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A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole

(cucumber/*Nicotiana silvestris*/*Nicotiana tabacum*/plant defense/secretion)

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ABSTRACT **Tobacco contains different isoforms of chitinase (EC 3.2.1.14), a hydrolase thought to be involved in the defense against pathogens. Deduced amino acid sequences for putatively vacuolar, basic chitinases differ from the homologous extracellular, acidic isoforms by the presence of a C-terminal extension. To examine the role of this C-terminal extension in protein sorting, *Nicotiana silvestris* plants were stably transformed with chimeric genes coding for tobacco basic chitinase A with and without the seven C-terminal amino acids. In plants expressing unmodified chitinase A, the enzyme activity was low in the intercellular wash fluid but high in protoplasts and isolated vacuoles. In contrast, in plants expressing mutant chitinase lacking the C terminus, the activity was high in the intercellular wash fluid but low in protoplasts. *N. silvestris* plants were also transformed with similar constructions coding for a structurally unrelated, extracellular cucumber chitinase. In plants expressing unmodified cucumber chitinase, its activity was present in the intercellular wash fluid and absent from protoplasts. In plants expressing cucumber chitinase with the C-terminal extension from tobacco chitinase A, activity was low in intercellular wash fluids but high in protoplasts and vacuoles. These results demonstrate that the C-terminal extension of tobacco chitinase A is necessary and sufficient for the vacuolar localization of chitinases and, therefore, that it comprises a targeting signal for plant vacuoles.**

The vacuole of the mature plant cell is a large organelle used for storing water, ions, and metabolites (1, 2). It is also a lytic compartment resembling the lysosome of animals and the vacuole of fungi—e.g., of yeast (1–3). Many typical lysosomal hydrolases in plants and fungi are found in the vacuole and the exoplasmic (intercellular) space (1, 4). This raises the question whether hydrolases are specifically targeted to the vacuole or to the exoplasmic space.

Polypeptides destined for the lysosome or vacuole and for the exoplasmic space carry an N-terminal signal peptide that targets the nascent polypeptide to the lumen of the endoplasmic reticulum and is subsequently removed cotranslationally (5, 6). Plant signal peptides correctly target polypeptides to the endoplasmic reticulum of animal cells and yeast and vice versa (review, ref. 6). In eukaryotes, the default pathway of polypeptides that have entered the endoplasmic reticulum is secretion to the exoplasmic space, and retention in the endomembrane system or targeting to the lysosome or vacuole requires additional information (5–7).

The nature of the signal for lysosomal or vacuolar targeting has been studied extensively in animals and yeast and found to differ between the two groups of organisms. A mannose-6-phosphate group on oligosaccharide side chains is necessary and sufficient for lysosomal targeting in animals (8), whereas the targeting information for yeast vacuoles is contained in a short N-terminal domain of vacuolar propeptides (9, 10).

To date, evidence for specific vacuolar targeting signals in plants has come primarily from studies of storage proteins accumulating in specialized vacuoles, present in seeds, embryonic tissue, or vegetative storage organs (review, ref. 6). Typically, these proteins accumulate only in vacuoles and have no secreted homologues. It has been shown that storage proteins from various plants, when constitutively expressed in transgenic tobacco, accumulate in the vacuole of the mature leaf (11–13). Furthermore, deletion of an N-terminal propeptide of sporamin (13) or of a C-terminal propeptide of barley lectin (14) caused secretion of the mutated proteins in transgenic tobacco, demonstrating that the propeptides of these storage proteins carry sequences necessary for vacuolar targeting.

To address the question of vacuolar targeting of lytic enzymes in plants, we have chosen chitinase (EC 3.2.1.14), a hydrolase induced by ethylene and pathogenesis that is thought to be involved in defense against pathogens (15). Several chitinases have been cloned and sequenced (16). On the basis of their amino acid sequences, three classes can be distinguished (16). Class I chitinases have an N-terminal cysteine-rich domain following the signal peptide, which is homologous to hevein, a vacuolar polypeptide from *Hevea* latex (17, 18). One member of this class, the basic chitinase of bean, has been shown to be localized in the vacuole (19, 20). Class II chitinases, initially described as "pathogenesis-related proteins," are similar to class I chitinases but lack the cysteine-rich domain at the N-terminal end and a short extension at the C-terminal end (21). Class II chitinase of tobacco is known to be located in the intercellular space (21). Class III chitinases have an entirely unrelated amino acid sequence (22). One member of this group, the pathogen-induced acidic chitinase of cucumber, is located in the intercellular space (23).

