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Biochemical and cytological aspects of genetical and acquired resistance in the rice blast interaction

U. Schaffrath, M. Thieron, H. Scheinpflug and H.J. Reisener
Inst. Bio III (Pflanzenphysiologie), RWTH Aachen (Germany)

Abstract. Economically, “rice blast” caused by *Pyricularia oryzae*, Cav. (*Magnaporthe grisea*, Barr) is the most important disease in rice plant cultivation. Resistance reactions of the plants have been investigated by bio- and histochemical means. Lignification of penetrated cells has been described as the earliest resistance reaction in infected plants, occurring much earlier than hypersensitive cell death and accumulation of phytoalexin.

A glycoprotein (mol. wt. 156 kDA) derived from *P. oryzae* cell walls was purified to homogeneity. Activity of the water-soluble elicitor is not affected by enzymatic digestion of the protein core, but is completely abolished after modification of the carbohydrate moiety by periodic treatment. The carbohydrate moiety consists mainly of mannose with some galactose and glucose. After application onto rice leaves, activation of enzymes involved in lignification were observed. Injection into intercellular spaces of rice leaves resulted in increased autofluorescens of cell walls, presumably due to the deposition of lignin or lignin-like substances. All resistance reactions that are triggered in incompatible interactions are induced as well by the Pyr-elicitor.

In addition, infiltration of intercellular spaces of rice plants followed by inoculation with a virulent race of *P. oryzae* established local acquired resistance. The crucial event that prevents plant from infection is induced lignification of the first penetrated cell. Furthermore, acquired resistance could be obtained in rice plants treated with several chemical substances. By inhibition of PAL and CAD, two essential enzymes of lignin biosynthesis, it could be proved that the mechanism of resistance in all these cases is induced lignification.

Physiological changes occurring in treated plants prior to challenge inoculation may play a role in the onset of induced disease resistance. These events are likely to be involved in lipid metabolism that is associated with lipoxygenase activity. In fact, all resistance inducing agents under investigation led to an increase in lipoxygenase activity. This may contribute to various defense mechanisms by induction of phytoalexin production or further conversion into jasmonic acid or thaumatin.

Acquired resistance is a phenomenon with excellent prospects, because plant protection by induction of the plants own defense reactions, without need of toxic compounds, may have great ecological benefit and public acceptance. Our further interest will be focused on identification of markers that help finding more resistance inducing chemical substances and on the elucidation of the mode of action of acquired resistance in rice.

I – Introduction

Inducible responses, known as local or systemic acquired resistance, that allow plants to defend themselves from pathogen infection, has become a subject of increasing interest (Kessmann et al., 1994). Although this phenomenon has been described for a long time (Chester, 1933; Ross, 1961), only little information is available on the underlying mechanisms. Even for rice, several biological and chemical substances are known that apparently protect it against the blast fungus, *Pyricularia oryzae*, Cav., by stimulating the plants' own defense responses.

In incompatible interactions alterations occur among penetrated rice cells that are characterized by a high affinity to aniline-blue (Thieron et al., in press) and a high autofluorescens under UV light (Koga *et al.*, 1994). It has been demonstrated that the aniline-blue reaction is due to induced lignification or the accumulation of lignin-like substances in the effected cells. This is the crucial resistance reaction of rice plants against *P. oryzae* that occurs much earlier than the hypersensitive cell death or the accumulation of phytoalexins (Thieron, 1993). Here, we report on a comparative study of plants possessed genetically or acquired resistance. By that, we try to elucidate whether induced lignification is also the essential mechanism in rice plants showing acquired resistance.

But how is the establishment of acquired resistance in rice gained? Metabolic changes occurring in treated plants prior to challenge inoculation are expected to play a role in the onset of induced disease resistance. Recently, genes have been cloned and identified that are induced in rice after treatment with *Pseudomonas syringae,* a bacteria that is known to establish acquired resistance in rice (Reimann et al., 1992; Reimann and Dudler, 1993). Here, we report on biochemical investigations of rice plants treated with a set of different biotic and chemical substances that are known as resistance inducing agents in rice.

