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Hyphal in vitro growth of the arbuscular mycorrhizal fungus *Glomus mosseae* is affected by chitinase but not by β-1,3-glucanase

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Abstract Purified basic chitinase or β-1,3-glucanase or a combination of the two enzymes were applied to hyphae of the arbuscular mycorrhizal fungus *Glomus mosseae* grown in vitro. Chitinase applied to the hyphal tip produced an inhibition of hyphal extension, lysis of the apex and alterations of the growth pattern of the fungus. No effect was observed, however, when chitinase was applied to subapical parts of the hyphae or when glucanase was applied to any part of the hyphae. Application of a combination of the two enzymes to the hyphal tip produced an effect similar to that of chitinase alone.

Keywords Chitinase · Glomales · Glucanase · Hypha · Inhibition · Mycorrhiza

Introduction

The two hydrolases chitinase and glucanase are strongly induced by pathogens in plant tissues (Meins et al. 1992; Boller 1993). Because of their inhibitory effect, alone or in combination, on the in vitro growth of a number of saprophytic and pathogenic fungi (Schlumbaum et al. 1986; Mauch et al. 1988; Ludwig and Boller 1990; Arlorio et al. 1992; Sela-Buurlage et al.1993), they are thought to be involved in plant defense against fungal infections. This is supported by the increased resistance of transgenic plants constitutively expressing chitinase and/or β-1,3-glucanase towards a number of fungal pathogens (Broglie et al. 1991; Vierheilig et al. 1993; Yoshikawa et al. 1993; Zhu et al. 1994; Lusso and Kuc 1996).

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The role of hydrolases during formation of the mycorrhizal symbiosis is less clear (see review by Dumas-Gaudot et al. 1996). In plants colonized by arbuscular mycorrhizal fungi (AMF), an initial weak induction and later suppression of the activity of the two enzymes has been reported (Spanu et al. 1989; Lambais and Mehdy 1993; Vierheilig et al. 1994; Volpin et al. 1994). In vitro studies with ectomycorrhizal and ericoid mycorrhizal fungi revealed that the two hydrolases have no effect on the hyphal extension of these fungi (Arlorio et al. 1991).

In transgenic tobacco plants inoculated with the AMF *Glomus mosseae*, the formation of the mycorrhizal symbiosis was reduced only in plants constitutively expressing high levels of PR 2, an acidic, extracellular β-1,3-glucanase. High levels of other hydrolases, however, showed no effect on mycorrhization (Vierheilig et al. 1993, 1995).

In the present work, we were interested in whether or not the in vitro growth of the AMF *G. mosseae* is affected by chitinase and β-1,3-glucanase, alone or in combination.

Materials and methods

Surface-sterilized spores (Mosse 1962) of *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) were placed in Petri dishes on water agar (2%, pH 7). Approximately 10 days after germination, the rate of hyphal extension was determined by registering the position of the hyphal tip using a dissecting microscope with a micrometer scale at 1-h intervals. Hyphae with approximately the same growth rate were chosen for the experiments. Hydrolases were applied either to hyphal tips or to subapical parts of hyphae (distance behind tip approximately 5 mm).

The effect of purified tobacco (*Nicotiana tabacum*) basic chitinase [activity measured according to Boller et al. (1983) was 1197 nkat/mg protein] and basic β-1,3-glucanase [activity measured according to Mauch et al. (1984) was 140 nkat/mg protein] on hyphal extension was tested. Preliminary experiments with the NH_4HCO_3 buffer (20 mM, pH 5.5) used as solvent for the enzymes showed no effect on hyphal growth (results not shown) (see also Vierheilig et al. 1996). The purified enzymes (chitinase 86 µg/ml, glucanase 130 µg/ml), a combination of the two enzymes with the same concentrations of each enzyme (chitinase 86 µg/ml, glucanase 130 µg/ml) and the NH_4HCO_3 buffer as a