Comparison of class I and class II chitinases prompted us to examine the possibility that the N-terminal cysteine-rich domain or the C-terminal extension of class I chitinase is important for vacuolar localization. Using site-directed mutagenesis and constitutive expression of mutant chitinases in *Nicotiana silvestris*, we report here that the short C-terminal extension of tobacco chitinase A functions as a vacuolar targeting signal. It is necessary for the correct vacuolar targeting of chitinase A and sufficient to target an unrelated, normally secreted chitinase of class III to the vacuole.

MATERIALS AND METHODS

Plant Material. *N. silvestris* L. plants were grown from seed in a greenhouse. Transgenic plants derived from shoot

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Abbreviation: ICF, intercellular wash fluid.

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culture were raised in soil in a greenhouse under BL2 containment as recommended by the National Institutes of Health guidelines.

Plasmid Constructions and DNA Transformation of *N. silvestris*. Constructions were based on plasmid pSCH10 (24), containing the coding sequence for tobacco chitinase A under the control of the expression signals for the 35S transcript from cauliflower mosaic virus, using the following oligonucleotides for site-directed mutagenesis (25):

M1 = TTTGGAAATTGACTCTTAGTCG
M2 = CCGCTCTTCGGATCCGGCTGG
M3 = CATCTTCTAGATTTAGTCTC
M4 = CTGCCTCGGCTGATCAATGTGG
M5 = CAGCATCGGTGATCAGGAAGCTC
M6 = CCAGAGATCTTTTGGGAAATGG.

Plasmid pSCM3 containing the sequence for tobacco chitinase A lacking the seven C-terminal amino acids was made by introduction of a stop codon seven codons upstream from the wild-type stop codon with M1. For plasmid pSCU1 containing the mature cucumber chitinase fused to the signal peptide of tobacco chitinase A, a cassette with the coding sequence of mature cucumber chitinase, constructed from a full-length cDNA clone (22) by introducing a *Bam*HI site at the end of the signal peptide coding sequence with M2 and a *Xba* I site in the 3′ noncoding sequence with M3, was cloned between a *Bcl* I site created by M4 at the end of the signal peptide coding sequence of pSCH10 and the *Xba* I site of its parent vector pGY1 (24). For plasmid pSCU3 containing the sequence for cucumber chitinase extended with the C-terminal sequence of tobacco chitinase A, a similar cassette, obtained by introducing into the cucumber chitinase cDNA clone (22) the same *Bam*HI site and a *Bcl* I site at the stop codon with M5, was cloned into pSCH10 between the *Bcl* I site created as above and a *Bgl* II site followed by a three-codon linker nine codons upstream from the stop codon created by M6. All constructions were cloned into the binary vector pCIB200 containing a plant-selectable chimeric *NOS/NPTII* gene and introduced into *N. silvestris* by *Agrobacterium*-mediated leaf disk transformation; this was followed by regeneration of kanamycin-resistant plantlets as described (24). Control plants were transformed with the vector pCIB200 without insert (24).

Preparation of Intercellular Wash Fluids (ICFs), Homogenates Depleted of Intercellular Fluid, and Total Homogenates. For extraction of ICF, leaves were cut into 4-cm strips, infiltrated under vacuum with 50 mM sodium citrate at pH 5.5, blotted dry with filter paper, rolled, and introduced into empty syringes. These were placed in centrifuge tubes and centrifuged at 1000 × *g* for 10 min. The ICF eluted was collected at the bottom of the centrifuge tube. The leaf strips were then ground in a mortar and pestle with *ca.* 1 g of quartz sand and 2 ml of the same buffer per 1 g of fresh weight; this was followed by centrifugation at 14,000 × *g* for 5 min to obtain ICF-depleted homogenates. The same procedure was used for freshly harvested leaves to obtain total homogenates.