II – Methods

1. Fungal and plant material, growth conditions and inoculation

Races No. 007 (H-373), No. 031 (TH-6772) and No. 003 of P. *oryzae* were used. While races 007 and 003 exhibit compatible interactions with the rice cv. Jukkoku, and both are incompatible with cv. Kusabue, race 031 is incompatible with cv. Jukkoku and compatible with cv. Kusabue. The rice *(Oryza sativa,* L.*) cv.* Kusabue carries the resistance gene Pi-k, while cv. Jukkoku possesses the resistance gene Pi-a. Plants were grown in soil (5 x 5 x 4,5 cm^3 pots) in a phytotron maintained at 23 ± 1°C with 70-80% RH. The photoperiod lasted for 14 h and the light intensity was 18 000 lux.

Oat-meal starch agar (30 g 1^{-1} oat-flakes, 20 g 1^{-1} agar-agar, 10 g 1^{-1} starch and 2 g 1^{-1} yeast-extract) was used to cultivate the fungus. After incubation at 27°C for two weeks, aerial mycelia were removed and synchronous sporulation was stimulated by further incubation under UV light (310-360 nm). For inoculation the concentration of conidia was adjusted to 5 x 10^5 per ml of the spray solution (1 g 1^{-1} gelatine, 0,5 g 1^{-1} sodium oleate). At the 4-leaf-stage (12 days after sowing), plants were inoculated by spraying the conidial suspension onto the leaves. After a 24 h-incubation in a dark moist chamber (26 ± 1°C, 100% RH), plants were kept in a light-moist chamber (24 ± 1°C, 90% RH) under the light regime described above.

Preparation of the Pyr-elicitor was done as described previously (Schaffrath et al., 1995).

2. Biochemical analysis

For determination of POX activity, the method of Putter (1970) was modified. The enzyme extract (100 µl) was incubated with 2.8 ml of phosphate-buffer containing 18 mM guajacol and the reaction was started by adding 100 µl of H_2O_2-solution (0,1% v/v). Production of tetraguajacol was measured in a spectrophotometer at 470 nm. Enzyme activity was expressed in nkat/mg protein. Chitinase activity was determined according to the method of Wirth and Wolf (1990) with CM-chitin-RBV (Fa. Löwe) as substrate. Lipoxygenase activity was measured with α-linoleic acid as substrate (Ocampo et al., 1986) with 0,15% Tween 20 (C. Bohland, personal communication), and activity of cinnamyl-alcohol dehydrogenase was determined using a method of Wyrambik and Grisebach (1975) modified by Moerschbacher et al. (1988).

3. Aniline-blue staining

Whole leaf staining with aniline-blue was performed as described by Peng et al. (1986).

