# Bacterial Chitinase Is Modified and Secreted in Transgenic Tobacco

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## ABSTRACT

The chiA gene of Serratia marcescens codes for a secreted protein, bacterial chitinase (ChiA). We have investigated the modifications and the cellular location of ChiA when it is expressed in transgenic tobacco plants. Immunoblots on total leaf protein probed with antibody to ChiA showed that when the bacterial chitinase is expressed in plants, it migrates as a series of discrete bands with either the same or a slower mobility than the secreted bacterial protein. Analysis of the vacuum infiltrate of leaves expressing ChiA showed that the modified forms of the protein are enriched in the intercellular fluid. Media recovered from suspension cultures of cell lines expressing the chiA gene were also enriched for the modified forms of ChiA. Washed protoplasts, however, contained only the nonmodified form. The molecular weight of these polypeptides is reduced by treatment with glycopeptidase F but not with endoglycosidase H. Treatment of the suspension cultures with tunicamycin also leads to reduction in the molecular weight of the chitinase bands. We suggest that some of the ChiA protein is N-glycosylated and secreted when expressed in plants, and that the modifications are complex glycans. These results show that a bacterial signal sequence can function in plant cells, and that protein secretion from plant cells probably operates by a default pathway.

Secretion of proteins from animal cells is a process which, while not fully understood, has been well characterized. Secretion is known to involve the entrance of the protein to the endomembrane system (which requires the presence of a signal sequence on the protein), movement of the protein through the ER and the Golgi body, and fusion of secretory vesicles with the plasma membrane (20, 22). At least two different pathways exist: the regulated pathway where release of proteins is mediated by extracellular signals, and the constitutive pathway where proteins move out of the cell at a constant rate (6). Evidence is now strong that the process of secretion via the constitutive pathway requires no further signals other than the initial signal sequence and may thus be considered a default or bulk-flow pathway (25). The role of other modifications in protein secretion are less clear. It is known that although many secreted proteins are glycosylated, the requirement for glycosylation varies according to the protein (for examples, see ref. 9, and references therein).

In contrast, protein secretion by plant cells is less well characterized, partly due to the lack of suitable model systems. Studies on protein secretion from plant cells are also complicated by the presence of the cell wall. Studies to date have focused on the proteins found in the media of cells grown in suspension, on cell wall proteins, and more recently on proteins found in the intercellular fluid (IF[1]) following pathogen challenge (the PR proteins) (30). A number of secreted proteins have been cloned and sequenced and shown to possess typical signal sequences (7, 23). It is not clear whether the default pathway for protein secretion exists in plant cells, or whether there are specific secretory signals on plant secreted proteins. The role of glycosylation in protein secretion in plants is also not understood. Many secreted and vacuolar proteins are not glycosylated. It is known, moreover, that transport to protein bodies (which also occurs via the ER/Golgi) of proteins which are normally glycosylated is not dependent on the presence of glycans (4, 18), and that inhibition of glycosylation in suspension cultures of carrot does not necessarily inhibit the secretion of proteins which are normally secreted in the glycosylated form (8).

A technique which has proven useful in dissecting many transport pathways within cells is the study of the behavior of foreign or chimeric proteins (8). The demonstration that bacterial β-lactamase is secreted by Xenopus oocytes provided strong evidence for the default hypothesis (32). We describe here a system which uses this approach to study protein secretion in plant cells. Our reporter protein is a bacterial chitinase (ChiA) from Serratia marcescens, which has been cloned and expressed in plant cells under a variety of promoters (11, 13, 14, 27). We show here that a proportion of this protein is modified, probably by N-glycosylation, and transported out of plant cells in which it is expressed, showing that a bacterial signal sequence can function in plant cells.

