Elicitation of β-1,3-Glucanase and Chitinase Activities in Cell Suspension Cultures of *Ascochyta rabiei* Resistant and Susceptible Cultivars of Chickpea *(Cicer arietinum)*

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β-1,3-Glucanase and chitinase activities have been analyzed in cell suspension cultures of an *Ascochyta rabiei* resistant ILC 3279 and a susceptible ILC 1929 cultivar of chickpea *(Cicer arietinum)* following treatment with an elicitor derived from this fungal pathogen. A facile method to determine both hydrolase activities in the cell culture medium was established.

Significantly higher constitutive and elicitor-induced levels of both hydrolase activities were found in the medium of the ILC 3279 cell culture. The release of these enzyme activities is not due to cell lysis, but rather the consequence of a secretion mechanism. The cells of the resistant line contained a 5 times higher level of chitinase activity in comparison to the ILC 1929 cell culture, whereas the latter cells possessed a 3 times higher β-1,3-glucanase activity.

The results are interpreted that accumulation of extracellular hydrolase activities may play an important role among the various plant defense mechanisms previously determined for the incompatible interaction between the resistant cultivar and its fungal pathogen.

Introduction

Resistance of higher plants against microbial pathogens is the result of constitutive and inducible defense mechanisms. After infection the accumulation of phytoalexins [1, 2] and the synthesis of PR proteins [3] are especially prominent reactions.

Recent investigations have identified different functions for PR proteins as β-1,3-glucanases (EC 3.2.1.6) and chitinases (EC 3.2.1.14) [4–6]. These hydrolytic enzymes are thought to play an important role in the plant defense system, due to their ability to inhibit fungal growth by degrading components of the mycelial cell wall [7, 8].

During our investigations [9, 10] on biochemical aspects of the interaction between the chickpea *(Cicer arietinum)* plant and its main fungal pathogen, *Ascochyta rabiei,* cell suspension cultures of a resistant (ILC 3279) and a susceptible (ILC 1929) cultivar have been employed with great benefit. Upon elicitor treatment these cell cultures were clearly shown to express cultivar-specific differences in antimicrobial defense reactions and may therefore be used as suitable model systems.

Here we report on the induction of β-1,3-glucanase and chitinase activities in cell suspension cultures of the different cultivars of chickpea following treatment with an elicitor derived from *Ascochyta rabiei.* Special attention was placed on the possible accumulation of these hydrolase activities in the cell culture medium of the resistant and susceptible cells. We provide evidence that both hydrolases are excreted into the culture medium. Due to their considerable accumulation in the medium of the ILC 3279 cell culture line, we suggest that these extracellular enzymes could be involved in the plant defense system against the attacking pathogen.

Materials and Methods

Chemicals

Regenerated [^{3}H]chitin was prepared as described [11]. Chitosan was purchased from Sigma (Munich, F.R.G.) and [^{3}H]acetic anhydride (1.9 GBq/mmol) from New England Nuclear (Frankfurt, F.R.G.).

For the β-1,3-glucanase assay laminarin and 4-hydroxybenzoic acid hydrazide were obtained from Sigma (Munich, F.R.G.).

All other chemicals were *p.a.* grade.

Abbreviations: PR proteins, pathogenesis-related proteins; ILC, international legume chickpea; TCA, trichloroacetic acid.

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Cell cultures

The cell cultures of the chickpea cultivars ILC 3279 and ILC 1929 were propagated on modified PRL-4c-medium [12] as described [13]. Transfer of 3 g cells into new medium was carried out every 7 days. The suspension cultures were grown in the dark at 25 °C. The 200 ml flasks containing 40 ml medium were shaken at 120 rpm.

Elicitor

The elicitor was prepared from a strain of *Ascochyta rabiei* according to Kessmann and Barz [14].

For elicitation 3 mg of the elicitor was dissolved in 1 ml water. The solution was autoclaved and added to the cell culture under sterile conditions 3 days after transfer of the cells into new medium. Controls were given 1 ml water.

Preparation of enzyme extracts

The treatment of cells and medium for the preparation of enzyme extracts was identical for the chitinase and β-1,3-glucanase assay. Cells were separated from the medium by filtration. The medium was stored at −20 °C until further preparation.