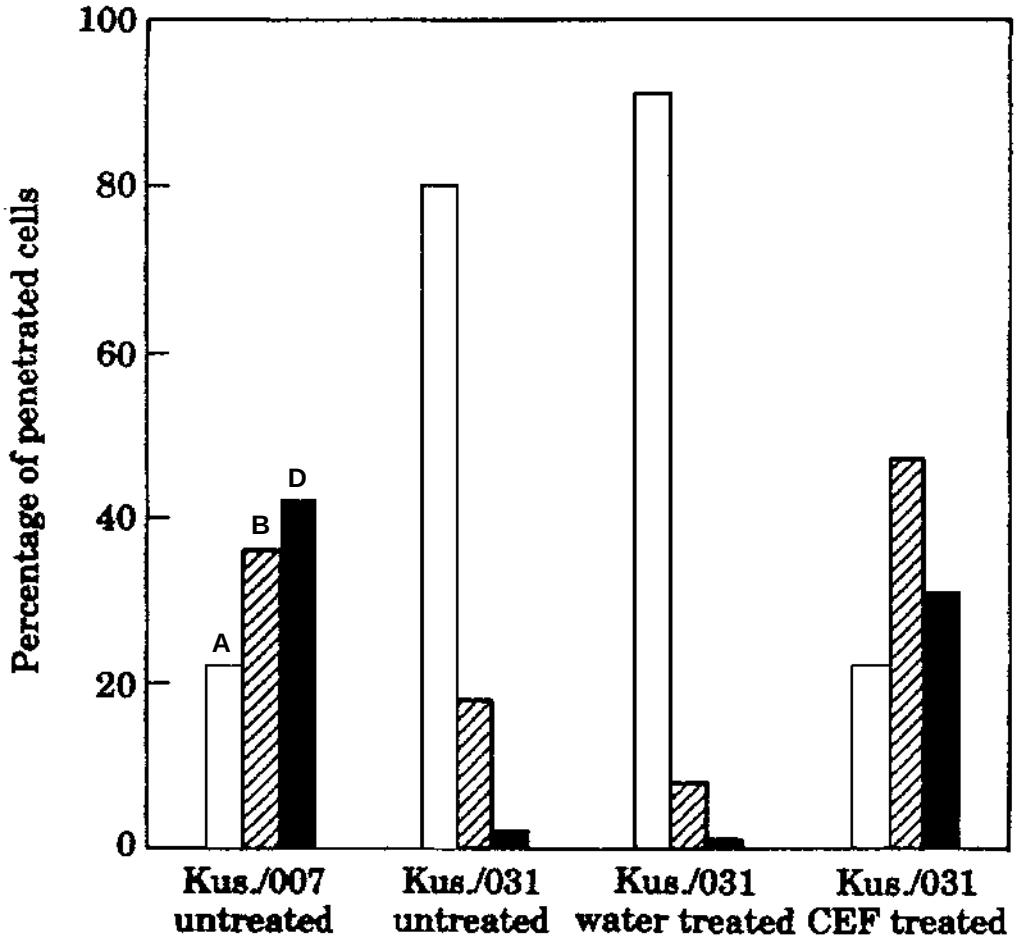
III – Results and discussion

It has been demonstrated that induced lignification is the crucial mechanism in rice plants with genetical resistance (Thieron, 1993; Thieron et al., in press). Penetrated cells of resistant plants exhibit a very fast lignification that restricts fungal development to a small invading hyphae within the first cell. Even in susceptible plants lignification occurs, but this event at the 3rd and 4th day of infection is too late, and therefore it has no influence on the outbreak of the disease.

We have investigated rice plants showing acquired resistance by using the aniline-blue staining tech-

nique. In this experiment the Pyr-elicitor was used as an inducing agent (Schaffrath et al., 1995). Intercellular spaces of rice leaves were infiltrated with an elicitor-active fraction using the Hagborg-device (Hagborg, 1970), and plants were inoculated after evaporation of the solution (4 h) with a virulent race of *P. oryzae.*

Fig. 1. Different cellular reactions of rice plants cv. Kusabue to penetration of the virulent race 031 of



Pyricularia oryzae **can be discriminated after aniline-blue staining.**
Reactions type A indicates no obvious host response, while type B and D indicate strong host responses correlated with lignification or accumulation of lignin-like substances in the cell walls (type B) or in the walls and the cytoplasm (type D) of invaded cells. Leaves were harvested 48 h after inoculation and stained with aniline-blue. CEF, crude elicitor fraction.

Inoculated leaves were harvested 48 hours after inoculation and stained with aniline-blue (Fig. 1). With this staining technique a discrimination of cellular reaction types (A, B and D) can be performed, and their distribution correlates with resistance and susceptibility. Typically incompatible interaction (Kusabue/race 007) are characterized by a high percentage (approx. 80%) of reaction type B and D at the second day of infection, while compatible interactions (Kusabue/race 031) exhibit a high percentage of unstained cells (type A). In contrast, elicitortreated and inoculated leaves showed a high percentage (75%) of cell-types B and D (Fig. 1). This indicates an active lignification response after penetration of the fungus like in an incompatible interaction. Water pre-treated control plants showed only 10% of stained cells, which is typical for compatible interactions. This control shows that the Hagborg-device itself has no influence on the lignification. The results of this experiment reveal that the lignification response in rice plants with acquired resistance occurs in the same manner as in rice plants genetically resistant. This histochemical results were in agreement with biochemical investigations concerning induced enzyme activities (Thieron, 1993; Schaffrath, 1994; Schaffrath et al., 1995).

