development was seen in control experiments where susceptible wheat plants were treated with inhibitors prior to inoculation with the stem rust fungus.

DISCUSSION

One of the major problems in the research on plant resistance responses to pathogens is the question of whether an observed change in the host metabolism is causally involved in restricting pathogen growth, or whether it merely represents a direct or indirect consequence of a different, unknown resistance mechanism. One way of addressing this question is the application of metabolic inhibitors of the suspected resistance reaction to genetically resistant plants, which should convert an incompatible host/parasite interaction into a more compatible one. This approach has been followed in different plant pathogen combinations, the most widely used inhibitors being those of phenolic metabolism (8, 14, 16, 19, 20, 23, 27, 29, 31). Generally, by applying inhibitors such as glyphosate, AOA, AOPP, or APEP, pathogen-induced synthesis of phenolic phytoalexins or lignin-like compounds could be blocked or delayed in these studies. The concomitantly observed increase in pathogen development in these inhibitor-treated plants suggested a causal relationship between phytoalexin production or lignification and resistance.

In highly resistant wheat leaves, phytoalexins appear not to be involved in the very efficient restriction of avirulent races of the wheat stem rust fungus (24). Rather, the observed hypersensitive necrotization of penetrated host cells has been implicated in resistance. It has been shown that the hypersensitive cell death in wheat is correlated with the rapid cellular deposition of lignin or lignin-like compounds (7, 22, 24, 30), accompanied by an increase in several enzyme activities involved in the production of lignin precursors (10, 21).

Lignin deposition is generally regarded as a rather slow process, appearing only late in the infection cycle. Thus, it is mostly thought to be a consequence of cell death and not causally involved in hypersensitive resistance. But, thorough studies of the time course of events in the hypersensitive reaction of wheat to the stem rust fungus have shown that host cells lignify within 2 to 3 h after being penetrated by a fungal haustorium, and that lignification precedes the observed retardation of fungal growth in resistant plants compared to near-isogenic susceptible plants (24, 30). The concluded cause/consequence relationship between lignification and resistance has been strengthened by experiments with the competitive PAL inhibitor AOA (24, 30) and is further corroborated by the results presented here.

In the interpretation of *in vivo* effects of metabolic inhibitors, two main aspects have to be considered. Firstly, the specificity of the applied inhibitor for the metabolic step to be inhibited, and, secondly, the specificity of the inhibited step for the metabolic process to be investigated, will determine how accurately the results will pinpoint participation of the pathway in the physiological phenomenon studied. In both of these aspects, the CAD inhibitors are clearly superior to the PAL inhibitors. However, the specificity of the CAD inhibitors in vivo remains to be demonstrated. OH-PAS and NH_2-PAS are pseudo-irreversible suicide inhibitors of CAD (*i.e.* they are bound and metabolized by the enzyme, and the enzymatic action exposes a Zn^{2+}-complexing sulfine group thus inactivating the enzyme) (6) compared to the purely competitive PAL inhibitors, AOA, AOPP, and APEP. Generally, such inhibitors are thought to be ideally suited for *in vivo* investigations.

The similar influence on host cell necrosis and fungal development of all PAL inhibitors tested, AOA, AOPP, and APEP, strongly suggests that all three act via their inhibitory effect on PAL (18), implying a general involvement of phenylpropanoid metabolism in resistance. As OH-PAS and NH_2-PAS are thought to specifically inactivate enzymes which are specifically involved in lignin precursor biosynthesis, the results with the CAD inhibitors show a causal relationship between the production of cinnamic alcohols and resistance.

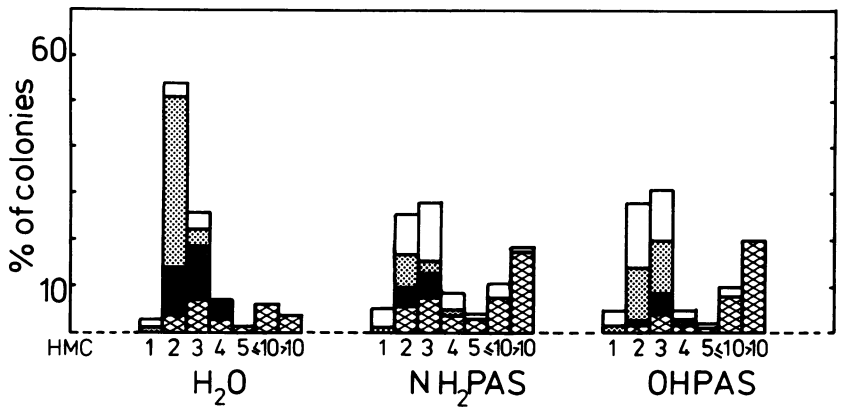

Figure 3. Influence of the CAD inhibitors, NH_2-PAS and OH-PAS, on fungal growth and host cell necrosis. Percentages of fungal colonies with n haustorial mother cells in highly incompatible interactions between wheat (cv Prelude-Sr5) and stem rust 7 d after inoculation. Inhibitors were applied via the cut-ends of the seedlings. Colonies associated with no □, epidermal (▨), epidermal and mesophyll (▩), or mesophyll (▧) necrosis. The differences between water- and inhibitor-treated leaves were shown to be significant ($P < 0.001$); the differences between the inhibitors were not ($P \gg 0.05$).

All the observations in this study were made on lactophenol/ethanol-extracted and, thus, dead tissues. Consequently, no firm conclusions can be made concerning the relationship between host cell death and the above discussed events of lignification and resistance. If lignification is considered a consequence of cell death, the following scenario would be consistent with our finding that lignification represents a causal factor in resistance: the penetrated host cell dies by some unknown mechanism, cell death leads to lignification which in turn prevents further fungal development. However, a somewhat different scenario is also possible, in which the cytoplasmic polymerization of cinnamic alcohols itself, as a chain reaction involving free radicals, represents the mechanism of active cell death, restricting growth of the biotrophic parasite. In all of our experiments, we found a strict correlation between lignification and collapse of penetrated host cells; all collapsed cells were lignified. If cell collapse is regarded as an indication of cell death, this correlation underscores the possibility of the second scenario. (However, if cells died without collapsing, such cells might not have been distinguishable in our preparations for microscopy from cells that had been living.) Nevertheless, we want to emphasize the possibility that induced lignification throughout the cytoplasm of the penetrated host cell might well be the mechanism of active, hypersensitive cell death in this host/parasite system. While the increased fungal growth upon inhibition of lignification clearly demonstrates a direct cause/consequence relationship between lignification and resistance, the proposed causal relationship between cell death and resistance (13) can not be concluded from these experiments; both events may be independent results of the lignification process.

Our results clearly show that the induction of the lignin biosynthetic pathway leading to the production of monomeric lignin precursors is causally related to resistance of wheat to the stem rust fungus. However, it remains to be determined whether the subsequent polymerization of these monomers to the three-dimensional network of the lignin polymer itself is a prerequisite for resistance.

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