A *Streptomyces* chitosanase is active in transgenic tobacco

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Summary. Growth inhibition towards *Rhizopus nigricans*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Verticillium albo-atrum* and *Pythium ultimum* was observed in vitro using a purified chitosanase from an actinomycete, *Streptomyces* sp. strain N174. The corresponding gene, with its own signal peptide, was inserted into pBI121.7 shuttle vector to transform tobacco. Transgenic plants were analysed for chitosanase activity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis assay. Two major and one minor active electrophoretic forms were detected in transgenic tobacco. Some chitosanases were recovered not only in leaf homogenates but also in leaf intercellular fluid extracts. One chitosanase electrophoretic form migrated very closely to the purified *Streptomyces* mature protein while the others corresponded to molecules of higher molecular mass. The N-terminus sequence was determined for one of the three chitosanase forms. It exhibited a different signal peptide cleavage site when compared to the mature chitosanase from *Streptomyces*. This is the first report on the expression of an active chitosanase gene with antimicrobial potential in plants.

Abbreviations: aa, amino acid; CIP, calf intestinal phosphatase; CM, carboxymethyl; GUS, β-glucuronidase; IF, intercellular fluid; MS, Murashige and Skoog; PAGE, polyacrylamide gel electrophoresis; PR, pathogenesis-related; PVDF, polyvinylidene difluoride; SP, signal peptide.

Introduction

Chitosan, a polymer of β-1,4-D-glucosamine, represents a structural component in the cell walls of zygomycetes occurring mostly in association with chitin, a linear homopolymer of β-1,4-N-acetyl-D-glucosamine residues (Araki and Ito, 1988). Chitosan is also present in smaller amounts in cell walls of other classes of fungi (Davis and Eveleigh, 1984). Commercially available chitosan is usually obtained by alkaline deacetylation of shellfish chitin. Chitosan and its derivatives have potential industrial, biomedical and agricultural applications. Diverse microbial chitosanases, which are glycosyl hydrolases degrading chitosan, have been purified and characterized (Monaghan, 1973; Zikakis, 1984; Pelletier and Sygusch, 1990; Masson et al. 1993).

Recently, by using a PAGE assay, chitosanases were detected in plant IF extracts and reported as PR proteins (Grenier and Asselin, 1990). Like chitinases and β-1,3-glucanases, chitosanases might be involved as defense proteins by degrading the cell walls of fungal pathogens (El Ouakfaoui and Asselin, 1992 a, b; Dumas-Gaudot et al. 1992; Cuero and Osuji, 1993; Sharma et al. 1993). Several eukaryotic chitinases (Broglie et al. 1991; Neuhaus et al. 1991; Vierheilig et al. 1993), hen lysozyme (Trudel et al. 1995) and β-1,3-glucanases (Sela-Buurlage et al. 1993) have been expressed constitutively in transgenic plants for conferring increased resistance to various pathogens. Among prokaryotic hydrolases, a chitinase from *Serratia marescens* (Howie et al. 1992) was also used for this purpose. However, no chitosanase has been expressed in transgenic plants until now.

Chitosanases were found widely distributed in higher and lower plants (El Ouakfaoui and Asselin, 1992 a). Interestingly, some chitosanase molecular forms were specific to developmental stages and/or organs (El Ouakfaoui and Asselin, 1992 b). Up to now, no amino acid or gene sequence has been reported for plant chitosanases. Two chitinase/chitosanase isoforms have recently been purified from *Citrus* (Osswald et al. 1993). To our knowledge, only three microbial chitosanase gene sequences have been determined: one from *Bacillus circulans* MII-K1 (Ando et al. 1992); one from *Streptomyces* sp. strain N174 (Masson et al. 1994) and one from *Nocardiooides* sp. N106 (R. Brzezinski, submitted for publication; GSDB accession number L40408). It was overexpressed in *Streptomyces lividans* and purified as an extracellular mature endochitosanase of 238 amino acids (Fink et al. 1991; Boucher et al. 1992). This chitosanase specifically hydrolysed chitosan (without any activity toward chitin or CM-cellulose). In this report, the antifungal potential of the *Streptomyces* sp. strain N174 chitosanase was investigated and the expression of this actinomycete gene in transgenic tobacco was analysed.
Materials and Methods

**Fungal growth inhibition assay.** Purified Streptomyces sp. strain N174 chitosanase produced by the recombinant strain Streptomyces lividans [pRL270] (Masson et al. 1993) was tested against Rhizopus nigricans, Fusarium oxysporum f. sp. radicis-lycopersici (FORL), Verticillium albo-atrum and Pythium ultimum for growth inhibition on 10% (w/v) polycrystalide slab gels previously immersed in 500 mL Yeast Peptone Dextrose (YPD) broth and autoclaved for 20 min at 121°C. The sterile gel slabs were then placed in Petri dishes (150 mm diameter) and air dried for 5 min under sterile air flow. The fungi were inoculated in the center of gel slabs and radial growth was allowed at room temperature for 1 day (R. nigricans, P. ultimum), 2 days (FORL) and 7 days (V. albo-atrum). Purified chitosanase was applied in front of the growing fungi. The plates were kept at room temperature for fungal growth.