Crude cell extracts were obtained by homogenizing 3 g cells with sand in a mortar. 6 ml buffer (100 mM K_2HPO_4/KH_2PO_4, pH 8.0 containing 1.4 mM mercaptoethanol) and 1.5 g Dowex 1 × 2 (phosphate-form) were added. Centrifugation for 15 min at 26,000 × *g* resulted in a clear supernatant which was filtered through glasswool and stored at −20 °C until used for enzyme assays.

After thawing the medium was centrifuged (5 min, 3000 × *g*) and subsequently brought to 95% saturation with solid $(NH_4)_2SO_4$. Centrifugation (30 min, 42,000 × *g*) yielded a precipitate, which was dissolved in 2.5 ml 100 mM potassium-acetate buffer (pH 5.3). The enzyme solution was desalted on Sephadex G-25 (PD 10, Pharmacia, Freiburg, F.R.G.) and stored at −20 °C.

Enzyme assays

β-1,3-Glucanase activity was measured as described by Kombrink *et al.* [15], except that 4-hydroxybenzoic acid hydrazide [16] was used as colour reagent to determine the amount of released reducing sugars. Glucose was used as a standard. Enzyme and substrate alone were included as controls. One katal (kat) is defined as the enzyme activity catalyzing the formation of 1 mol glucose equivalents/s.

Chitinase activity was determined using the radiometric assay according to Boller *et al.* [17]. The reaction mixture (total volume 250 µl) contained 120 µl 100 mM potassium phosphate buffer, 30 µl enzyme solution, and 100 µl [^{3}H]chitin suspension (2.9×10^5 cpm). After incubation for 10 min at 37 °C the reaction was stopped by adding 250 µl 10% TCA, and the suspension was centrifuged at 3000 × *g* for 4 min. From the supernatant 200 µl were carefully removed and radioactivity determined by liquid scintillation counting after adding 4 ml of Hydroluma (Baker, Groß-Gerau, F.R.G.). Enzyme activity was calculated as described by Boller *et al.* [17] and is expressed as Unit (U). One Unit is the amount of radioactivity in cpm × 10^{-3} × min^{-1} released by a defined amount of enzyme solution. Controls with enzyme solution or substrate alone were carried out.

Protein determination

Protein content was determined using the method described by Bradford [18], employing bovine serum albumin as a standard.

Results

β-1,3-Glucanase and chitinase enzyme activities of chickpea cell suspension cultures of a resistant and a susceptible cultivar were determined in the tissue extracts and in the medium following treatment with an elicitor preparation derived from the fungal pathogen *Ascochyta rabiei*. Addition of the elicitor to the cell culture occurred on day 3 of the growth cycle. The response of the two cell culture lines towards elicitor treatment was significantly different.

β-1,3-Glucanase activity

In the cell extract of the resistant cell culture (ILC 3279) β-1,3-glucanase showed a 2.8-fold increase compared with the control 56 h after elicitor application, whereas in the susceptible cell culture (ILC 1929) a 2-fold increase of enzyme activity was found (Fig. 1A). However, the level of induced and constitutive enzyme activity (control) was three times higher in the cells of ILC 1929 than in ILC 3279. In addition enzyme activity in the cells of ILC 1929 increased very rapidly and the maximum level was already reached after 12 h.

