Increased Lipoxygenase Activity is Involved in the Hypersensitive Response of Wheat Leaf Cells Infected with Avirulent Rust Fungi or Treated with Fungal Elicitor

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The hypersensitive reaction in incompatible wheat-rust interactions is characterized by an increase in lipoxygenase activity detectable as early as 28 h after penetration of the pathogen. In contrast, lipoxygenase activity in the compatible interaction did not increase until the onset of sporulation.

Lipoxygenase activity also increased following treatment of wheat leaves with an elicitor fraction from germ tubes of *Puccinia graminis tritici.*

Introduction

Host cell necrosis is recognized as one of the most prominent defense mechanisms against biotrophic parasites such as the rust fungi. Light and electron microscopic studies have demonstrated that in some incompatible interactions rapid localized host cell death accompanies this type of response to the invasion of an avirulent pathogen [1–4].

Alterations in membrane structure resulting in leakage of electrolytes and serious disorder to cellular control mechanisms would be expected to occur concomitantly with cell death [see review 5]. Membrane disorganization may result from lipoxygenase-catalyzed peroxidation of membrane lipids; this includes the peroxidation of the *cis,cis*-1,4-pentadiene systems commonly found in unsaturated fatty acids to the corresponding conjugated hydroperoxides. We therefore investigated the activity of lipoxygenase in susceptible and resistant wheat cultivars inoculated with *Puccinia graminis tritici* or, alternatively, with a nonpathogenic organism, *Puccini coronata avenae.* In addition, results are presented concerning the effect of an elicitor fraction isolated from germ tubes of wheat stem rust uredospores on the activity of lipoxygenase.

Abbreviations: Pgt, *Puccinia graminis tritici;* Pca, *Puccinia coronata avenae.*

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Materials and Methods

Host and pathogen

Wheat plants were grown in a growth chamber at 20 °C, 50–60% relative humidity, 16 h light regime and 15 klux. Primary leaves of 7 day-old plants were inoculated with uredospores or treated with elicitor. The following wheat cultivars were used throughout the experiments: *cv.* Marquis, which is susceptible to *Puccinia graminis tritici* (Pgt), race 32, and which exhibits hypersensitivity to the non-pathogen *Puccinia coronata avenae* (Pca); the corresponding, nearly isogenic line of *cv.* Marquis which carries the Sr5 gene for resistance against Pgt; *cv.* 417/65 which is highly resistant to Pgt, race 32.

Uredospores of Pgt, race 32, were collected from infected leaves of wheat *cv.* Little Club, and stored either at 4 °C for a few days or frozen in liquid nitrogen at −192 °C where they maintained their infectivity over long periods. Uredospores of Pca were collected from oat *cv.* Pc40 and stored under the same conditions as Pgt.

Plants were infected by spraying them with a suspension of uredospores in Freon 112 (100 mg uredospores/50 ml Freon). Incubation took place in a plastic cage used as a humidity chamber, which was carefully sealed to maintain high humidity conditions during the incubation period (16 h of darkness, followed by 4 h of light). Then the plants were taken out of the chamber and left to stand under standard conditions. Penetration of the stomata by the germinated rust uredospores is presumed to commence

with the onset of the light period (time 'zero' in the Figures; [6]).

Enzyme assay

Crude enzyme extracts were prepared from two primary leaves of wheat at the times indicated. The plant material was weighed, and ground in liquid nitrogen with a pestle and mortar. Precooled potassium phosphate buffer (0.1 M, 1 mM EDTA, pH 7.5) was added (4 volumes/weight) and the extracts further homogenized. Samples were subsequently centrifuged at 28,000 × *g* for 15 min at 2 °C. Lipoxygenase activity was measured in the supernatant fraction.

Enzyme activity was measured spectrophotometrically [7]. An antioxidant was not added since it has been reported that this disturbs spectrophotometric assays of lipoxygenases [8]. All measurements were carried out in triplicate on independent samples.

The enzyme reaction mixture contained 1 ml citrate phosphate buffer (0.1 M, pH 6.2; at this pH, wheat lipoxygenase was found to have its maximum activity, data not shown) and 100 µl of substrate solution prepared according to [7] (2.5×10^{-3} M linoleic acid in 0.05 M phosphate buffer, pH 9, containing 0.25% Tween 20). The reaction was started by the addition of 20 µl of enzyme extract. The reaction was found to be linear for at least 20 min. One enzyme unit is defined as the amount of enzyme which produces a change in absorption of 0.001 min^{-1} at 234 nm; this corresponds to a catalytic activity of 0.667 nkat (absorption coefficient of the peroxide is 25.000 $M^{-1}cm^{-1}$; [9]).