## MATERIALS AND METHODS

### Plasmid Constructions

Construction of the fusions which enable expression of the chiA gene in plant cells has been described elsewhere. Briefly, construct 1866 contains the chiA gene under the control of the promoter and the 3' polyadenylation sequence of the nopaline synthase (nos) gene from Agrobacterium tumefaciens (27). Construct pJJ2104 contains the bacterial chiA gene under the control of the 35S promoter from cauliflower mosaic virus, with the 3' polyadenylation sequence being derived from the nos gene (11). The chiA gene in construct 1781 is expressed from the promoter of the most highly expressed ribulose bisphosphate carboxylase small subunit (rbcS) gene from petunia, and has the polyadenylation site from the same gene (14). All of these fusions have been cloned

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[1] Abbreviations: IF, intercellular fluid.

into binary vectors suitable for *Agrobacterium*-mediated transformation of plant cells (as described in the above references). pJJ3499 is a binary vector containing the bacterial β-glucuronidase gene under the control of the 35S promoter from cauliflower mosaic virus, with the 3' polyadenylation signal from the *ocs* gene of *Agrobacterium tumefaciens* (J Jones, unpublished data).

## Production of Transgenic Tobacco Plants

Transformation of tobacco (*Nicotiana tabacum*, cv SR1 or Wisconsin 38) was by cocultivation of *Agrobacterium* containing the appropriate binary vector with tobacco protoplasts followed by selection of transformed callus and regeneration of plants as described (17, 29). The constructs 1866 and 1781 (*nos* and *rbcS* promoter fusions with *chiA*) were transformed into cultivar Wisconsin 38; pJJ2104 (35S promoter fusion with *chiA*) and pJJ3499 (35S promoter fusion with β-glucuronidase) were transformed into cultivar SR1. Several plants were assessed for levels of ChiA by immunoblotting, and a single plant with a high level of expression of ChiA was chosen for each of the three constructs described.

## Protoplast Preparation and Suspension Culture Methods

Protoplasts were prepared as described (1) from leaves of *in vitro* grown plants, by digestion with Macerase (0.25%) and Cellulysin (1.0%), both obtained from Calbiochem. Protoplasts were collected by flotation on 0.4 M sucrose. To establish suspension cultures, protoplasts were serially diluted in a succession of media of decreasing osmotic strengths as described elsewhere (29). When the osmoticum reached 0.1 M, plates were placed on a rotary shaker at 50 rpm and 28°C. The incubation medium was changed to a 1:1 mix of MSP and RMNO (defined below) after 4 d, and to RMNO after a further 4 d. Cells were pooled from five plates 4 d later, transferred to a sterile 250 mL flask containing 25 mL RMNO, and shaken at 100 rpm under low light illumination at 28°C. From this point, 8 g of cells were transferred to 40 mL fresh RMNO every 4 d. MSP medium is Murashige-Skoog medium (19) containing in addition naphthalene acetic acid and 6-benzylaminopurine (both at 0.1 μg/mL). RMNO is MS medium containing in addition 2,4-dichlorophenoxyacetic acid (0.1 μg/mL), IAA (3 μg/mL) and kinetin (0.04 μg/mL).

## Protein Analysis

Production of antibody to the bacterial chitinase protein, and methods for SDS-PAGE and immunoblotting have been described (27). Protein concentrations were estimated by the method of Bradford (5) using the Bio-Rad protein assay kit. β-Glucuronidase activities were determined fluorometrically as described (12).

## Preparation of Protein Extracts

IF from leaves was prepared by vacuum infiltration (21). Total leaf protein was extracted in 0.1 M sodium citrate buffer pH 5.0, as described elsewhere (3). To extract the protein from suspension cultures, 40 mL of culture were spun at 1500 rpm in a Beckman TJ-6 centrifuge for 10 min to gently pellet the cells. The cell pellet (15–20 mL packed cell volume) was washed with 5 mL culture medium and this wash was combined with the supernatant from the spin. The cells were ground in liquid nitrogen and extracted using the same protocol as was used for total leaf protein. Proteins from the combined medium and wash were collected by Amicon pressure dialysis as described elsewhere (8). To extract the IF from callus grown on solid media, 3 to 4 g of callus was placed in a 10 mL syringe with a 1.5 mL Eppendorf at the tip, and the whole assembly was spun for 10 min at 1500 rpm in a Beckman TJ-6 centrifuge. Approximately 1 mL of fluid was recovered from 3 to 4 g of callus. Extractions for determinations of β-glucuronidase activity were prepared as described elsewhere (12).