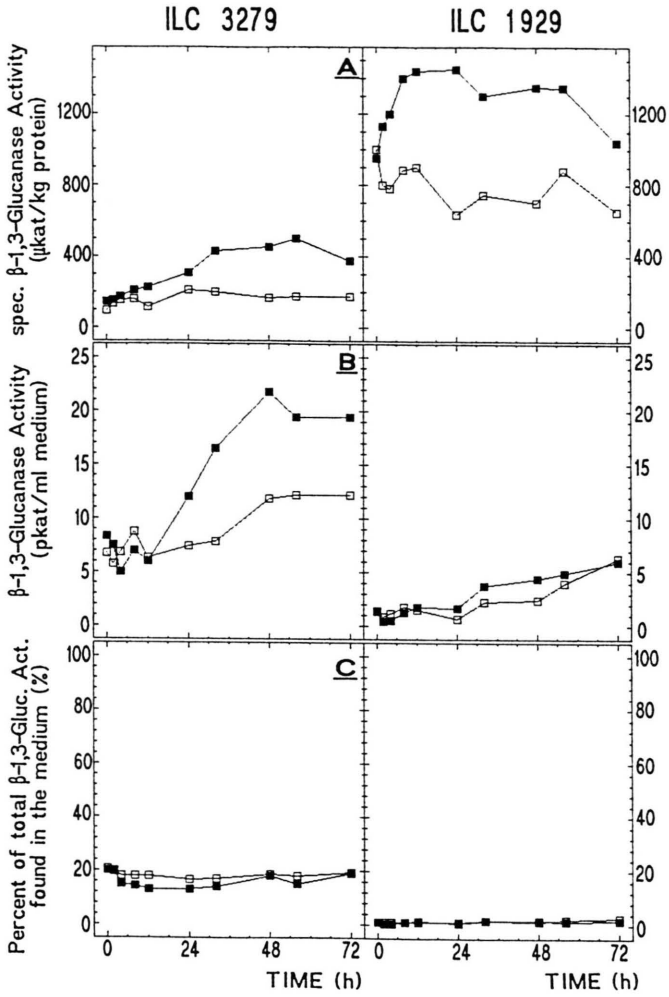

Fig. 1. Changes of β-1,3-glucanase activities in cell suspension cultures established from resistant (ILC 3279) and susceptible (ILC 1929) cultivars after application of *A. rabiei* elicitor (■). Controls (□) received water. A: enzyme activity in the cells; B: enzyme activity in the culture medium; C represents the distribution of total β-1,3-glucanase activity (data not shown) between cells and medium.

The medium of the ILC 3279 cell culture revealed a rapid increase of enzyme activity after a lag phase of 12 h and reached a maximum level about 36 h later (Fig. 1 B). The increase in enzyme activity was approximately 2 times higher than in the untreated controls. In contrast, cells of ILC 1929 did not show a significant increase of enzyme activity in the medium. Compared with the cell culture of the susceptible cultivar the amount of elicitor-caused enzyme activity secreted into the medium by the ILC 3279 cell culture is 5 times higher. Furthermore the amount of constitutively secreted β-1,3-glucanase activity (controls) in ILC 3279 cells is 3 times higher than in the cells of ILC 1929.

Total enzyme activity of cells and medium was separately calculated (data not shown). On this basis the distribution of β-1,3-glucanase activity between cells and medium can be demonstrated (Fig. 1 C). After elicitation the percentage of activity in the medium of ILC 3279 cells declined slightly due to the early increase of activity in the cells relative to the medium. Subsequently it returned to the same level as in the beginning. In the control 17 to

