Planta (2002) 214: 848–852
DOI 10.1007/s00425-001-0709-x

ORIGINAL ARTICLE

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Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes?

Received: 21 April 2001 / Accepted: 22 October 2001 / Published online: 18 January 2002

Abstract In shoot tip-derived tissue cultures of Scots pine (*Pinus sylvestris* L.), browning and subsequent degeneration of the culture is accompanied by lipid peroxidation and lignification of cells, which are characteristic features of a plant defense reaction. Since chitinases are enzymes acting primarily in plant defense, their expression was studied in pine callus in order to elucidate the defense reaction. Chitinases were present diversely in tissue cultures originating from shoot tips and embryos of *P. sylvestris*, in contrast to *Pinus nigra* embryogenic callus, where production of chitinases or browning was not detected. Because endophytic microbes had earlier been detected in buds of Scots pine, their subsequent presence in the tissue cultures was considered a potential cause of the defense reaction. Therefore, the presence of endophytes in the tissue cultures was examined by in situ hybridization. Endophytes were found to colonize heavily in 45% of the tissue cultures of *P. sylvestris* and to form biofilms, while the *P. nigra* callus was not found to contain any microbes. The endophytes seemed to propagate uncontrollably once a tissue culture of *P. sylvestris* was initiated. Regardless of the high level of chitinase production in the callus, the control of the endophytes presumably becomes inadequate during the tissue culture of *P. sylvestris*.

Keywords Chitinase · Defense reaction · Endophyte · *Pinus* (defense) · Tissue culture

Introduction

Plant defense systems are comprised of a number of different enzymes, of which probably only a fraction are known today. Chitinases are the largest group, having diverse roles in plant defense. The chitinases are especially interesting because their substrates do not originate from plants, but rather from fungal cell walls and the exoskeleton of arthropods (Collinge et al. 1993).

Although the main role of chitinases is in defense, primarily acting on fungal pathogens, they also have a non-defensive role in plant development. The basic chitinases belonging to class I become activated in tobacco explants during flower formation, but not during the formation of vegetative shoots (Meeks-Wagner et al. 1989; Neale et al. 1990). In other plant species, such as cucumber, petunia, and *Arabidopsis*, different chitinases work in distinct organs of the flower, or are activated differentially during flower development (Lawton et al. 1994; Lotan et al. 1990; Trudel et al. 1989; Samac and Shah 1991). Roots, fruits, and seeds are other tissues in which high chitinase activities have been detected (Graham and Sticklen 1994; Salzman et al. 1998; Regalado et al. 2000). Some of the chitinases detected in seeds may only be present in seeds, and not in the mature plants (Graham and Sticklen 1994).

Specific chitinases belonging to class II are produced during somatic embryogenesis (De Jong et al. 1992; Mo et al. 1996; Dong and Dunstan 1997; Domon et al. 2000), and chitinases are also found in cell-suspension cultures (Kragh et al. 1991; Masuta et al. 1991; Arie et al. 2000). Whereas several studies have concentrated on chitinases in elicited or induced suspension or tissue cultures (Masuta et al. 1991; Mason and Davis 1997; Popp et al. 1997; Wu et al. 1997; Jayasankar et al. 2000), the non-induced, non-embryogenic callus culture has, to our knowledge, been examined only rarely for chitinase production (Mayer et al. 1996; Domon et al. 2000). However, browning or blackening is a typical feature in tissue cultures of woody plants. This phenomenon is also continually detected in the tissue cultures derived from shoot tips of mature Scots pine (*Pinus sylvestris* L.; Laukkanen et al. 1997). The browning of a tissue culture is caused by oxidation of phenols, which occurs as a result of cellular degradation (Lee and Whitaker 1995).

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In tissue cultures of Scots pine, browning is accompanied by lipid peroxidation and lignification of cells, which are symptoms of high oxidative stress (Laukkanen et al. 2000) and characteristic features of the plant defense reaction (Lee and Whitaker 1995; Keller et al. 1996).

