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Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: Evidence for a strictly compartmentalized plant defense system

(electroblotting/microsequencing/pathogenesis-related proteins/*Pseudomonas syringae*/salicylic acid)

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ABSTRACT **β(1,3)-Glucanases are a class of hormonally and developmentally regulated plant hydrolytic enzymes, which are also induced upon pathogen infection, suggesting a role of these hydrolases in the defense response to pathogens. We have purified several β(1,3)-glucanases present in tobacco leaves from control plants and from plants treated with salicylic acid or infected with *Pseudomonas syringae* and studied in detail the subcellular localization of the different isoforms. Partial protein sequence analysis demonstrated that each of the different isoforms had a unique amino acid sequence and was therefore encoded by a different gene. We have also demonstrated that two of these isoforms, similar to the cytokinin/auxin-regulated isoforms previously isolated from tobacco cell suspensions, are located in the central vacuole. Upon salicylic acid treatment or *P. syringae* infection, three secreted isoforms are induced, belonging to the so-called pathogenesis-related proteins. These pathogenesis-related β(1,3)-glucanases are all distinct from each other and also different from the vacuolar isoforms. We demonstrate that the vacuolar isoforms are not secreted to the extracellular spaces of the plant following pathogen infection, suggesting that any function they play in the plant defense response is restricted to an intracellularly coordinated defense process.**

Infection of plants by pathogens (viruses, bacteria, or fungi) is accompanied by a drastic metabolic change (1). In many cases, this defense reaction is accompanied by the induction of the synthesis of a specific set of proteins, preferentially accumulating in the extracellular spaces: the so-called pathogenesis-related (PR) proteins (2–4). The synthesis of these proteins is considered part of a general defense response to pathogens, although a detailed picture of their action has not emerged yet. Recently, several PR proteins have been identified as β(1,3)-glucanases and chitinases (5, 6), two classes of hydrolytic enzymes capable of inhibiting pathogen growth, presumably through degradation of their cell walls (7).

In addition, intracellular β(1,3)-glucanases and chitinases have also been identified (8, 9). Their synthesis is regulated during development by a variety of factors including the combined action of cytokinins and auxins, ethylene, or pathogen infection (9–11). As such, these intracellular β(1,3)-glucanases and chitinases could also be considered as plant defense proteins. Their precise role in the defense response is not clear, although a hypothesis has been forwarded that several intracellular plant defense proteins (such as proteinase inhibitors and chitinases) would be secreted upon pathogen infection to inhibit further spreading of the pathogen (12).

To evaluate the participation of β(1,3)-glucanases in the plant defense system to pathogens, we have carried out a detailed protein–chemical characterization of the β(1,3)-glucanases (both intra- and extracellular) present in tobacco leaf tissue under different physiological conditions. This has allowed correct isotyping of the different β(1,3)-glucanases according to a set of specific amino acid substitutions. Parallel subcellular fractionation analysis has provided data about the exact localization of the respective isoforms in the cell. This information is essential for correctly allocating the forthcoming β(1,3)-glucanase genomic and cDNA clones and identifying the signals that control specific targeting. Also, these analyses have allowed us to test the hypothesis whether the secretion of the intracellular β(1,3)-glucanases is a major feature of the plant defense response to pathogen infection.

MATERIALS AND METHODS

Isolation of the Vacuolar Proteins of Tobacco Protoplasts. Protoplasts of sterile-grown *Nicotiana tabacum* var. Petit Havana SR1 plants were prepared as described (13). Vacuoles were stained with neutral red (0.1% solution) before disrupting the protoplasts by addition of 10 vol of lysis buffer (0.1 M K_2HPO_4/3 mM EDTA/1 mM dithiothreitol, pH 8.0). Next, the cell lysate was filtered and layered on top of a 5% Ficoll cushion prepared in lysis buffer. The lysate was covered with a fresh layer of buffer. The vacuoles floated to the top of the gradient during centrifugation at 1500 × *g* for 30 min and were collected with a Pasteur pipette.