The protein content was assayed using the Biuret method, taking BSA as standard. It was found that fresh extracts could be rapidly frozen in liquid nitrogen and stored at −20 °C for at least two weeks without any measurable loss of enzyme activity.

Isolation and injection of elicitor fraction

Germinated uredospores of Pgt were used as the elicitor source, as described elsewhere [30]. Briefly, germ tubes were ground in distilled water at 4 °C in a cold mortar and the homogenate was centrifuged at 65,000 × *g* for 30 min. Lipid material was extracted with chloroform/methanol (2:1) and the remaining insoluble material was lyophylized. This material was resuspended in water and again centrifuged at 65,000 × *g* for 30 min. The resulting clear supernatant was found to contain elicitor activity as judged by its ability to stimulate phenylalanine ammonia-lyase and to cause lignification after injection into leaves [10].

Elicitor solution was injected into primary leaves of 7 day-old wheat plants using a hypodermic syringe [11]. The plants were incubated in the growth chamber under the conditions described above. The concentration of the elicitor solutions and the times at which lipoxygenase was measured are indicated in Figures 2 and 3.

Fluorescence microscopy

Leaf sections were stained according to the method described by Rohringer *et al.* [12] and viewed with a Zeiss epifluorescence microscope (filter combination: BP 400−440/FT 460/LP 470). Uvitex 2B (Ciba Geigy) instead of Calcofluor was used as fluorochrome. Colony length was measured with the aid of a built-in microscale.

Results

Lipoxygenase activity in inoculated plants and uninoculated controls

We found remarkable differences between the lipoxygenase evolution in healthy and that in rust-infected wheat leaves. Whereas there were no major changes in lipoxygenase activity in uninoculated control plants throughout the course of the experiments, the activity increased in all plants inoculated with rust fungi. However, the increase in activity differed, depending on the type of interaction: the incompatible interactions (*cv.* 417/Pgt and *cv.* Marquis/Pca) showed an early rise in lipoxygenase activity whilst the lipoxygenase activity in the compatible interaction did not increase until the onset of sporulation (Fig. 1, A + B).

Microscopic examination of the autofluorescence of infected cells revealed that incompatibility in the various interactions studied was characterized by a hypersensitive reaction. Hypersensitivity could be detected 24−48 h after inoculation. In general, only 2−4 haustorium mother cells were formed in incompatible interactions (Table I), and the colonies did not expand beyond the stage reached two days after inoculation.