Since we have detected both fungal and bacterial endophytes in the buds of Scots pine (Pirttilä et al. 2000, and our unpublished data), we became interested in how these microbes react once a tissue culture is initiated from the buds. Additionally, since we have found that *Hormonema dematioides* frequently grows in pine tissue cultures (unpublished data), we wanted to explore whether it remains in the tissue cultures latently. Our hypothesis was that this fungal endophyte causes a defense reaction and the subsequent browning and death of the tissue cultures (Laukkanen et al. 2000). The expression of chitinases was studied in concert with the microbial detection in order to elucidate the reaction occurring in the tissue cultures of *P. sylvestris*.

Materials and methods

Tissue culture

Buds ($n = 240$) of Scots pine (*Pinus sylvestris* L.) were collected in October 2000 from healthy-looking trees, growing on a natural stand in Oulu (65° N; 25°30′ E). The seeds ($n = 120$; Forelia Oy, Rovaniemi, Finland) were allowed to imbibe water overnight before sterilization. The buds and seeds were surface-sterilized for 1 min in 70% ethanol and for 20 min in 6% calcium hypochlorite. After rinsing, the brown bud and seed scales were removed aseptically. The apices were separated from the buds and placed on a modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962; Hohtola 1988), except that the inorganic nitrogen source was ammonium nitrate (2 mM), the sucrose concentration was 2%, and arginine and glucose were omitted. Embryos were separated from the megagametophytes, and placed on a K medium (Krogstrup 1986). Within a week, fungal growth was detected in 39% of the bud-derived tissue cultures, which were subsequently discarded. Samples for the protein isolation were taken on days 0, 4, 12, 28 and 35, and for in situ hybridization, on day 10 from start of the culture. Additionally, long-term cultures of embryogenic calli originating from immature embryos of *Pinus nigra* Arnold, were grown as a control (Salajová et al. 1999) on a DCR medium (Gupta and Durzan 1985).

Extraction of soluble proteins

The samples (0.5 g) were ground in liquid nitrogen and homogenized with a mortar and pestle in 2 ml of extraction buffer, which was comprised of 50 mM Tris-HCl (pH 8.65), 1 mM dithiothreitol (DTT; Sigma) and 25% (w/v) insoluble polyvinylpolypyrrolidone (Polyclar AT; Serva). Additionally, the samples were homogenized with an electric grinder (Ystral) for 1 min. The homogenates were placed on ice for 40 min, and vortexed 5–10 times during the incubation. The samples were then centrifuged (1 h, 25,000 *g*, +4 °C), and the supernatant was filtered through glass fiber (Whatman), and 0.22-μm PVDF (Millipore) filters. Finally, the samples were concentrated by centrifuging for 1 h (2,500 *g*) at +4 °C in Ultrafree CL filters (Millipore).

Immunoblotting

The protein electrophoresis and western blotting were performed as described by Laukkanen et al. (1999). Polyclonal antibodies raised against *Beta vulgaris* L. chitinases classes II and IV were used at a dilution of 1:200 to recognize chitinases in the pine tissues.

Fixation of tissue culture specimens

Altogether, 20 callus samples originating from buds and seeds (10 samples per origin) of *P. sylvestris* were detached from the medium after 10 days of growth. In addition, two samples of *P. nigra* callus were prepared. The samples were dissected aseptically into pieces of 2×3 mm, or smaller. The specimens were fixed, dehydrated, cleared through an ethanol *t*-butanol series and embedded in paraffin (Pirttilä et al. 2000).

In situ hybridization

The paraffin-embedded specimens were sectioned, the sections were baked on silane-coated slides, and the paraffin was removed (Pirttilä et al. 2000). Three probes were hybridized per sample: eubacterial probe E11 (Pirttilä et al. 2000), the *Hormonema dematioides*-specific probe HD13 (5′-TCCTTCCGGACAAGGTGATGAAC-3′), and the *Rhodotorula minuta*-specific probe RM6 (5′-TGAGTCATTAAAAACCTCATC-3′). A negative control without a probe was included each time. The slides were treated prior to hybridization, and hybridized under maximum stringency conditions, as described earlier (Pirttilä et al. 2000). The detection was performed with the DIG Nucleic Acid Detection Kit (Roche), after which the slides were viewed under bright-field illumination. All in situ hybridizations were performed aseptically. Negative and positive control (sense and antisense) hybridizations with an RNA-probe targeted to plant 25S rRNA (Wanner and Gruissem 1991) were also performed.