The vacuolar proteins were extracted by lysing the organelles with 0.2 M Na_2CO_3. The tonoplast vesicles were precipitated by centrifuging the lysate for 30 min at 20,000 × *g*. The proteins present in the supernatant were then concentrated by deoxycholate/trichloroacetic acid precipitation (14).

To assay the purity of the vacuoles, the isolated fraction was first analyzed under the light microscope and then monitored for the presence of the following contaminants: chlorophyll (chloroplasts) (15), DNA (nuclei) (16), fumarase (mitochondria) (17), glucose-6-phosphate dehydrogenase (cytosol) (18), and catalase (peroxisomes) (19). Acid phosphatase activity was taken as a vacuolar marker (20). β(1,3)-Glucanase activity was measured as described by Ashwell (21).

Induction and Isolation of the Extracellular PR Proteins of *N. tabacum*. The PR proteins were extracted from leaves of *N. tabacum* plants (grown in the greenhouse) by use of the vacuum infiltration technique (22). Untreated plants were used as a control. The salicylic acid treatment was carried out as described by Hooft van Huijsduijnen *et al.* (23). The *Pseudomonas syringae* infection was done with a late-logarithmic bacterial culture grown in LPG medium (0.3% yeast extract/0.5% Bacto peptone/0.5% glucose) diluted to 10^7 bacteria per ml prior to injection into the leaves (0.3–0.5

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Abbreviations: PR, pathogenesis-related; P4VMP, poly(4-vinyl-*N*-methylpyridinium iodide).

ml per inoculation site). The extracellular proteins were isolated 2 days after bacterial infection.

Chromatographic Purification of the Extracellular $\beta(1,3)$-Glucanases Induced on Different Stress Stimuli. First, the extracellular PR proteins were dialyzed exhaustively against water and 15 mM Tris·HCl (pH 8). The extracts were then applied on a DEAE-Sepharose column equilibrated with 50 mM Tris·HCl (pH 8) before eluting the proteins with a 200-ml linear salt gradient (0–0.5 M NaCl in 50 mM Tris·HCl, pH 8). Fractions of 5 ml were collected and assayed for $\beta(1,3)$-glucanase activity. The fractions containing activity were further analyzed by reverse-phase HPLC using a C_4 column (4.6 × 250 mm; Baker) equilibrated in 0.1% trifluoroacetic acid (TFA). The proteins were eluted with a linear acetonitrile gradient (0–70% acetonitrile in 0.1% TFA over 70 min) and detected by adsorption at 214 nm. Peaks were collected manually in Eppendorf tubes and further analyzed for the presence of $\beta(1,3)$-glucanase by $NaDodSO_4$/PAGE followed by immunodetection (24). For the identification of the vacuolar isoforms, a total extract of salicylic acid-induced plants was first separated by DEAE-Sepharose (as described above) and the void volume was analyzed by reverse-phase HPLC.

$NaDodSO_4$/PAGE, Electroblotting onto Poly(4-Vinyl-*N*-Methylpyridinium Iodide) (P4MVP)-Coated Glass-Fiber and Immobilon Membranes, and Amino Acid Sequence Analyses of the Electroblotted Proteins. The deoxycholate/trichloroacetic acid-precipitated vacuolar and PR protein fractions were separated by $NaDodSO_4$/PAGE according to Laemmli (25) using 15% and 17.5% polyacrylamide gels, respectively. For amino acid sequencing of the different PR $\beta(1,3)$-glucanases upon electroblotting, the extracellular protein fractions were separated by 10% polyacrylamide gels. The separated proteins were transferred either onto P4MVP-coated glass-fiber or onto Immobilon membranes as described by Bauw *et al.* (26) and stained with fluorescamine (1 mg/liter of acetone) and amido black, respectively.