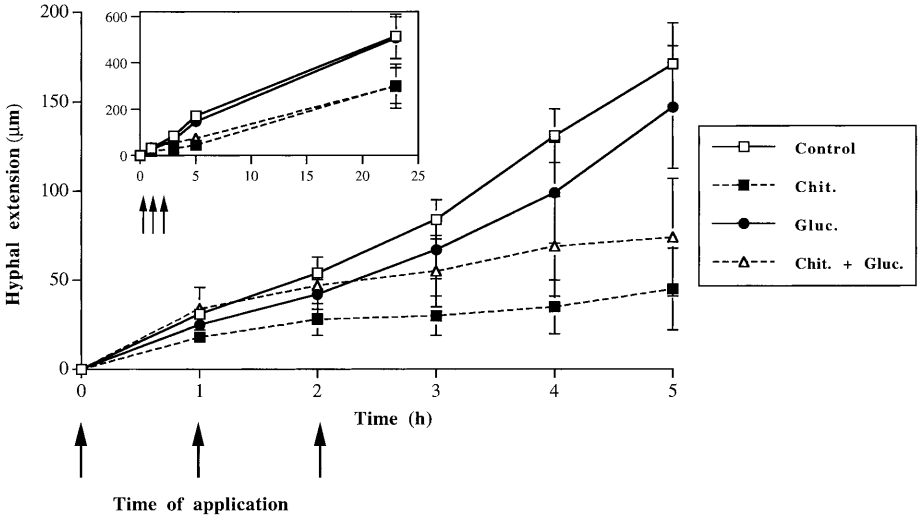

Fig. 1 Effect of chitinase and glucanase, alone or in combination, on hyphal extension of *Glomus mosseae*. Enzymes were applied to the hyphal apex at the beginning of the experiment (0 h) and after 1 h and 2 h (*arrows*). Hyphal extension measurements were made at the beginning of the experiment (0 h) and 1 h, 2 h, 3 h, 4 h, 5 h, and 23 h after the first application (0 h). Data represent means and standard errors for eight individual hyphae

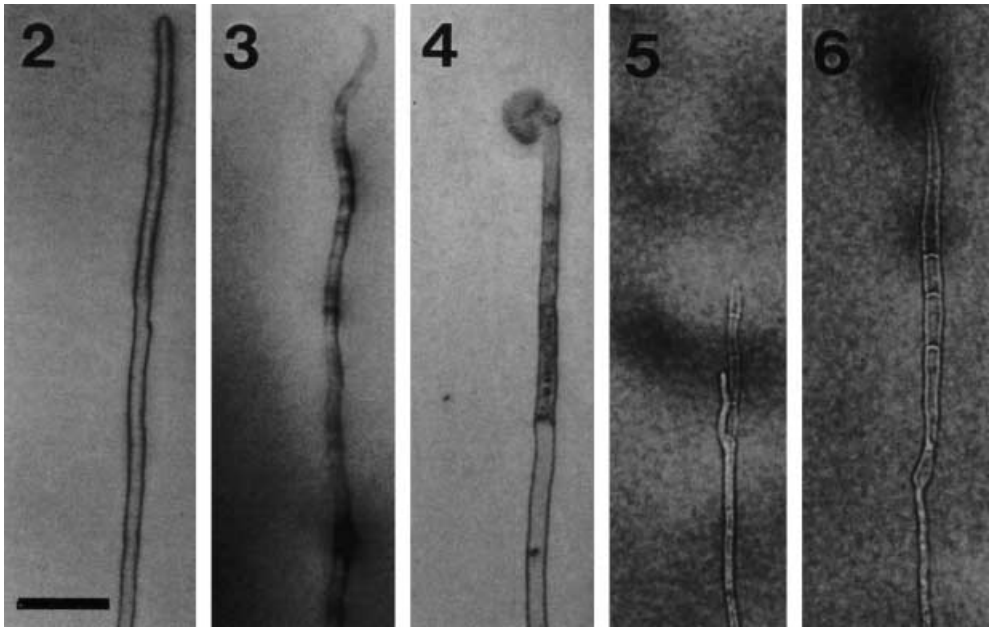

Fig. 2 Hyphae of *Glomus mosseae* after application of chitinase to the apex. Growth pattern of control hypha; *bar* 50 µm

Fig. 3 As Fig. 2. Growth pattern after application of chitinase; *bar* 50 µm

Fig. 4 As Fig. 2. Swelling of the apex after application of chitinase; *bar* 50 µm

Fig. 5 As Fig. 2. Septation of the hyphal apex after application of chitinase and subsequent formation of a new apex; *bar* 100 µm

Fig. 6 As Fig. 2. Septation of the hyphal apex after application of chitinase; *bar* 100 µm

control were applied to hyphal tips or subapical parts of hyphae. The test solutions (2 µl per treatment) were applied repeatedly to the hyphae at times 0 h, 1 h, and 2 h. Hyphal extension was measured every hour until 5 h after the first application and again 23 h after the first application.