Scanning electron microscopy (SEM)

Callus samples were obtained after 42 days of growth and fixed in FAA (formalin:acetic acid:ethanol, 10:5:85, by vol.). The samples were dried with a critical point drier, covered with gold and viewed with a Jeol JEM 100B scanning electron microscope.

Results and discussion

When proteins were isolated from intact pine buds, there were only three very weak bands of sizes 23, 25 and 36.5 kDa detected with the antibody raised against class-IV chitinase in the soluble protein fraction (Fig. 1A). The intensity of the bands became modestly higher in the 4-day-old callus samples, and very strong protein expression was detected with the chitinase class-IV antibody in the 12-day-old samples. In addition to the three proteins, immunologically active proteins of 31 and 142 kDa were present in the 12-day-old pine tissue cultures. Proteins of 23, 25, 31 and 142 kDa were subsequently detected in the callus samples of 28 and 35 days using the chitinase class-IV antibody (Fig. 1A). A chitinase of 25 kDa was earlier detected in the non-embryogenic callus of *Pinus caribaea* (Domon et al. 2000) and in the embryogenic callus of Sweet orange (*Citrus sinensis* L.; Möder et al. 1999).

Using the chitinase class-II antibody, proteins of 110–150 and 63 kDa were recognized in the soluble fractions isolated from intact pine buds (Fig. 1B). These proteins were found subsequently in the callus samples until 12 days of growth, in addition to which the antibody

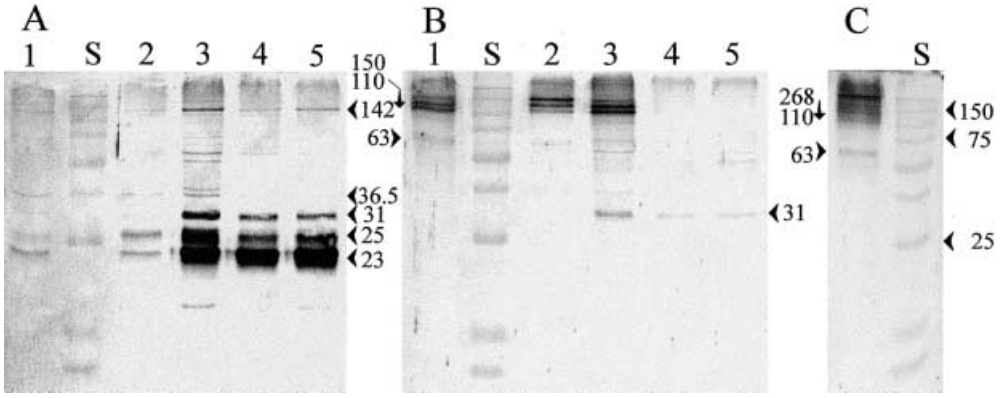

Fig. 1A–C Western blot analysis of proteins extracted from buds and tissues during callus culture of Scots pine (*Pinus sylvestris*). Polypeptides were transferred onto nitrocellulose membrane after protein gel electrophoresis. An antibody raised against chitinase classes IV (**A**) and II (**B**) recognized some proteins (*arrow*). *Lane 1* Pine buds, *lanes 2–5* 4-, 12-, 28-, and 35-day-old tissues from callus cultures. **C** Western blot analysis of proteins extracted from *Hormonema dematioides*, using the chitinase class-II antibody. *Lane S* Prestained standards of 250, 150, 100, 75, 50, 37, 25, 15 and 10 kDa (Bio-Rad)

recognized proteins of 31 and 57 kDa in the 12-day-old callus samples. However, most of these proteins were no longer detected after 12 days. In the 28- and 35-day-old samples, only one protein of size 31 kDa was detected using the type-II chitinase antibody (Fig. 1B).