Preparation of Mesophyll Protoplasts and Vacuoles. Protoplasts were obtained by overnight digestion of leaf slices with 0.4% (wt/vol) Macerozyme R10 (Serva)/0.6% (wt/vol) Cellulysin (Calbiochem) in K3M [mannitol adjusted to 500 mosM, macronutrients (26) at half concentration, pH 5.6]. The protoplasts were filtered through a 100-μm steel sieve, mixed with half a volume of 0.6 M sucrose, overlaid with K3M, and collected by floatation in a low-speed centrifuge. The protoplasts were washed once by low-speed centrifugation in K3M. Vacuoles were isolated by floatation of protoplasts through a polycation/polyanion step gradient (27) in K3M as follows. Protoplasts were suspended in 2.5 ml of 20% (wt/vol) Ficoll (pH 6.5). They were overlaid with 2 ml of 15% (wt/vol) Ficoll containing 0.7% (wt/vol) DEAE-dextran (pH 6.5), 2 ml of 10% (wt/vol) Ficoll containing 0.3% (wt/vol) of dextran sulfate (pH 8.0), 2 ml of 6% (wt/vol) Ficoll containing 0.3% (wt/vol) dextran sulfate (pH 8.0), and 4 ml of 0% Ficoll (pH 8.0). The gradients were centrifuged in a Kontron TST41.14 swing-out rotor at 3500 rpm for 15 min; this was followed by centrifugation at 40,000 rpm for 100 min. The vacuoles accumulated at the 0–6% Ficoll interface.

Immune Blot Analysis. Samples of homogenates from leaf tissue and from protoplasts of each transformant (containing equal amounts of total protein) were loaded on adjacent lanes, separated by $NaDodSO_4$/10% (wt/vol) polyacrylamide gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). The blots were incubated with 5% (wt/vol) milk powder in phosphate-buffered saline and then probed with a mixture of an antiserum against tobacco chitinase A (17) and an antiserum against cucumber chitinase (22); this was followed by incubation with a goat anti-rabbit antibody coupled to horseradish peroxidase (Immune-Blot, Bio-Rad) and then with H_2O_2 and the color development reagent (4-chloro-1-naphthol) as specified by the supplier (Bio-Rad).

Measurement of Protein, Chitinase, and Marker Enzymes. Protein concentration was measured according to Bradford (28). Chitinase activity was determined with [^{3}H]chitin as a substrate as described (29). The activity of cucumber chitinase was measured in the presence of 2 μl of antiserum against tobacco chitinase (17), an amount sufficient to inhibit the endogenous basic chitinase of *N. silvestris* by at least 99%. Control experiments showed that this antiserum did not affect the activity of cucumber chitinase. The vacuolar marker, α-mannosidase, and the extravacuolar markers hexosephosphate isomerase and chlorophyll, respectively, were measured as described (3), except that DEAE-dextran was used to precipitate the residual dextran sulfate before measuring hexose-6-phosphate isomerase. The vacuolar marker α-mannosidase was used to normalize the comparison between protoplasts and vacuoles (3).

RESULTS

Chitinase Activities in ICFs. Preliminary experiments demonstrated that deletion of the N-terminal cysteine-rich domain of the basic tobacco chitinase affected neither its intracellular localization nor its enzymatic activity when constitutively expressed in transgenic *N. silvestris* plants (data not shown). Therefore, we concentrated on the possibility that the short extension at the C-terminal end of vacuolar chitinases carried the vacuolar targeting signal. To test our working hypothesis, we constructed chimeric genes encoding either tobacco class I chitinase A (24) with or without its C-terminal extension of seven amino acids or a structurally unrelated, extracellular cucumber class III chitinase (22) with its natural C terminus or with the nine C-terminal amino acids from tobacco chitinase A added through a three-codon linker (Fig. 1). These constructions were placed under control of the promoter for the 35S transcript from cauliflower mosaic virus and introduced into the genome of *N. silvestris* by *Agrobacterium*-mediated transformation. Chitinase localization was examined in *N. silvestris* plants containing these genes and in control plants transformed with the vector without insert.