**Chitosanase construct.** The chitosanase gene from Streptomyces sp. strain N174 was previously cloned and sequenced (Fink et al. 1991; Masson et al. 1994). For this study, the clone pRL270 (Masson et al. 1993) was digested with HindIII/Xhol generating a 1607 bp fragment containing the chitosanase gene. This fragment was subcloned in pBluescript SK(-) (Stratagene) digested with the same restriction enzymes, resulting in pSN9. This last clone was used to engineer pSN9C, the chitosanase gene with its own 40-aa signal peptide. Clone pSN9 was digested by HindIII and NdeI in order to eliminate the non-coding sequence upstream the chitosanase gene. After end gap filling-in and circularization, a HindIII site was regenerated next to the ATG of the chitosanase gene. Following EcoRI digestion (BRL) transformation, one clone identified as pSN9C was selected and confirmed by HindIII/Xhol digestions.

**Plasmid constructs for plant transformation.** The plant transformation vector pBI121.7 originated from commercial pBl121 (Clontech) in which the GUS gene has been removed (Trudel et al. 1992). The EcoRl/ClaI fragment from pSN9C and the dephosphorylated (CIP) BamHI linearized vector were treated with T4 DNA polymerase and dNTP to blunt the ends, before overnight ligation. A BamHI restriction site was regenerated downstream the chitosanase gene. Clones obtained after EcoRI/DH5aF’ (BRL) transformation, one clone identified as pSN9C was selected and confirmed by HindIII/Xhol digestions.

**Tobacco transformation.** Constructs pB12C and pB19Cr were transferred in Agrobacterium tumefaciens LB4404 harboring Ti plasmid pAL4404 using standard triparental mating procedures (Horsch et al. 1985) with the pRK2013 conjugative plasmid in E. coli HB101. Tobacco (Nicotiana tabacum L. cv. Xanthi-nc) leaf pieces were inoculated with transformed A. tumefaciens using nurse culture plates with MS-104 medium (Horsch et al. 1985). Resulting kanamycin-resistant calli were propagated in vitro and regenerated plants were transferred to soil and grown under normal greenhouse conditions (Trudel et al. 1992).

**Chitosanase PAGE assay.** Leaf homogenates [1:3 (w/v)] prepared in 2.5% (w/v) SDS, 15% (w/v) sucrose, 25 mM sodium phosphate buffer (pH 7.0) and 1% (v/v) β-mercaptoethanol, were heated at 100°C for 3 min and clarified at 10,000g for 10 min at 4°C. Leaf IF extracts were prepared by performing vacuum infiltration of leaf pieces in 50 mM sodium phosphate buffer (pH 6.5) and low speed centrifugation (Parent and Asselin, 1984). Protein concentration and detection of chitosanase activity after 15% (w/v) PAGE under denaturing (SDS) conditions were performed as previously described (Grenier and Asselin, 1990).

**Chitosanase lysozyme assay.** Leaf IF extracts were tested for chitosanase activity in 1% (w/v) agarose slab gels containing 0.05% (w/v) of chitosan (Fluka Chemical Corp.) as substrate. Chitosan was solubilized in 20% (v/v) acetic acid overnight and the pH was adjusted to 5.0 with 1 N NaOH before it was added to the agarose gel. The gel contained 10 mM sodium acetate buffer (pH 5.0) and 1% (v/v) purified Triton X-100 (Grenier and Asselin, 1990). Gel slabs (30 mL) were poured on 15 x 10 cm glass plates. Wells of 3.5 mm in diameter contained 10 μL samples. Incubation was for 16 h at 37°C in closed boxes. Lysis zones were visualized by transparency against a black background or by Coomassie Blue R-250 staining (Grenier and Asselin, 1990).

**Protein purification for microsequencing.** Chitosanase forms were purified by successive native and denaturing PAGE according to Trudel and Asselin (1994). Leaf IF extracts were prepared from pB19C R2 progeny transgenic tobacco. The IF was concentrated (10X) and a first electrophoretic separation was performed using eight preparative native 15% (w/v) polyacrylamide gels (0.75 mm thick) run at pH 4.3. After Coomassie Blue staining, chitosanase bands were excised in the denaturing buffer (Trudel and Asselin, 1994). Gel slices were washed in distilled water and equilibrated. The eight gel slices were subjected to preparative SDS-PAGE using four 15% (w/v) polyacrylamide (1 mm thick) gels. After Coomassie Blue staining, the three chitosanase bands were excised and each set of bands was stacked on top of one denaturing 15% (w/v) polyacrylamide gel (1.5 mm thick). The proteins (2-3 μg each) were then electroblotted on PVDF membrane (Trudel and Asselin, 1994). Protein N-terminus sequencing was undertaken by automated Edman degradation using Applied Biosystems Sequencer 473A (Service de Séquence de Peptides de l’Esq du Québec, Centre Hospitalier de L’Université Laval, Ste-Foy, Canada). The purified chitosanase from Streptomyces sp. strain N174 was subjected to the same sequential PAGE steps as a control.

**Results**

**Fungal growth inhibition assay**

Growth inhibition was observed with 2.5 μg of purified Streptomyces sp. strain N174 chitosanase towards Rhizopus nigricans and with 25 μg towards Verticillium albo-atrum, Fusarium oxysporum f. sp. radicis-lycopersici and Pythium ultimum (Fig. 1). No inhibition was observed with the chitosanase buffer alone.

**Chitosanase analysis in transgenic tobacco**

Using the PAGE assay, chitosanase activity was already detected in transformed R0 callus homogenates (data not shown). Subsequently, chitosanase activity was tested in leaves from the R0 transformants that had been grown in soil for two weeks. Leaves of the R1 and R2 (self-pollinated) tobacco progeny were also tested for chitosanase activity. No unusual phenotype was observed with