## Characterization of Modified Proteins

Endo-β-*N*-acetylglucosaminidase H (endoglycosidase H) and glycopeptide-*N*-glycosidase (glycopeptidase F) were both obtained from Boehringer Mannheim. Cleavage with glycopeptidase F was carried out as described elsewhere (26), using 2 units of enzyme to cleave 200 μg total protein. Cleavage with endoglycosidase H was carried out as described elsewhere (28), using 10 milliunits of enzyme to cleave 200 μg total protein.

## RESULTS

### Bacterial Chitinase Exists in Forms with Different Mol Wt in Transgenic Tobacco

Protein extracts were made from lower leaves of transgenic tobacco plants which were expressing the *chiA* gene. One hundred μg of protein were loaded onto 10% SDS polyacrylamide gels with the purified bacterial ChiA protein and mol wt standards as markers. Following electrophoresis, the proteins were transferred to nitrocellulose and probed with the ChiA antibody. The results are shown in Figure 1. The ChiA protein isolated from bacteria migrates with a mol wt of 58,000, which is the expected size of the mature protein following cleavage of the signal sequence (13). Most of the ChiA expressed in plants migrates at the same mol wt as the bacterial protein, while the remainder of the protein migrates in two to three discrete bands of progressively higher mol wt. The estimated increase in mol wt between the bands is ca. 2,000. Essentially the same pattern of bands is seen irrespective of the promoter from which the *chiA* gene is being expressed (compare lanes 3, 4, and 7 in Fig. 1). No cross-reacting bands are seen in extracts from a nontransformed plant (lane 10), implying that the bands seen with a lower mol wt than the ChiA protein are degradation products.

### Washed Protoplasts Contain Only the Lowest Mol Wt Form of ChiA

We considered two possible explanations for the origin of the higher mol wt forms of the ChiA protein. One was that they represented the full-length protein (without cleavage of
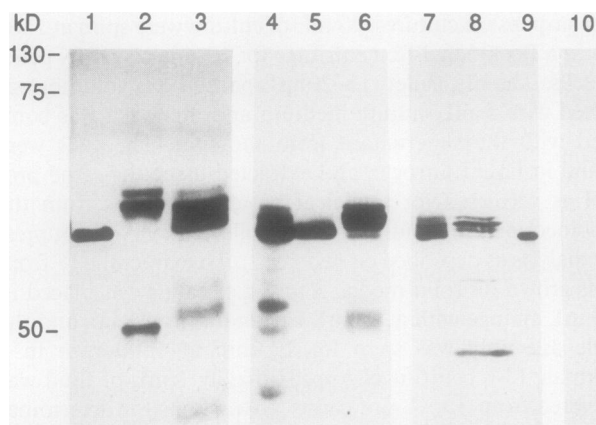
**Figure 1.** Production of different mol wt forms of ChiA in transgenic plants. Each lane contains 200 μg protein except for lanes 1 and 9, which each contain 100 ng of pure ChiA from *E. coli*. Lane 2: IF from 1866 plant; lane 3: total leaf protein from 1866 plant; lane 4: total leaf protein from 2104 plant; lane 5: protein from protoplasts of 2104 plant; lane 6: IF from 2104 plant; lane 7: total protein from 1781 plant; lane 8: IF from 1781 plant; lane 10: IF from nontransformed plant.

the signal sequence) and correct and aberrant cleavage products thereof. The other was that the higher mol wt forms were modified forms of ChiA, the most likely candidate for the modifications being *N*-glycosylation, as ChiA contains four potential *N*-glycosylation sites (Asn-X-Ser/Thr). To see whether there was differential secretion by plant cells of the different forms, protoplasts were prepared from leaves as described in "Materials and Methods." These were thoroughly washed to remove any intercellular proteins and then extracted for total remaining protein. Comparison of the protein profiles from total leaf extracts and from washed protoplasts (Fig. 1, lanes 4 and 5) clearly shows that only the lowest mol wt form of ChiA is present inside the protoplasts.