NH_2-terminal amino acid sequence determination of the blotted proteins was performed as described by Bauw *et al.* (26). Internal sequencing of NH_2-terminally blocked proteins was performed after either chemical cleavage (Asp-Pro) or proteolytic degradation (trypsin) of the immobilized proteins (27). The amino acid sequence analysis was performed using a 470A gas-phase sequenator equipped with a 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems).

RESULTS

The Central Vacuole Contains at Least Two $\beta(1,3)$-Glucanases. It has been reported that intracellular chitinases accumulate in the central vacuole of the cell (12). As $\beta(1,3)$-glucanases and chitinases are known to be coregulated and their cellular localization to be identical in many species (6, 9), we have investigated whether the intracellular $\beta(1,3)$-glucanases are also sequestered in the vacuoles.

Vacuoles of tobacco leaf protoplasts were isolated, and light microscopic and biochemical marker analysis (for markers, see *Materials and Methods*) indicated that the purified organelle fraction was free of any major contamination, with the exception of a low level of chloroplasts (<5%). In addition to high acid phosphatase activity, typical of vacuolar extracts, high $\beta(1,3)$-glucanase activity could also be detected. The soluble vacuolar protein fraction was analyzed by one-dimensional $NaDodSO_4$ gel electrophoresis, and a fluorescamine staining of the electroblots showed the presence of an abundant 35-kDa protein that was further characterized by amino acid sequence analysis (Fig. 1*A*). This 35-kDa protein was blocked NH_2-terminally. Thus, peptides were generated by *in situ* trypsin digestion, separated by reverse-phase HPLC, and characterized by amino acid sequence analysis.

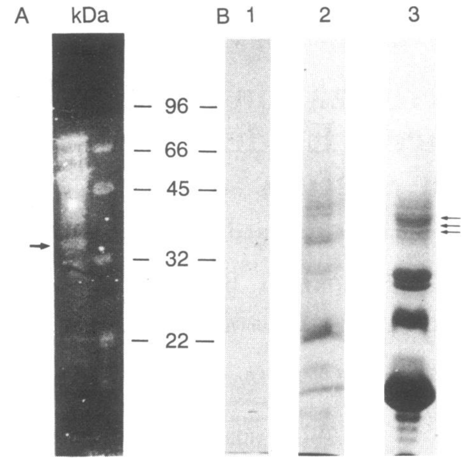

FIG. 1. $NaDodSO_4$/PAGE of the vacuolar and the extracellular protein fractions. (*A*) The vacuolar proteins were electroblotted onto P4MVP-coated glass-fiber membranes and stained with fluorescamine. (*B*) The extracellular protein fractions were transferred onto Immobilon membranes and stained with amido black. The $\beta(1,3)$-glucanases are indicated by the arrows. Lane 1, control plants; lane 2, *P. syringae*-infected plants; and lane 3, salicylic-acid-treated plants. Molecular mass markers are indicated in kDa.

In total, >20% of the mature protein sequence was covered and showed complete identity with the $\beta(1,3)$-glucanase encoded by the cDNA clone described by Shinshi *et al.* (28) (Fig. 2). Interestingly, one peptide matched exactly the COOH-terminal sequence of the latter $\beta(1,3)$-glucanase (including its COOH-terminal glycine), suggesting a similar processing event in both cases.

In one of the tryptic peptides we noticed the presence of an Asp-Pro sequence, which constitutes a preferential cleavage site in acidic conditions. To further extend the sequence homology, we have applied this chemical cleavage method to the electroblotted vacuolar $\beta(1,3)$-glucanase. Sequence analysis of the *in situ* cleaved and unseparated fragments identified two residues at each cycle, correctly reflecting the presence of two Asp-Pro cleavage sites in the mature protein, as can be deduced from the corresponding cDNA clone (28). As one of the sequences was already (partially) covered by one of the tryptic peptides, the second sequence could be assigned by difference (Fig. 2). In this way, our homology studies were extended up to 30% of the entire sequence; the presence of two very similar vacuolar isoforms was revealed by the sequence microheterogeneity at one position (residue 192).