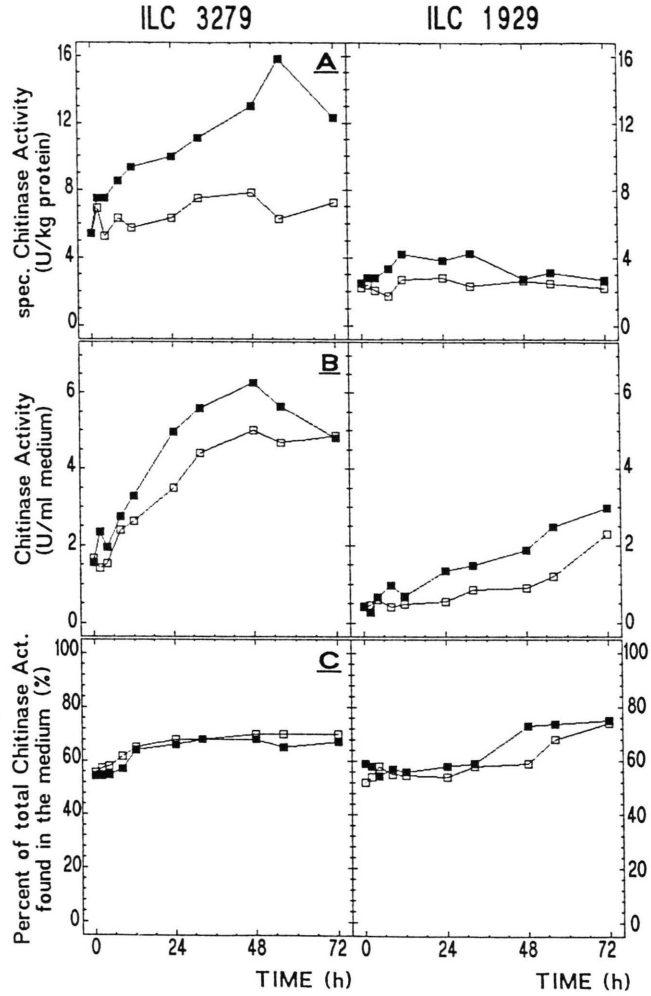

Fig. 2. Changes of chitinase activities in cell suspension cultures established from resistant (ILC 3279) and susceptible (ILC 1929) cultivars after application of *A. rabiei* elicitor (■). Controls (□) received water. A: enzyme activity in the cells; B: enzyme activity in the culture medium; C represents the distribution of total chitinase activity (data not shown) between cells and medium.

20% of total β-1,3-glucanase activity were found in the medium. In contrast, in ILC 1929 cells only a very small portion of β-1,3-glucanase activity was present in the medium (1–2%) both in the elicitor-induced as well as in the control probes.

Chitinase activity

In the cell culture of the resistant cultivar the characteristics of chitinase induction in the cells are similar to that shown for the β-1,3-glucanase. The maximum of chitinase activity was measured 56 h after elicitation with a 2.8-fold increase compared with the control (Fig. 2A). In contrast, ILC 1929 cells showed only a very slight increase. The maximum level was again reached after 12 h and subsequently it declined to the level of the controls. Thus, ILC 3279 cells revealed a 5 times higher intracellular chitinase activity than ILC 1929 cells 56 h after elicitation. In addition the constitutively expressed enzyme activity in the resistant cultivar was 3 times higher.

After addition of elicitor the enzyme activities in the medium of ILC 3279 cells increase very rapidly (Fig. 2B), but with almost no quantitative differ-

ence in comparison to the control. The cell culture ILC 1929 did not show such a significant difference between induced and constitutive chitinase activity in the medium, because there only a very slight increase of enzyme activity could be measured. Comparing this activity with the value found for the cell culture ILC 3279, the level of chitinase activity is 3–4 times lower in the cell culture of the susceptible cultivar.

The predominant portion of total chitinase activity (55–70%) was found in the medium (Fig. 2C). In both cell culture lines no significant difference between elicitor-treated probes and controls were found. The increase in total chitinase activity in the medium was either due to a more rapid increase of enzyme activity in the medium (ILC 3279) or to a decrease of enzyme activity in the cells concomitant with a slow increase in the medium (ILC 1929).

Treatment with the elicitor preparation caused no negative effect on several growth parameters of the cell culture, such as fresh weight and protein content of the cells (data not shown). Browning reactions of the cells were also not observed.

Discussion

The cell suspension cultures of chickpea *(Cicer arietinum)* serve as a simplified model system to study various biochemical defense reactions of coordinately induced cells after treatment with an elicitor.

The accumulation of pterocarpan phytoalexins in chickpea cell cultures as well as in plants of the two different cultivars demonstrated significant cultivar-specific differences [10, 19]. Furthermore, the expression of elicitor-induced enzymes involved in pterocarpan biosynthesis [20] and of a 18 kDa PR protein [21] were found to be much more pronounced in ILC 3279 cells than in cells from the susceptible cultivar. This study was carried out to detect possible differences in β-1,3-glucanase and chitinase activities between plant cell suspension cultures derived from the resistant and the susceptible chickpea cultivars.

Different basic and acidic isoforms of both hydrolases have been detected in several plants [4–6, 22]. In intact plants of tobacco and bean the basic isoforms are located in the central vacuole, whereas the acidic isoforms are found in the intercellular fluid [23, 24]. In plant cell cultures the culture medium can be regarded as the intercellular space. We have now demonstrated that an appreciable amount of hydrolase activity is released into the medium by the ILC 3279 cell culture in clear contrast to the ILC 1929 cell culture (Fig. 1, 2B). After a short lag-phase β-1,3-glucanase and chitinase activities increase simultaneously and reach maximum level about 48 h after induction. This coordinated pattern of hydrolase induction has also been found in various plants [25–27]. This induction kinetic is considered to be an important prerequisite for an effective inhibition of fungal growth in infected tissues since both hydrolases act synergistically in the degradation of fungal cell walls [7].