## IF Contains Predominantly the Upper Mol Wt Forms of ChiA

Vacuum infiltration of leaves is a standard method for the recovery of intercellular proteins (21, 24). Use of cytoplasmic marker proteins has been used to show that the fraction being recovered in these experiments is genuinely secreted as opposed to being due to leakage from damaged cells. We carried out control experiments on plants which expressed high levels of the *Escherichia coli* β-glucuronidase protein, which has become widely used as a reporter gene for the analysis of transgenic plants and which is cytoplasmically located (R Jefferson, personal communication). The total and specific activities for β-glucuronidase were determined for vacuum infiltrates and whole cell extracts (Table I). These results show that only 0.15% of the total amount of β-glucuronidase activity is found outside the cell. Comparison of specific activities shows that some leakage occurs, but 70% of the protein recovered in the IF is genuinely secreted protein. We also showed that the IF prepared from plants which had been treated with salicylic acid was strongly enriched for the PR proteins, which are known to be secreted by plant cells (not shown). Thus we believe that the fraction recovered from

**Table I.** *β-Glucuronidase Activity in Cell Extracts, IF, and Conditioned Media from Suspension Cell Cultures*

Protein extracts were made for the various tissues and fractions as described in "Materials and Methods." The total activity is the total units of activity recovered from an individual leaf (lines 1 and 2). Figures shown are the mean of at least two independent determinations. MU = 4-methylumbelliferone; ND = not determined.

| Experiment | Total Activity | Specific Activity |
|---|---|---|
| | *μmol 4-MU/min* | *μmol 4-MU/min/mg protein* |
| 1. Total leaf extract | 46.1 | 4.02 |
| 2. IF from leaf | 0.07 | 1.21 |
| 3. Total cell extract (suspension cells) | ND | 0.85 |
| 4. Conditioned medium (suspension cells) | ND | 0.037 |

plant leaves following vacuum infiltration does represent secreted proteins, albeit with some contamination with cytoplasmic proteins.

Vacuum infiltration was used to recover the IF from the leaves of the tobacco plants which expressed ChiA. Comparison of the proteins from IF with those in total leaf extracts by immunoblotting shows clearly that the upper molecular weight forms of the ChiA protein are selectively enriched, suggesting that they are being secreted from the cell (Fig. 1, lanes 2, 6, and 8).

We also investigated the IF produced by callus, grown either in suspension culture or on solid media. Intercellular protein was recovered from conditioned media or from solid callus as described in "Materials and Methods." Two hundred μg protein was electrophoresed and visualized by immunoblotting. The results are shown in Figure 2, tracks 2 and 3, with IF from leaf for comparison. The gel shows that only higher mol wt forms of ChiA are seen in the IF derived from callus. In a parallel control experiment, measurement of the specific activity of β-glucuronidase released into the medium from suspension cultures of cells from plants transformed with pJJ3499 suggested that the leakage of cytoplasmic proteins into the medium was small (*ca.* 4% of the total intercellular protein). This may account for the difference in band pattern seen in leaf IF as opposed to conditioned medium.