Comparison of rice plants with genetically and acquired resistance by histochemical and biochemical investigations shows a close correlation between the occurrence of cellular lignification and resistance against the blast fungus. The bearing of induced lignification was unequivocally proofed by inhibition of

enzymes involved in lignin-biosynthesis. We blocked both the first enzyme of phenyl propanoic pathway (PAL, phenylalanine ammonia lyase) and an exclusive enzyme of lignin-biosynthesis (CAD, cinnamyl alcohol dehydrogenase) with specific inhibitors. While AOPP is a competitive inhibitor of PAL (Carver et al., 1991), NH2PAS inhibits the CAD (Grand et al., 1995).

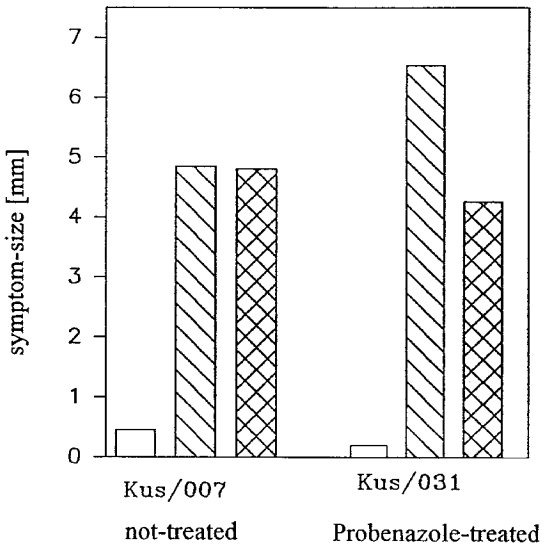
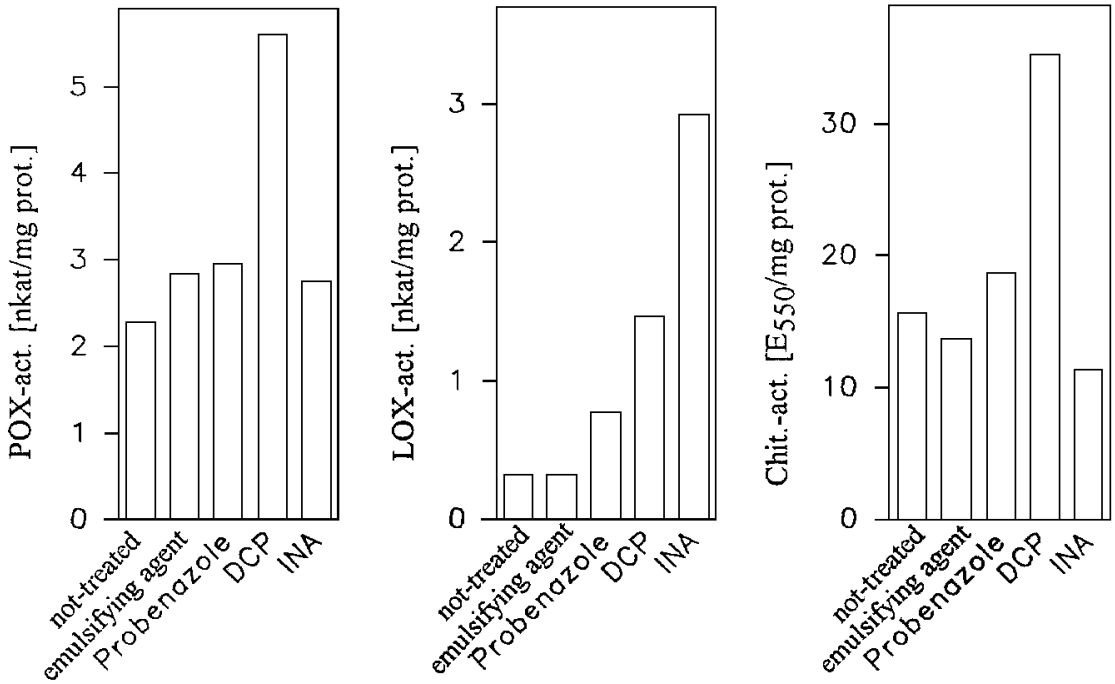


Fig. 2. Rice plants cv. Kusabue were treated with Probenazole 5 days after sowing by soil drench.