Hyphal extension and morphological alterations were observed using a Zeiss Axioplane microscope. The four treatments (1) control, (2) chitinase, (3) glucanase, (4) chitinase and glucanase were tested on four hyphae from the same spore with approximately the same growth rate. The experiment was repeated three times using a minimum of eight replicates per treatment. Results were similar in all experiments. Means and standard errors of one experiment are shown.

Results

Application of glucanase to hyphal tips had no effect on the hyphal extension rate (Fig. 1) or on the hyphal growth pattern (images not shown). In the treatment with purified chitinase applied to the hyphal tip, a clear growth inhibition was observed 3 h after the first application (Fig. 1). The combination of chitinase and β-1,3-glucanase produced an effect similar to chitinase alone, although this was only apparent 5 h after the first application (Fig. 1).

After application of chitinase (chitinase alone or in combination with glucanase) to the hyphal tip, several alterations of the hypha were observed. Whereas control hyphae grew straight (Fig. 2), chitinase-treated hyphae grew with a wave-like pattern (Fig. 3). Some hyphal tips showed balloon-like swellings indicating lysis of the apex (Fig. 4). Other hyphae stopped tip growth and resumed growth subapically from a newly branched apex (Fig. 5). Some hyphae continued to grow after chitinase application, but the hyphal portion formed after the treatment had a greater diameter than that formed before the treatment; finally the newly formed portion septated (Fig. 6). Application of chitinase or glucanase to subapical portions of a hypha showed no effect at the site of application or at the tip.

Discussion

In a study by Arlorio et al. (1991), hydrolytic enzymes had no inhibitory effect on ectomycorrhizal and ericoid mycorrhizal fungi. This contrasts with our results with the AMF *G. mosseae*. Here, application of chitinase, alone or in the presence of glucanase, produced effects similar to those reported for a number of saprophytic and pathogenic fungi (Schlumbaum et al. 1986; Mauch et al. 1988; Ludwig and Boller 1990; Arlorio et al. 1991, 1992; Sela-Buurlage et al. 1993). Hyphae treated with chitinase showed growth inhibition accompanied by swelling and lysis of the apex and the hyphal growth pattern was altered. In some cases, a septum and a new apex were formed, indicating that cell-wall synthesis was not affected and that the fungus could adapt to the presence of the hydrolytic enzyme; this was also shown previously for saprophytic and pathogenic fungi by Ludwig and Boller (1990) and Boller et al. (1983).

Despite the presence of β-1,3-glucan in the cell walls of fungi of the order Glomales (Balestrini et al. 1994; Gianinazzi-Pearson et al. 1994; Lemoine et al. 1995), the application of β-1,3-glucanase to hyphae of *G. mosseae* had no effect on hyphal extension. This concurs with the absence of any visible hydrolytic effect of β-1,3- glucanase reported for other fungi with β-1,3-glucan in their cell walls (Mauch et al. 1988; Ludwig and Boller 1990; Arlorio et al. 1991, 1992; Sela-Buurlage et al. 1993). For several fungal species, the combination of the two hydrolases was reported to be much more effective in terms of fungal growth inhibition than either enzyme alone (Broekaert et al. 1988, 1989; Mauch et al. 1988; Ludwig and Boller 1990; Sela-Buurlage et al. 1993). In our experiment, however, such a combination resulted in growth inhibition and lysis of the apex of *G. mosseae* similar to that with chitinase alone. This indicates that the observed hyphal extension inhibition was produced solely by chitinase.