## Higher Mol Wt Forms of ChiA Are Glycosylated

The results described above indicate that the ChiA protein enters the secretory pathway and may undergo a series of modifications en route to its intercellular location. As glycosylation is known to be a frequent modification of secreted proteins, and as ChiA contains potential glycosylation sites, we looked to see whether the ChiA protein was glycosylated when secreted from plant cells. Treatment with glycopeptidase F, which cleaves *N*-linked glycans irrespective of whether they are high mannose or complex (26) was carried out on IF prepared by vacuum infiltration, as described in "Materials and Methods." SDS-PAGE followed by immunoblotting of the products of this reaction showed that the molecular weight of the chitinase was reduced by the treatment with glycopep-
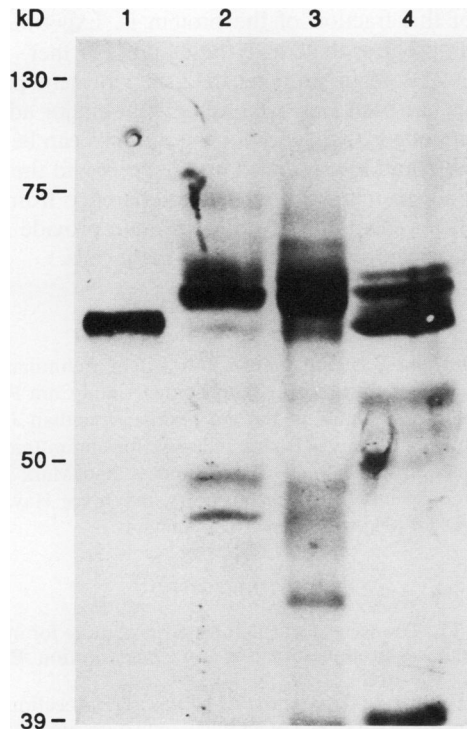
**Figure 2.** ChiA in different IFs. Lane 1: 100 ng purified ChiA from *E. coli*; lane 2: protein in conditioned medium from 2104 suspension culture; lane 3: protein in IF from 2104 callus; lane 4: protein in IF form 2104 leaf. All tracks except lane 1 contain 200 μg protein.
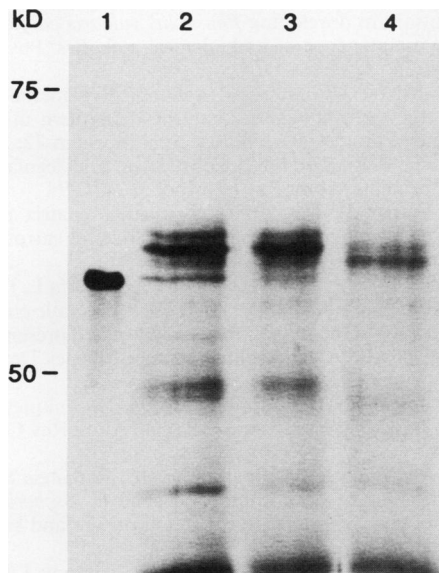


**Figure 3.** Glycopeptidase F cleavage of ChiA. Lane 1: purified ChiA; lane 2: IF from 2104 plant; lane 3: IF from 2104 plant incubated 24 h in glycopeptidase F buffer alone; lane 4: IF ffrom 2104 plant incubated with glycopeptidase F for 24 h. All lanes escept 1 contain 200 μg protein.

tidase F (Fig. 3). The mol wt was not reduced to that of the fastest migrating band, which we believe is the nonglycosylated form of the protein. This may be due to incomplete cleavage by the glycopeptidase F, although no further change

in band mobility occurred when the incubation time was increased to 48 h with addition of more glycopeptidase F at 24 h (data not shown). Treatment with endoglycosidase H, which cleaves only high mannose residues, did not give any change in mol wt of the chitinase (data not shown). If yeast carboxypeptidase Y was added to preparations of IF and cleaved with endoglycosidase H, cleavage was seen, demonstrating that this enzyme was fully active in the presence of IF (data not shown). Thus, the *N*-linked glycans present on the ChiA protein have been modified, showing that the protein has passed through the Golgi apparatus en route to the cell surface.

We also investigated the effects of tunicamycin on modification and secretion of ChiA into the medium of suspension cultures. Cells (2.5 g) containing pJJ2104 were grown for 6 d in 25 mL growth medium (RMNO, see "Materials and Methods") with or without the addition of 5 μg/mL tunicamycin, an antibiotic which is commonly used as an inhibitor in glycosylation studies (4). Samples (1 mL) of medium were taken at 3 d and 6 d, and the washed cells were extracted for total protein after 6 d. Cells which had been treated with tunicamycin secreted 66% of the total protein secreted by untreated cells, possibly because of a decrease in overall protein synthesis which tunicamycin has been reported to cause (15). The media and cell samples were analyzed by immunoblotting. The results (Fig. 4) show that in the presence of tunicamycin, most of the ChiA has the same mol wt as the nonmodified bacterial protein, supporting the identification of the modification as *N*-glycosylation. The lack of glycosylation in the tunicamycin-treated cells does not appear to inhibit the secretion of the ChiA protein.
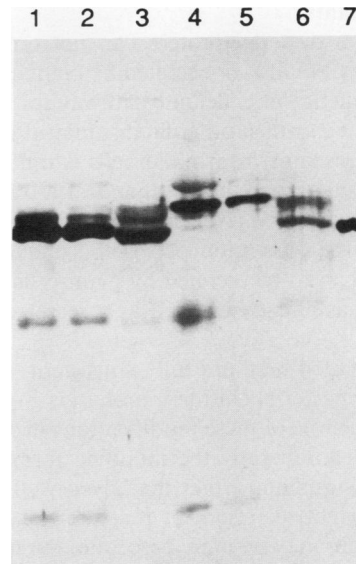