Taken together, these data provide evidence that the central vacuole contains at least two intracellular $\beta(1,3)$-glucanases similar to the hormonally regulated isoforms in *N. tabacum*.

Characterization of the PR $\beta(1,3)$-Glucanases Induced by Salicylic Acid and *P. syringae*. Recently it has been shown that several $\beta(1,3)$-glucanases are present in the PR protein fraction accumulating in the extracellular spaces upon different pathogen infections (5). However, it is still an open question whether these extracellular $\beta(1,3)$-glucanases are secreted vacuolar enzymes or different, newly synthesized isoforms. Therefore, we have characterized the extracellular $\beta(1,3)$-glucanases induced by different agents (tobacco mosaic virus, *P. syringae*, ethephon, and salicylic acid). Treatment with salicylic acid produced the highest induction (data not shown) and the corresponding PR fraction was therefore taken for detailed analysis.

Extracellular protein fractions were obtained by using the vacuum infiltration technique (22). Possible contamination of these fractions by intracellular proteins was checked by using glucose-6-phosphate dehydrogenase activity as marker. The

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          1                                                50
cDNA      MAAITLLGLLLVASSIDIAGAQSIGVCYGMLGNNLPNHWEVIQLYKSRNI
VAC(35)
PR(37)                                  IANNLPSDQDVINLY
PR(36)                                  HANNLPSDQDVINLY
PR(35)

          51                                              100
cDNA      GRLRLYDPNHGALQALKGSNIEVMLGLPNSDVKHIASGMEHARWWVQKNV
VAC(35)       LYDPNHGALQALKGSNIEVMLGLPN                WWVQK
PR(37)        IYYPETNVF                                 ANGWVQDNI
PR(36)        IYNPDTNVFNALRGSNIEIILDVPLQDLQSL-TDPSRANGWVQDNI
PR(35)        IYDPDQPTLEALR

          101                                             150
cDNA      KDFWPDVKIKYIAVGNEISPVTGTSYLTSFLTPAMVNIYKAIGEAGLGNN
VAC(35)
PR(37)    INGFP     YIAVGNEVSP?NEI
PR(36)    IN?FPDVK                       ?PAMQNVYNALAAAGLQDQ
PR(35)              YIAVGNEVSP?NENS

          151                                             200
cDNA      IKVSTSVDMTLIGNSYPPSQGSFRNDARWFTDPIVGFLRDTRAPLLVNIY
                                                   N
VAC(35)                                  DPIVGFLRDT APLLVNIY
                                                   R
PR(37)
PR(36)    IK
PR(35)

          201                                             250
cDNA      PYFSYSGNPGQISLPYSLFTAPNVVVQDGSRQYRNLFDAMLDSVYAALER
VAC(35)   PYF?Y
PR(37)
PR(36)
PR(35)

          251                                             300
cDNA      SGGASVGIVVSESGWPSAGAFGATYDNAATYLRNLIQHAKEGSPRKP-GP
VAC(35)   SGGASVGIVVSESGWPSAGAFG??YDNAA
PR(37)    AGGPNVEIIVSESGWPSEGNSAAT         NLIDHVK
PR(36)    AGGQNVEIIVSESGWPSEGNSAAT
PR(35)                               TYNNNLISHVK     APSGP

          301                                             350
cDNA      IETYIFAMFDENNKNPELEKHFGLFSPNKQPKYNINFGVSGGVWDSSVET
VAC(35)                                   YNINFG
PR(37)        LFNMFDENQK      HFGLFSPDQR  YQLNFN
PR(36)          AMFDENNKEGDIEKHFGLFSPDQR  YQLNFN
PR(35)    IETYVFALFDEDQKDPEIEK            YQISFN