ICF fractions and ICF-depleted homogenates were prepared from leaves of transformed plants. Typical results are given in Fig. 2; the experiments were performed in at least two repetitions with at least two transformed plants per construction, yielding essentially the same results. ICF fractions contained <5% of the protein of ICF-depleted homogenates and <1% of intracellular markers such as hexose-6-

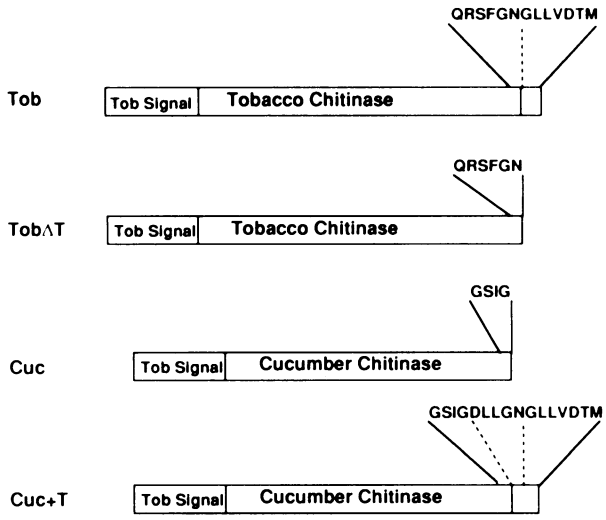

FIG. 1. Structure of the chitinase polypeptides encoded by the chimeric genes that were introduced into *N. silvestris* plants to study their localization. Tob is the wild-type tobacco chitinase A (18); TobΔT is Tob lacking seven C-terminal amino acids. Cuc is mature cucumber chitinase (22) fused to the signal peptide of tobacco chitinase. Cuc+T is Cuc modified by adding the nine C-terminal amino acids of the tobacco chitinase A to the C-terminal end of Cuc via a three-amino acid linker. Tob Signal, signal peptide of tobacco chitinase A.

phosphate isomerase or chlorophyll (data not shown). Leaves from control plants (Ctrl) had about the same low chitinase activity as untransformed plants (data not shown), most of which was found in the leaf homogenate rather than in the ICF fraction (Fig. 2*A*). Leaves from plants expressing the chimeric gene for wild-type tobacco chitinase (Tob) had up to 100 times higher chitinase activities than leaves from control plants (24). Only about 3% of this activity was found in the ICF fraction; most of it was present in the ICF-depleted homogenate (Fig. 2*A*). Leaves from plants expressing tobacco chitinase without its C-terminal extension (TobΔT) had a similarly high chitinase activity; however, about half of this activity was found in the ICF fraction, and only half remained in the ICF-depleted homogenate (Fig. 2*A*).

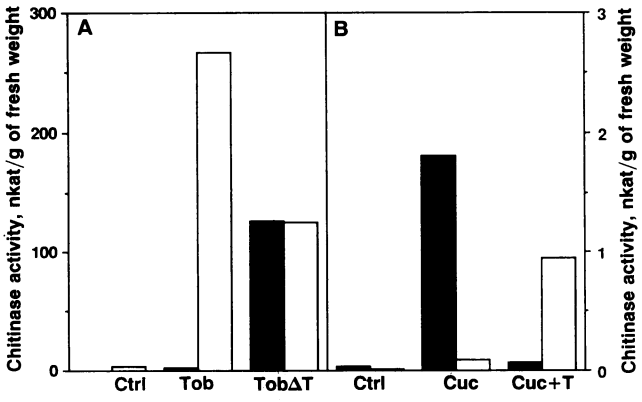

FIG. 2. Chitinase activities in the ICF fractions (solid bars) and in the ICF-depleted homogenates (open bars) of leaves from *N. silvestris* plants transformed with vectors containing no insert (control) or chimeric genes coding for the chitinases described in the legend to Fig. 1. (*A*) Control plants (Ctrl) and plants expressing tobacco chitinase with (Tob) and without (TobΔT) its C-terminal sequence. (*B*) Control plants (Ctrl) and plants expressing cucumber chitinase with (Cuc+T) and without (Cuc) the C-terminal sequence derived from tobacco chitinase. (*B*) Activities of chitinase were measured in the presence of antibodies against tobacco chitinase. nkat, Nanokatals.