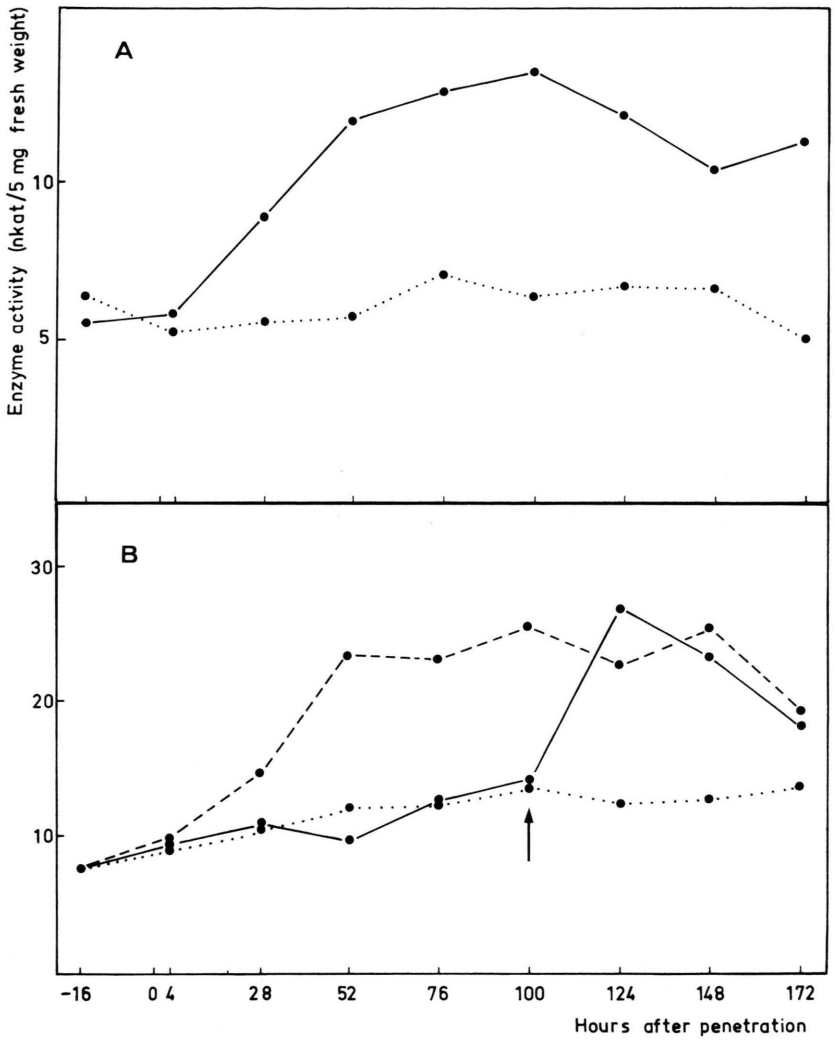

Fig. 1. Lipoxygenase activity in different wheat-rust interactions. Extracts were prepared at 24 h intervals starting 4 h after the beginning of the light period, *i.e.* after the start of penetration into the stomatal cavities.
A) *cv.* 417/65 infected with *Puccinia graminis tritici*, race 32 (●—● = incompatible interaction; ●·····● = uninoculated control plants).
B) *cv.* Marquis infected with *Puccinia graminis tritici*, race 32 (●—● = compatible interaction) or with *Puccinia coronata avenae* (●– – –● = incompatible interaction); (●·····● = uninoculated control plants). Arrow indicates beginning of sporulation in the compatible interaction.

Table I. Development of rust fungi in different wheat-rust interactions. Values represent colony diameter (μm) and number of haustorium mother cells (HMC) growing on wheat leaves at 24 h time intervals. Pgt = *Puccinia graminis tritici*, Pca = *Puccinia coronata avenae*. Standard deviations are given for $n = 200$ counts.

Hours after penetration	*cv.* Marquis				*cv.* 417/65			
	Pgt		Pca		Pgt		Pca	
	Diameter	HMC	Diameter	HMC	Diameter	HMC	Diameter	HMC
4	151 ± 12	6.8 ± 3.6	145 ± 20	2.2 ± 0.9	95 ± 14	1.8 ± 0.8	135 ± 30	1.9 ± 0.8
28	308 ± 40	> 20	177 ± 18	3.9 ± 1.3	106 ± 12	2.8 ± 2.3	140 ± 15	2.5 ± 1.2
52	650 ± 70	> 20	165 ± 15	4.6 ± 2.8	98 ± 13	2.0 ± 0.6	150 ± 35	2.8 ± 1.3

Lipoxygenase activity after treatment with a Pgt-elicitor fraction

Treatment with an elicitor fraction isolated from the germ tubes of uredospores of *Puccinia graminis* evoked a significant activation of lipoxygenase as measured both in a time course experiment (Fig. 2) and in a dose-response experiment (Fig. 3). In the latter, the response of *cv.* Marquis sr5, susceptible

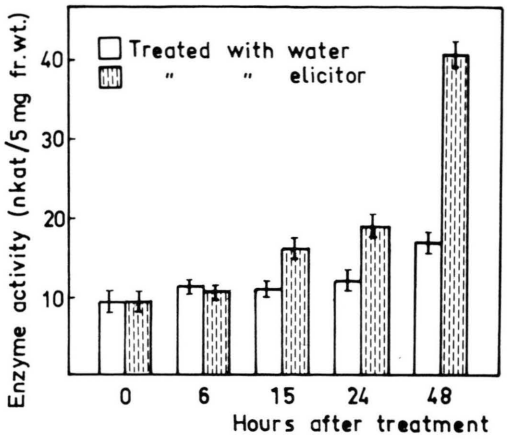

Fig. 2. Increase in lipoxygenase activity after injection of an elicitor fraction isolated from germ tubes of *Puccinia graminis tritici* into primary leaves of the wheat *cv.* 417/65. Concentration of elicitor was 400 µg sugar equivalents/ml.