In contrast, hydrolase activities found in the medium of the cell culture from the susceptible cultivar (Fig. 1, 2B) did not increase significantly. It is, therefore, assumed that in case of ILC 1929 the limited amounts of secreted enzyme activities will not significantly contribute to the plant defense system, because they might not be sufficient to inhibit fungal growth.

Several observations favour the assumption that release of hydrolase activities into the medium is the result of active secretion mechanisms and not of cell lysis. The viability of the cells remained intact during the growth cycle (data not shown) and cells and medium showed totally different electrophoretic protein patterns (data not shown). In case of significant cell damage intracellular proteins would have been detected as additional bands in the electrophoretic pattern of the medium. Intracellularly located intermediates of phytoalexin biosynthesis and other secondary constituents were not detected in the culture medium of elicitor-treated cells [28]. Finally, if release of hydrolase activities were due to cell lysis, approximately equal portions of β-1,3-glucanase as well as chitinase activities would have to be expected in the cell culture medium. However, as shown for ILC 1929 (Fig. 1, 2C) 70% of chitinase and only 1–2% of β-1,3-glucanase total activity were found in the medium. In cultured carrot cells Kurosaki *et al.* [29] found an active secretion of chitinase activities into the medium.

The role intracellular hydrolases play following infection is discussed controversially. Mauch *et al.* [30] suggest that during hypersensitive response in

bean tissue disruption of plasmamembrane and tonoplast causes the release of the hydrolases localized in the vacuole. The sudden flooding of high amounts of hydrolases into the intercellular space may then lead to lethal conditions for the invading pathogen. In contrast, Van den Bulcke *et al.* [24] showed that there is a strict compartmentalization between vacuolar and extracellular β-1,3-glucanase in tobacco. Furthermore hypersensitive response caused by infection with *Pseudomonas syringae* did not lead to a secretion of vacuolar isoforms into the intercellular space. This observation points to some other still unknown function in cellular metabolism for the vacuolar β-1,3-glucanase activity. We found most of the β-1,3-glucanase activity in the cells (Fig. 1C), whereas the portion of chitinase activity in the cells was low in comparison with the medium (Fig. 2C). This observation together with the fact that β-1,3-glucans but not chitin are a structural component of plant cells strengthens the assumption that β-1,3-glucanase may also be involved in different reactions of cellular metabolism [24, 31, 32].

The reason for the high level of β-1,3-glucanase activity in the cells of ILC 1929 (Fig. 1A) is at present unknown. An inhibition of transport of enzymes into the culture medium would only partly contribute to an accumulation inside the cells. Further investigations are required to elucidate this surprising finding.

The cell culture of the resistant chickpea cultivar produced a 5 times higher elicitor-induced chitinase activity in the cells compared with the ILC 1929 cell culture (Fig. 2A). The cells of ILC 1929 showed only a very slight induction after addition of the elicitor. Thus, ILC 3279 has the capacity to accumulate high amounts of chitinase activity in the cells. However, these chitinases have to be released in order to degrade the chitin in the fungal cell wall. This could be achieved by a transport out of the vacuole into the medium or by lysis of the cells in the hypersensitive response.

In summary, we found significant differences between cell cultures established from a resistant and a susceptible cultivar following elicitor treatment. This concerns primarily the different capacity of both lines to accumulate hydrolase activities in the culture medium. Similar studies dealing with a comparison between two different cultivars were carried out by several authors [22, 33–35]. They all correlated higher amounts of hydrolase activities with the incompatible interaction between a certain fungus and a resistant plant. Together with our findings it seems likely that β-1,3-glucanases and chitinases play a major role in recognition and defense of the invading pathogen.

Our further investigations will deal with the elucidation of the isoenzyme pattern in the cells and the medium of the two cell cultures with regard to number and molecular size of the enzymes, their isoelectric points and the secretion of constitutive and elicitor-induced isoforms. Subsequently we will also examine the situation in the intact plant by immunocytochemical localization studies during pathogenesis.

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