Proteins were not detected in calli originating from immature embryos of *Pinus nigra* with either of the antibodies (data not shown). In calli originating from mature embryos of *P. sylvestris*, the production of immunologically active proteins was similar to that detected in the shoot tip-originating calli (data not shown). For proteins detected with both class-IV and class-II chitinase antibodies, 12 days of growth appeared to be the turning point for activation, or suppression in the callus of *P. sylvestris*. The proteins recognized by type-IV chitinase antibody were present in the pine calli especially after 12 days of growth. In contrast, proteins detected with the class-II chitinase antibody were present until 12 days of callus growth, after which their production was mostly diminished. Because the endophytic fungus of pine buds, *Hormonema dematioides*, regularly colonizes the pine tissue cultures, this organism was studied for its own chitinase production, as well. *H. dematioides* produced 110- to 268-kDa proteins recognized by type-II chitinase antibody, and a protein of 63 kDa was also detected in the fungal protein fraction (Fig. 1C). At least three proteins of these sizes were detected with the same antibody in the shoot tip-originating callus of pine. Therefore, some of the proteins detected in calli derived from *P. sylvestris* may have originated from the endophyte *H. dematioides*. Because an antibody raised against a certain chitinase class may cross-react with other chitinase classes (Möder et al. 1999), the proteins detected in this study may represent other classes of chitinases, as well.

When the presence of endophytes was examined by in situ hybridization in the callus samples, they were detected in the tissue cultures of *P. sylvestris* (Fig. 2A), but not of *P. nigra* (Fig. 2B). Bacterial endophytes were detected with probe E11 (Pirttilä et al. 2000) in 95% of all the *P. sylvestris* callus samples. *Rhodotorula minuta* was detected in 50% of the bud-derived, and in 30% of the embryo-originating calli, using the probe RM6. *Hormonema dematioides* was detected in two bud-originating tissue cultures with probe HD13. *H. dematioides* ordinarily propagates on the tissue of *P. sylvestris* within a week of culture. Because the tissue cultures studied were 10 days old, *H. dematioides* apparently does not remain mainly latent in the cultures. The cultures of *P. sylvestris* were heavily colonized by the microbes in 45% of all the calli; a high colonization was detected in 50% of the bud-originating, and in 40% of the embryo-originating calli. There was also one bud-derived callus of *P. sylvestris* that was devoid of microbes. Because the samples were taken on day 10 when the calli are green and the differences in the callus sizes are small, the correlation of the microbial presence with the level of browning and the final callus size cannot be evaluated from this study.

Biofilms, which may be comprised of diverse populations of microorganisms (Costerton et al. 1999) and are also found on plant surfaces (Morris et al. 1997), were detected inside calli originating from both buds and embryos of *Pinus sylvestris* by in situ hybridization (Fig. 2C, D). Biofilms were also detected on the surface of bud-derived calli using scanning electron microscopy (Fig. 3). Metabolic interactions have already been observed to occur between microbial species in biofilms, both mutualistic and commensal relationships having been detected (Wolfaardt et al. 1994; Moller et al. 1998). According to the in situ hybridization, all the microbes examined were detected in the biofilms of the bud-derived calli, although not always simultaneously. Neither biofilms nor intensive metabolic activity of microbes was detected in another study of ours on pine buds during their natural, active growth, but substantially higher metabolic activity of the endophytes was detected in buds preparing for growth (data not shown). In that study, the endophytes in the bud tissues