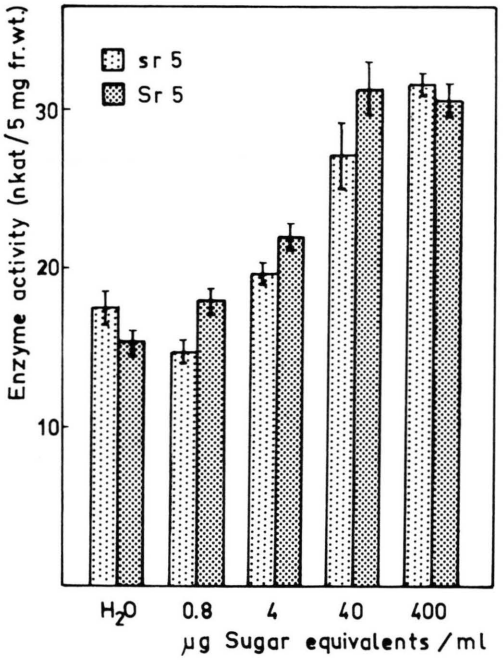

Fig. 3. Increase in lipoxygenase activity 48 h after injection of different amounts of elicitor fraction into primary leaves of the nearly isogenic lines of Marquis carrying the Sr5 allele for resistance to Pgt, or the corresponding sr5 allele conferring susceptibility. The leaves were treated with distilled water or with increasing amounts of the elicitor fraction as indicated.

to Pgt, was compared with the corresponding genetically-related line *cv.* Marquis Sr5 (nearly isogenic line carrying the Sr5 gene), resistant to Pgt race 32. As seen in Fig. 3 however, the two *cv.* Marquis lines did not differ significantly from one another as regards the response to the elicitor.

Discussion

The evolution of lipoxygenase activity was characterized by a late increase in the compatible interaction and an early rise, clearly detectable 28 h after penetration, in the incompatible interactions studied. In the latter, the maximum activity was reached within 52 h after penetration of leaf tissues by the rust fungus. At this time fungal colonies had formed 2–4 haustorium mother cells, and fungal growth was definitely inhibited in the incompatible interaction [13]. In contrast, in the compatible interaction lipoxygenase activity was not altered during the early stages of disease development although the fungus spread rapidly into the host tissue. Here, lipoxygenase activity was increased only at a later stage of development, namely, with the onset of sporulation (100 h after penetration).

Lipoxygenase activity also increased following treatment of wheat leaves with an elicitor fraction from germ tubes of Pgt, race 32. In this case, however, there was no expression of specificity as regards the response of the susceptible and resistant wheat lines.

The results indicate that an increase in lipoxygenase activity accompanies the early and rapid hypersensitive response in incompatible wheat-rust interactions and that it may be directly involved in the sequence of reactions leading to necrosis. Such an increase also appears to be linked to the disorganization of exhausted infected host cells in the late stages of the compatible interaction. Similar behaviour, *i.e.* an early and rapid response in incompatible interactions characterized by a hypersensitive reaction, and a late response in compatible interactions, has been reported for a number of enzymes and factors which are thought to be related to resistance in various host-parasite interactions, *e.g.* lignification in cucumbers infected with *Cladosporium cucumerinum* [14], hydroxyproline levels in cell walls of cucumbers infected with *Cladosporium cucumerinum* [15], accumulation of hydroxyproline-rich glycoprotein mRNA in bean hypocotyls infected with *Colletotrichum lindemuthianum* [16], and an increase in chalcone synthase mRNA activity in bean hypocotyls infected with *Colletotrichum lindemuthianum* [17]. It is apparent from this spectrum of

host responses that disease occurs when the resistance response of the host to the pathogen is suppressed [18].

Lipoxygenase activity has been reported to be linked to senescence [19], to the production of superoxide anions in senescing plant tissue [20], and also to the production of ethylene in senescence [21–24] which, in turn, may affect the response of stressed plant cells [25]. Lipoxygenase activity increased in the late stages of pathogenesis in tobacco infected with *Erysiphe cichoracearum* [26]. Inhibitors of lipoxygenase decreased phytoalexin synthesis in arachidonic acid-stressed potato tissue [27]. The possible involvement of cyanide-respiration in elicitor-induced sesquiterpenoid accumulation in potato [28] and the effect of inhibitors of cyanide-resistant respiration, which also inhibit lipoxygenase activity [29], has led Kuć and Preisig [18] to argue that lipoxygenase could be a mediator of elicitor action.

These results taken together with those presented here point to a fundamental role of lipoxygenase activity in senescence, stress response, and hostparasite interactions.

In addition, the results presented in this paper suggest that lipoxygenase is involved in the early hypersensitive response in wheat-rust interactions. These interactions are characterized by the activation of processes leading to lignification and formation of callose at about the same time as there is an increase in lipoxygenase activity [13]. At present, however, the sequence of events, and thus the actual role of lipoxygenase, is not clear and requires further investigation.

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