          351       361
cDNA      NATASLVSEM
VAC(35)
PR(37)
PR(36)
PR(35)
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FIG. 2. Amino acid sequence homology between the vacuolar [VAC(35)], the PR [PR(37), PR(36), and PR(35)], and the cytokinin/auxin-regulated $\beta(1,3)$-glucanases of tobacco (cDNA). The upper line shows the full cDNA-derived sequence encoding the nonprocessed $\beta(1,3)$-glucanase (28). The sequences shown are derived from tryptic peptides or from *in situ* Asp-Pro cleavage (underlined). Amino acids that could not be identified are indicated by question marks; gaps introduced in the amino acid sequence to allow correct alignment are indicated by dashes. The molecular masses of the different isoforms are indicated in kDa within parentheses.

absence of this activity was an indication that no major cell damage had occurred during the PR protein isolation (data not shown). Moreover, the extracellular protein fraction of control plants contained almost no protein and was free of any $\beta(1,3)$-glucanase activity (Fig. 1*B*, lane 1). This contrasts with the proteins present in the extracellular fraction of salicylic acid-treated plants (Fig. 1*B*, lane 3) and *P. syringae*-infected plants (Fig. 1*B*, lane 2). The striking difference in expression level and protein profile between both fractions is remarkable, suggesting that the induction of the different PR proteins is not strictly coregulated but governed by a set of distinct regulatory mechanisms. The $\beta(1,3)$-glucanases were identified by electrophoretic transfer (Western) blotting (data not shown). Three bands cross-react strongly with the antiserum (a 35-kDa, 36-kDa, and 37-kDa band; marked by arrows in Fig. 1*B*, lane 3). Corresponding electroblots onto Immobilon membranes were used for further protein–chemical characterization. As in the case of the other $\beta(1,3)$-glucanases, all secreted isoforms were found to be NH_2-terminally blocked. *In situ* trypsin digestion, followed by reverse-phase HPLC separation of the generated peptides, provided internal sequences that could be aligned with previous sequences of tobacco $\beta(1,3)$-glucanases (Fig. 2). Two of the secreted isoforms are >95% similar to each other, whereas the third one is only about 65% similar to them. The secreted isoforms also differ considerably from the intracellular variants, only 60% of the primary structure being conserved in both classes. These data show that the PR $\beta(1,3)$-glucanases represent a distinct class of newly induced proteins, each being the product of different structural genes. Notice that the COOH-terminal end of all three secreted $\beta(1,3)$-glucanases is at exactly the same position as that found for the intracellular isoform.

To exclude the possibility that the 35-kDa $\beta(1,3)$-glucanase band would have masked less abundant vacuolar $\beta(1,3)$-glucanases, we separated the PR protein fraction by ion-exchange chromatography on a DEAE-Sepharose column under conditions in which the intracellular isoforms are known not to bind to the resin (29). We found that all enzymatic activity present in the extracellular protein fraction was retained on the column, indicative of the acidic nature of PR $\beta(1,3)$-glucanases (5). By contrast, when vacuolar extracts were analyzed in a parallel experiment, all activity was recovered in the void volume in accordance with the basic character of these proteins (29). As such, none of the vacuolar $\beta(1,3)$-glucanase was present in the extracellular PR protein fraction.

As a third criterion for distinguishing between PR and intracellular $\beta(1,3)$-glucanases, we analyzed the extracellular extract by reverse-phase HPLC. Each of the peaks containing a $\beta(1,3)$-glucanase protein was assigned by Western blotting (Fig. 3*A*) and characterized by peptide analyses after *in situ* digestion (data not shown). Again, the vacuolar isoforms were absent in the extracellular protein fraction. This also demonstrates that the vacuolar $\beta(1,3)$-glucanases have a slightly shorter retention time (r_t) than the extracellular isoforms [r_t (vacuolar) = 48 min; r_t (PR 37) = 50 min; r_t (PR 36) = 48.5 min; r_t (PR 35) = 49 min] (Fig. 3*C*).