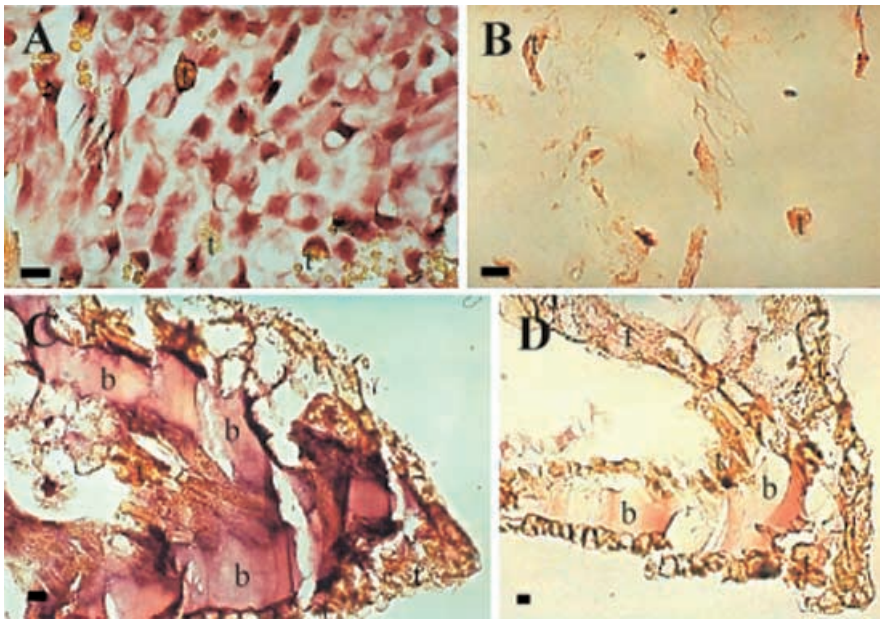

Fig. 2A–D In situ hybridization of *Pinus sylvestris* and *Pinus nigra* callus tissue with a digoxigenin-labeled, eubacterial oligonucleotide probe E11. **A, B** Hybridization with E11 of bud-derived callus tissue of *P. sylvestris* (**A**) and embryogenic callus tissue of *P. nigra* (**B**). For differentiation from the positively hybridized cells, the naturally brown cells of the pine tissue, which contain tannins and other phenolic compounds, are marked *t*. **C, D** Bud-derived callus tissue of *P. sylvestris* where biofilm (*b*) is detected, hybridized with E11 (**C**), and a negative control, hybridized without a probe (**D**). Bars = 20 μm

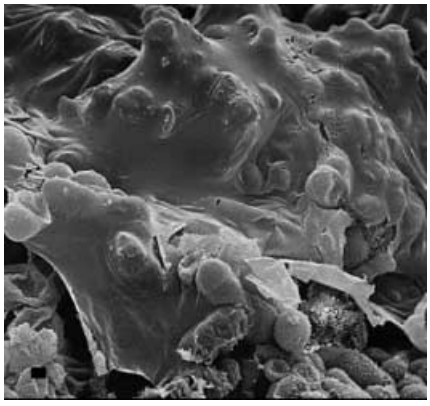

Fig. 3 Scanning electron micrograph showing biofilm on the surface of callus initiated from *Pinus sylvestris* buds. Bar = 20 μm

decreased in either amount or metabolic activity during the natural growth of the bud. Therefore, we suggest that the endophytes are under strict control in a growing pine bud, but become uncontrollable once a tissue culture is initiated from the bud.

Because chitinases are activated in plant tissue primarily as a reaction to microbial presence, the endophytes could readily be controlled by chitinases. The ectomycorrhizal fungus *Amanita muscaria* has actually been detected to induce chitinase activity both in its natural root environment and in a cell-suspension culture of *Picea abies* (Sauter and Hager 1989). Although the activation of chitinases in plant developmental organs has been detected in several studies (Graham and Sticklen 1994; Salzman et al. 1998; Regalado et al. 2000), to our knowledge their role in plant development has not been elucidated. In this study we discovered, that proteins recognized by two types of chitinase antibody are present in the callus of *Pinus sylvestris* during tissue culture, and that microbes grow partly uncontrollably in the callus, forming biofilms in the tissue. The control of the endophytes presumably becomes inadequate during the tissue culture. Proteins of the chitinase types studied, or microbes, were not detected in the embryogenic callus of *Pinus nigra*, for which browning is not typical either. Therefore, the production of certain chitinases in a callus culture of *P. sylvestris* may be correlated with the microbial presence, and microbes probably are, at least in part, responsible for eliciting the defense reaction occurring in the tissue cultures. However, since not all tissue cultures of *P. sylvestris* contained uncontrolled microbial growth, the prevalent browning, and subsequent death of the tissue cultures cannot be totally explained as a result of the microbial disequilibrium.

Acknowledgements We are grateful to Dr. Terezia Salajová (Institute of Plant Genetics, Nitra, Slovakia) for the kind gift of *P. nigra* callus, and to Dr. Jørn Dalgaard Mikkelsen (Maribo, Frö, Bioteknologi, Danisco A/S, Denmark) for generously providing the polyclonal antibodies against class-II and class-IV chitinases. We also thank Dr. Stanton B. Gelvin and Ms. Cindy Ruuskanen for revising the English in the manuscript. Ms. Taina Uusitalo is thanked for the technical assistance. This work was supported by the Kone Foundation, the Oskar Öflund Foundation, and the Ella and Georg Ehrnroot Foundation.

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