In our study, effects differed according to the portion of the growing hyphae to which chitinase was applied. Application to the apex resulted in the lytic effects described above, whereas subapical application showed no effect. These results are in agreement with previous reports. Mauch et al. (1988) suggested that chitinases are particularly efficient at the hyphal apex because in this extension region the freshly synthesized chitin is not yet covalently bound to glucans and, therefore, is accessible to the chitinase. This hypothesis was confirmed in a study with the fungus *Trichoderma longibrachiatum* (Arlorio et al. 1992), which shows a layering of the cell wall comparable to that in some endomycorrhizal fungi (Bonfante-Fasolo 1987; Bonfante-Fasolo et al. 1990).

The sensitivity of the chitin at the apex of the AMF *G. mosseae* to adverse effects has been reported previously. *Urtica dioica* agglutinin (UDA), a lectin known to inhibit the growth of several chitin-containing fungi (Broekaert et al. 1989), also inhibited the growth of *G. mosseae* when applied to the hyphal apex (Vierheilig et al. 1996). However, the modes of action of UDA and chitinase appear to differ. Whereas chitinase degrades the chitin-containing fungal cell wall (Arlorio et al. 1992), chitin-binding lectins seem to disturb the apical growth by binding or crosslinking chitin chains (Broekaert et al. 1989).

Our results show that the AMF *G. mosseae* when grown in vitro is not sensitive to β-1,3-glucanase. This seems to conflict with results of Vierheilig et al. (1995) showing that in transgenic tobacco plants enhanced levels of β-1,3-glucanase reduced root colonization by an AMF. This conflict may be resolved by considering the hydrolase isoform. The β-1,3-glucanase applied to growing hyphae in this study was of the class I type (basic, vacuolar), whereas in transgenic tobacco plants the β-1,3-glucanase negatively affecting root colonization was of the class II type (acidic, extracellular). It has been shown before that different isoforms of hydrolases exhibit different antifungal activities (Sela-Buurlage et al. 1993).

The localization of hydrolases in the plant may explain the contradictory results with chitinases. We found a clear inhibitory effect of the basic chitinase on AMF in vitro growth, whereas enhanced levels of basic chitinase in transgenic tobacco plants had no effect on root colonization (Vierheilig et al. 1995). In plants, the basic chitinase is localized in the vacuole (Neuhaus et al. 1991) and may not come into contact with AMF growing in the intercellular space. Thus, even enhanced enzyme levels in roots of AM host plants might not affect root colonization. Moreover, in plants constitutively expressing high levels of hydrolases, the AM fungus may adapt to the presence of these enzymes, as already observed for other fungi grown in the presence of hydrolases (Ludwig and Boller 1990; Boller 1993).

Little is known about the regulation of hyphal growth and fungus morphogenetic events during AMF association with roots. Two different hypotheses have been brought forward to explain fungal differentiation. Whereas Bago et al. (1996) suggested involvement of the fungal chitin synthase regulated by the host plant, Bonfante-Fasolo (1987) proposed chitinase as a regulatory factor. Our results confirm that the growth of an AMF can be affected by a chitinase. Further studies are needed, however, to determine whether the observed effects are of relevance to the formation of the AM association.

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References

Arlorio M, Ludwig A, Boller T, Mischiati P, Bonfante P (1991) Effects of chitinase and β-1,3-glucanase from pea on the growth of saprophytic and mycorrhizal fungi. G Bot Ital 125: 956–958

Arlorio M, Ludwig A, Boller T, Bonfante P (1992) Inhibition of fungal growth by plant chitinases and β-1,3-glucanases. Protoplasma 171:34–43

Bago B, Chamberland H, Goulet A, Vierheilig H, Lafontaine JG, Piché Y (1996) Effect of nikkomycin Z, a chitin-synthase inhibitor, on hyphal growth and cell wall structure of two arbuscular-mycorrhizal fungi. Protoplasma 192:80–92

Balestrini R, Romera C, Puigdomenech P, Bonfante P (1994) Location of a cell-wall hydroxyproline-rich glycoprotein, cellulose and β-1,3-glucans in apical and differentiated regions of maize mycorrhizal roots. Planta 195:201–209

Boller T (1993) Antimicrobial functions of the plant hydrolases, chitinase and β-1,3-glucanase. In: Fritig B, Legrand M (eds) Mechanisms of plant defense responses. Kluwer, Dordrecht, pp 391–400

Boller T, Gehri A, Mauch F, Vögeli U (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible functions. Planta 157:22–31