The results described above demonstrate that vacuolar $\beta(1,3)$-glucanases are not secreted after salicylic acid treatment, although in these plants considerable amounts of a different set of extracellular $\beta(1,3)$-glucanase are synthesized. To determine whether this is also the case after pathogen infection we performed a similar $\beta(1,3)$-glucanase isoform typing on tobacco plants that were inoculated with a strain of *P. syringae* known to induce the hypersensitive reaction in tobacco. All extracellular $\beta(1,3)$-glucanase activity was retained on a DEAE-Sepharose column, suggesting the absence of any vacuolar isoforms. Analysis of the same fraction by reverse-phase HPLC, combined with Western blot screening, identified the presence of the same isoforms as found upon salicylic acid treatment (Fig. 3*B*). Interestingly, the level of expression of the respective PR $\beta(1,3)$-glucanases was different in both cases. This suggests that the accumulation of these extracellular $\beta(1,3)$-glucanases is not strictly coordinated but rather subject to parallel, but not identical, regulatory mechanisms. In conclusion, these data provide evidence that the vacuolar $\beta(1,3)$-glucanases are also not secreted upon pathogen infection.

DISCUSSION

This study was undertaken to obtain a better insight in the role of $\beta(1,3)$-glucanases in the defense response of plants toward pathogens. Towards this aim, $\beta(1,3)$-glucanases were

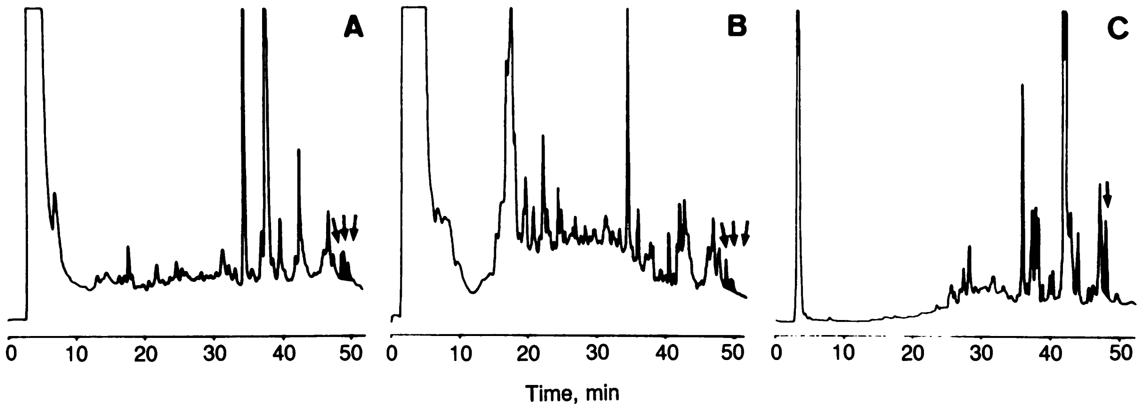

FIG. 3. Reverse-phase HPLC analysis of the extracellular PR fractions of salicylic-acid-treated and *P. syringae*-infected plants. The different $\beta(1,3)$-glucanases are indicated by the arrows. (*A*) Salicylic-acid-induced PR fraction. (*B*) *P. syringae*-induced PR fraction. (*C*) Analysis of the void volume upon separation by DEAE-Sepharose of a total protein extract of salicylic-acid-induced plants.

isolated from different subcellular fractions and characterized by about 30% of their primary structures. Two very similar isoforms were identified in the soluble vacuolar protein extract of protoplasts of sterile-grown tobacco plants. Their amino acid sequence is highly homologous with that of the previously reported hormonally regulated $\beta(1,3)$-glucanases isolated from tobacco cell suspension cultures (28).