To study the expression of cucumber chitinase in transgenic plants, an enzyme with a specific activity about 10 times lower than tobacco chitinase A (B. Iseli, J.-M.N., and T.B., unpublished observations), it proved necessary to measure chitinase activities in the presence of antibodies against tobacco chitinase to avoid interference by the endogenous *N. silvestris* chitinase. Under these conditions, very little chitinase activity was found in preparations from leaves of control plants (Ctrl); the ICF fraction contained a higher residual activity than the leaf homogenate (Fig. 2*B*). Under the same assay conditions, leaves from plants expressing the cucumber chitinase (Cuc), directed into the secretory pathway by way of the signal peptide from tobacco chitinase A, had up to 50 times higher chitinase activity. Most of this activity was found in the ICF (Fig. 2*B*), showing that cucumber chitinase is indeed secreted in transgenic *N. silvestris* plants. In contrast, most of the activity of cucumber chitinase with the C-terminal extension from tobacco chitinase (Cuc+T) was found in the ICF-depleted homogenate (Fig. 2*B*). These results show that the C-terminal extension of tobacco chitinase A prevents secretion of the structurally unrelated cucumber and tobacco chitinases into the intercellular space.

Chitinases in Protoplasts. The localization of the different transgene-encoded chitinases was further investigated by comparing specific activities and immunoreactivities between total leaf homogenates and protoplasts (Fig. 3). The specific activities of chitinase in homogenates and protoplasts from plants expressing the intact tobacco chitinase construction (Tob) were the same, demonstrating that most of the enzyme has an intracellular localization. In contrast, the specific activity of chitinase in protoplasts from plants expressing the construction lacking the seven C-terminal amino acids (TobΔT) was only 12.5% of that in leaf homogenates, much of which is probably due to the endogenous *N. silvestris* chitinase (see immune blots below). This indicates that most of the chitinase lacking the C-terminal extension is secreted and that the 50% of this chitinase remaining in the ICF-depleted leaves (Fig. 2) is probably trapped in the extracellular compartment. Cucumber chitinase behaved similarly: its specific activity in protoplasts was only 2.5% of that in leaf homogenates of plants expressing the cucumber chitinase without C-terminal extension (Cuc). However, the specific activity of cucumber chitinase was at least as high in

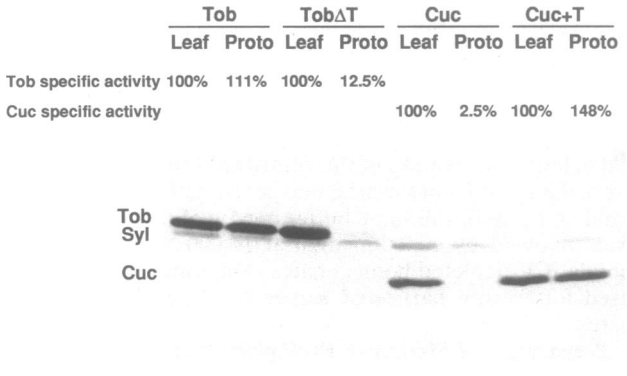

FIG. 3. Chitinases in homogenates from leaf tissue (Leaf) and protoplasts (Proto) from transgenic *N. silvestris* plants expressing the chitinases described in the legend to Fig. 1. Specific activities of chitinase measured in the absence (Tob specific activity) or in the presence of antibodies against tobacco chitinase (Cuc specific activity) are given in percent of the specific activities in leaf homogenates. Immune blot analysis of samples containing equal amounts of protein was performed with a mixture of antibodies against tobacco and cucumber chitinases. Tob, Syl, and Cuc, positions, in this sequence, of tobacco chitinase A, the endogenous *N. silvestris* chitinase, and cucumber chitinase, respectively.

protoplasts as in leaf homogenates of plants expressing the cucumber chitinase construction with the C-terminal extension of tobacco chitinase (Cuc+T).