Bonfante-Fasolo (1987) Vesicular-arbuscular mycorrhizae: fungus-plant interactions at the cellular level. Symbiosis 3:249–268

Bonfante-Fasolo P, Vian B, Perotto S, Faccio A, Knox JP (1990) Cellulose and pectin localization in roots of mycorrhizal *Allium porrum*: labelling continuity between host cell wall and interfacial material. Planta 180:537–547

Broekaert W, Van Parijs J, Allen AK, Peumans WJ (1988) Comparison of some molecular, enzymatic, and antifungal properties of chitinases from thornapple, tobacco, and wheat. Physiol Mol Plant Pathol 33:319–331

Broekaert W, Parijs JV, Leyns F, Joos H, Peumans WJ (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 245:1100–1102

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Dumas-Gaudot E, Slezack S, Dassi B, Pozo MJ, Gianinazzi-Pearson V, Gianinazzi S (1996) Plant hydrolytic enzymes (chitinases and β-1,3-glucanases) in root reactions to pathogenic and symbiotic microorganisms. Plant Soil 185: 211–221

Gianinazzi-Pearson V, Lemoine MC, Arnould C, Gollotte A, Morton JB (1994) Localization of β(1,3) glucans in spore and hyphal walls of fungi in the Glomales. Mycologia 86: 478–485

Lambais MR, Mehdy MC (1993) Suppression of endochitinase, β-1,3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. Mol Plant Microb Interact 6:75–83

Lemoine MC, Gollotte A, Gianinazzi-Pearson V (1995) Localization of β(1,3) glucan in walls of the endomycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and *Acaulospora laevis* Gerd. & Trappe during colonization of host roots. New Phytol 129:97–105

Ludwig A, Boller T (1990) A method for the study of fungal growth inhibition by plant proteins. FEMS 69:61–66

Lusso M, Kuc J (1996) The effect of sense and antisense expression of the PR-N gene for β-1,3-glucanase on disease resistance to fungi and viruses. Physiol Mol Plant Pathol 49: 267–283

Mauch F, Hadwiger LA, Boller T (1984) Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol 76:607–611

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3 glucanase. Plant Physiol 88:936–942

Meins F, Neuhaus JM, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In: Boller T, Meins F Jr (eds) Genes involved in plant defense. Springer, Vienna, pp 245–282

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27: 745–751

Neuhaus JM, Sticher L, Meins F, Boller T (1991) High-level expression of a tobacco chitinase gene in *Nicotiana sylvestris*. Susceptibility of transgenic plants to *Cercospora nicotianae* infection. Plant Mol Biol 16:141–151

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324:365–367

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, Van den Elzen PJM, Cornelissen JC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3 glucanases exhibit antifungal activity. Plant Physiol 101:857–863

Spanu P, Boller T, Ludwig A, Wiemken A, Faccio P, Bonfante-Fasolo P (1989) Chitinase in roots of mycorrhizal *Allium porrum*: regulation and localization. Planta 177:447–455

Vierheilig H, Alt M, Neuhaus JM, Boller T, Wiemken A (1993) Colonization of transgenic *Nicotiana sylvestris* plants expressing different forms of *Nicotiana tabacum* chitinase by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant Microb Interact 6:261–264

Vierheilig H, Alt M, Mohr U, Boller T, Wiemken A (1994) Ethylene biosynthesis and activities of chitinase and β-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. J Plant Physiol 143:337–343

Vierheilig H, Alt M, Lange J, Gut-Rella M, Wiemken A, Boller T (1995) Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Appl Environ Microbiol 61:3031–3034

Vierheilig H, Iseli B, Alt M, Reikhel N, Wiemken A, Boller T (1996) Resistance of *Urtica dioica* to mycorrhizal colonization: a possible involvement of *Urtica dioica* agglutinin. Plant Soil 183:131–136

Volpin H, Elkind Y, Okon Y, Kapulnik Y (1994) A vesicular-arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defense response in alfalfa roots. Plant Physiol 104:683–689

Yoshikawa M, Tsuda M, Takeuchi Y (1993) Resistance to fungal diseases in transgenic tobacco plants expressing the phytoalexin elicitor-releasing factor β-1,3-endoglucanase from soybean. Naturwissenschaften 80:417–420

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. Bio/Technol 12:807–812