Treatment of plants with salicylic acid or infection with *P. syringae* provoked the induction of a separate class of $\beta(1,3)$-glucanases, consisting of at least three different isoforms. These PR $\beta(1,3)$-glucanases accumulate extracellularly and are distinct from the vacuolar isoform, indicating that they are encoded by different structural genes. Interestingly, the vacuolar isoforms are not secreted in situations in which the extracellular $\beta(1,3)$-glucanase are highly induced (salicylic acid treatment or *P. syringae* induction). This was confirmed by (*i*) the absence of vacuolar $\beta(1,3)$-glucanase-specific peptides in tryptic digests of $NaDodSO_4$/PAGE-separated PR proteins, (*ii*) the lack of vacuolar $\beta(1,3)$-glucanase activity in the extracellular protein fractions upon separation of vacuolar and PR isoforms by ion-exchange chromatography, and (*iii*) the absence of vacuolar-specific $\beta(1,3)$-glucanases in reverse-phase HPLC analyses of the extracellular protein fractions.

Although the physiological relevance of salicylic acid-induced reactions is limited (30), due to the poor characterization of its function *in vivo*, the results obtained upon *P. syringae* infection are undoubtedly significant. Indeed, the hypersensitive reaction elicited upon infection of tobacco with *P. syringae* is one of the most efficient defense reactions of plants against pathogen infection (31). Thus, the fact that no vacuolar $\beta(1,3)$-glucanase is secreted in a situation in which hypersensitive reactions occur indicates that any function of the vacuolar $\beta(1,3)$-glucanase (and presumably all vacuolar proteins) in the defense response will be restricted to an intracellularly coordinated defense program.

It is difficult to assign a function for strictly intracellularly compartmentalized $\beta(1,3)$-glucanases. Although $\beta(1,3)$-glucan (or callose) is deposited in the cell wall during several developmental processes and also following pathogen infection (32, 33), no evidence has been found for the presence of $\beta(1,3)$-glucans in any intracellular compartment. Nevertheless, the presence of these vacuolar isoforms in total leaf protein extracts of sterile plants showing no sign of any environmental stress (unpublished results) suggests they have a constitutive level of expression and, as such, they could play a basic role in cellular metabolism. The fact that the expression of the intracellular $\beta(1,3)$-glucanases is regulated by the concerted action of cytokinins and auxins possibly reflects a role of these enzymes in cell growth and cell division (e.g., degradation of internalized β-glucans of the primary cell wall) (34). However, further experiments will be needed to elucidate the actual role of the vacuolar $\beta(1,3)$-glucanases in cellular metabolism.

From the data presented here it is clear that at least two classes of $\beta(1,3)$-glucanases can be identified upon pathogen infection of tobacco: the first class is localized in the central vacuole of the cell, has a constitutive level of expression, and has been shown to be highly induced upon pathogen infection (11); the second one accumulates in the extracellular spaces of the plant and is stress dependent. It is generally accepted that the secretion of proteins is a default pathway (35), whereas proteins that are sequestered in specific cellular organelles contain distinct targeting signals (for review, see ref. 36). Interestingly, we found that the vacuolar $\beta(1,3)$-glucanase undergoes COOH-terminal processing, removing a 22-amino acid COOH-terminal peptide. Currently, it is not known whether this COOH-terminal extension has any function in the targeting of the intracellular $\beta(1,3)$-glucanases to the central vacuole. However, recent data have shown that correct compartmentalization of peroxisomal proteins and endoplasmic reticulum proteins is governed by COOH-terminal amino acid residues (37, 38). By fusing this COOH-terminal region to reporter genes and analyzing the localization of the fusion protein in transgenic plants it should be possible to determine whether or not this COOH-terminal region has any function in the targeting of the vacuolar $\beta(1,3)$-glucanase.

In summary, we have characterized five $\beta(1,3)$-glucanases of tobacco. Three of them are induced upon salicylic acid treatment or pathogen infection and all three accumulate in the extracellular spaces of the plant. The two other isoforms are localized in the central vacuole of the plant cell and are not secreted upon salicylic acid treatment or during the hypersensitive reaction in response to *P. syringae*. From this, we can conclude that vacuolar defense proteins remain intracellular upon pathogen infection and, as such, their function in the defense response will be restricted to intracellularly mediated defense processes.

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