**Figure 4.** Effects of tunicamycin on ChiA modification and secretion in suspension cultured cells. Lanes 1 to 3 are from 2104 suspension cultures treated with tunicamycin (5 μg/mL). Lanes 4 to 6 are from nontreated 2104 suspension cultures. Lanes 1 and 4: protein from 1 mL conditioned medium at d 6; lanes 2 and 5: protein from 1 mL conditioned medium at d 3; lanes 3 and 6: 200 μg protein from cells at d 6; lane 7: 150 ng purified ChiA.

## DISCUSSION

Because of the difficulty in establishing whether genuine protein secretion is occurring in plant cells, we have used three different approaches in this paper. Analysis of IF indicates that although it is contaminated with cytoplasmic proteins, it is enriched for secreted proteins. The conditioned medium from suspension cells shows very low contamination with cytoplasmic proteins and thus proteins which appear in it are probably secreted. The absence in washed protoplasts of a particular protein or form of a protein which is known to be present in whole leaf tissue is further evidence for an intercellular location for that protein. The results from all three of these analyses are consistent with secretion of at least a fraction of the chitinase protein, this fraction being the modified form. The absence of these modified forms from protoplasts suggests that their half-life inside the cell is short.

It has previously been shown that signal sequences at the N termini of secreted proteins will function across species barriers, including plant signal sequences working in bacteria (10) and animal cells (31). The secretory apparatus can apparently tolerate a high degree of degeneracy in the signal sequence (16) although not all signal sequences are interchangeable between species (2). As ChiA is secreted into the periplasm of E. coli (PA Lund, unpublished data) and possesses a typical signal sequence which is known to be cleaved when the protein is expressed in bacteria (13) it is likely that this signal is also functioning in transgenic tobacco to mediate the entry of ChiA into the secretory pathway. Formal proof of this must await studies on the behavior of ChiA proteins expressed without a signal sequence, and establishment of the precise site of cleavage of the signal sequence. We believe this is to be the first demonstration of a bacterial signal sequence functioning in plants.

The fact that a bacterial protein can not only enter the secretory pathway but also be secreted by plant cells strongly supports the existence of a default pathway for secretion in plants. The extent to which other factors may affect secretion of heterologous proteins from plant cells is unknown. Such factors may include physical and chemical attributes which have not yet been defined (e.g., solubility, degree of folding, and charge), or the possession of specific retention signals. The ability of ChiA to be secreted by plant cells may reflect the fact that is has already evolved as a secreted protein in bacteria.

The data presented here are fully consistent with the hypothesis that the bacterial chitinase protein is N-glycosylated by plant cells. The role of these modifications in secretion are not yet clear. Predominantly the modified forms are found outside the cells, implying either that glycosylation is a prerequisite for efficient secretion, or that the nonglycosylated form of the protein may be more susceptible to the action of intercellular proteases. Reconstruction experiments have so far failed to demonstrate any such differential stability (R Lee, P Lund, unpublished results). However, the data from the tunicamycin treatments show that the nonglycosylated form of the protein can be secreted by these cells. It is also not clear why a proportion of the ChiA has its signal sequence cleaved but fails to become glycosylated, nor what the cellular location of this fraction of the protein is. Experiments are currently in progress to address these points further.

The use of foreign proteins to dissect protein pathways within cells has been well established. One major advantage of this technique is that genes for the proteins can be manipulated in vitro and reintroduced into the cell, and the mutant proteins studied in the absence of interference from native proteins. The system described here should provide a useful tool for studying protein secretion by plant cells.

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