11 days after sowing intercellular spaces of the youngest leaves were infiltrated with inhibitor solutions (AOPP 0.lrnM; NH2-PAS 0.l rnM), while control plants were water-infiltrated. After 6 hours untreated rice plants were inoculated with the avirulent race 007, and Probenazole treated plants were inoculated with the virulent race 031. Measurement of symptom size was done 5 days after inoculation. Water-infiltrated (☐); CAD-inhibitor (☐); PAL-inhibitor (☐).

Both inhibitors were applied to genetically as well as acquired resistant rice plants prior to inoculation. Estimation of the disease was done on the fifth day after inoculation by measurement of symptom size (Fig. 2). Water-infiltrated control plants that are genetically resistant to race 007 have very small necrotic spots on the inoculated leaves. Infiltration with both enzyme-inhibitors led to an increase in symptom size up to 5 mm, and to a change in symptom type from small necrosis to blast symptoms with a grey sporulation area. Thus, genetical resistance is broken by inhibition of lignin-biosynthesis. In addition, acquired resistance of rice plants against the virulent race 031 is abolished after blocking lignification. In fact, the findings that not only inhibition of PAL (i.e., a common enzyme of secondary metabolism) but also inhibition of CAD (an exclusive enzyme of lignin-biosynthesis) broke both types of resistance, evidenced the importance of the lignification response for resistance.

Fig. 3. Rice plants cv. Jukkoku were treated 7 days after sowing with different chemical inducers by soil



drench; water-treated plants and plants treated with the emulsifying agent serve as controls. LOX-, POX- and chitinase activity were measured 7 days after treatment.

Up to now, we know the mechanism of defense in rice against *P. oryzae.* In genetically resistant rice plants, this capability is based on genome information, but how is resistance established in immunized plants that were previously susceptible? It appears to be reasonable that pretreatment with any inducer sensitizes the host cells to respond faster on pathogen attack. The onset and establishment of the acquired resistant state in rice plants might base on physiological changes that occur after pre-treatment and before challenge inoculation. Firstly, we looked at different enzyme activities each related to a typical resistance reaction in rice:

- ❏ peroxidase activity (POX), which is involved in lignification reaction;
- ❏ chitinase activity, which is a so-called “pathogenesis-related protein”;
- ❏ lipoxygenase activity (LOX), which is involved in membrane alterations and phytoalexin production.

The biotic inducer “Pyr-elicitor” increased all of this enzyme activities, and it is compared in the following experiment to three different chemical inducing agents (Fig. 3).

- ❏ 2,2-dichloro-3,3-dimethylcyclopropanionic-caboxylic acid (DCP);
- ❏ 2,6-dichloro-isonicotinic acid (INA);
- ❏ Probenazole (syn. Oryzemate).

All chemicals were suspended in an emulsifying agent and applied to the plants by soil drench. Not treated plants and plants treated with the emulsifying agent were serving as controls. In general, there is no common set of enzyme activities that is triggered by each inducer in the same manner. While pop stimulates all three enzyme activities, INA and Probenazole only induces LOX-activity. But while Probenazole doubles this activity, INA increases it to a 10-fold higher level. In this experiment each of the chemicals behave differently. However, all inducers share the induction of LOX-activity, so one may speculate that acquired resistance in rice requires the lipoxygenase pathway. Stimulation of LOX-activity is generally known as a response to membrane alterations and therefore may serve as a marker of any interaction between a resistance inducer and the plant cells. In addition, increasing of LOX-activity is known as an autocatalysing process, and thereby it is an excellent mean for amplifying a signal. Supplementary studies revealed that rice plants treated with Probenazole respond much stronger upon application of the Pyr-elicitor than control plants (Thieron, 1993). This enhancement of elicitor recognition may lead to a faster onset of defense responses.

The phenomenon of acquired resistance provides an excellent tool for further progress in chemical disease control. Because the inducing agents are active via the plants own defense mechanisms, these compounds require no toxic properties. Therefore, they are of great ecological benefit and will have a high public acceptance. Further work has to be done on developing more resistance inducing agents and on the elucidation of the mode of action in detail.

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