Because of their different molecular weights, it was possible to examine the identity and distribution of the chitinases detected by activity measurements in parallel experiments by immune blotting using a mixture of antibodies against tobacco and cucumber chitinase (Fig. 3). Immune blots from leaf homogenates showed a weak band representing the endogenous chitinases of *N. silvestris* and, in addition, a strongly immunoreactive band at the positions expected for tobacco chitinase A and cucumber chitinase, respectively, regardless of the presence and absence of the C-terminal extension. Protoplasts had similar levels of the same immunoreactive bands when the plant expressed chitinases with the C-terminal extension. However, these immunoreactive bands were completely absent from the protoplasts when the constructions lacked a C-terminal extension. As expected, the immunoreactive band corresponding to endogenous basic chitinase of *N. silvestris* had similar levels in leaf homogenates and protoplasts and contributed most of the antigen in protoplasts from plants expressing TobΔT.

Chitinases in Vacuoles. To verify the vacuolar localization of the intracellular chitinases, we isolated vacuoles from the protoplasts, using a floatation technique in combination with polycation-induced lysis of protoplasts (Table 1). The vacuolar marker α-mannosidase (2, 3) was used as a reference for the comparison with the protoplasts. This enzyme showed a high activity in vacuole preparations. The two extravacuolar markers hexose-6-phosphate isomerase and chlorophyll had low activities in the vacuole preparation. This contamination arose mainly from protoplasts surviving lysis, as checked by microscopic examination; mitochondria, plastids, and vesicles derived from the endoplasmic reticulum and Golgi are expected to sediment during the floatation procedure (3, 27). The complete tobacco chitinase and the cucumber chitinase carrying the additional C-terminal sequence had about the same activity per 10^6 vacuoles as per 10^6 protoplasts, indicating that both were entirely localized in the vacuoles. These results were confirmed by immune blot analysis (data not shown).

DISCUSSION

We have used constitutive expression of genetically engineered chitinases in *N. silvestris* as a tool to study vacuolar targeting of hydrolases in plants. *N. silvestris* contains only one resident form of class I chitinase (24). *Nicotiana tabacum*, an amphidiploid plant thought to have arisen from a hybrid of *N. silvestris* with *Nicotiana tomentosiformis*, contains two isoforms of class I chitinase, differing about 2000 Da in molecular mass (17). We have chosen the cDNA of tobacco chitinase A, which corresponds in size to the *N. tomentosiformis* chitinase (M. van Buuren, J.-M.N., H. Shinshi, J. Ryals, and F.M., unpublished data), as a basis for our study in order to be able to distinguish the transgenes from the resident chitinase in *N. silvestris*.

We have studied the localization of chitinases in ICF and vacuoles of transgenic plants. Pulse–chase data to be reported elsewhere provide evidence that these enzymes, which are highly stable under various conditions (15, 16), are transported through the endomembrane system and accumulate at their final destination. The data presented here show that constitutively expressed chitinase A accumulates in the vacuole, as the resident *N. silvestris* chitinase. This demonstrates that the vacuolar sorting mechanism is correctly operating in the presence of a high rate of synthesis of vacuolar chitinase, a situation found naturally in tobacco leaves only upon induction by pathogens (30, 31). Thus, as in the case of heterologous storage proteins (11–13), the leaf cells have an efficient constitutive sorting system for proteins targeted to the vacuole. On the other hand, cucumber class III chitinase, which is secreted in pathogen-infected cucumber leaves (23), is also secreted when expressed constitutively in *N. silvestris* leaves, demonstrating correct localization in the heterologous system in the absence of pathogen stress. Taken together, these data indicate that the vacuolar or extracellular localization of each isoform of chitinase is a fixed property dependent upon its amino acid sequence.

Our results show that the seven C-terminal amino acids encoded by the mRNA for basic chitinase are *necessary* for correct targeting to the vacuole. Work to be reported elsewhere (L.S., J.-M.N., T.B., J. Hofsteenge, and F.M.) demonstrates that these seven amino acids are lacking at the C terminus of the mature tobacco chitinase, indicating that it is processed during or after sorting. Analogous results have been described for modified proteins that are normally localized in protein storage vacuoles. The barley seed lectin with a C-terminal propeptide deleted (14) and the *Ipomoea* tuber storage protein, sporamin, with its N-terminal propeptide deleted (13) are secreted to the extracellular space.

Furthermore, our results demonstrate that the nine C-terminal amino acids of the tobacco chitinase propeptide, when attached to cucumber chitinase through a three-amino acid linker, are *sufficient* for correct vacuolar targeting of this otherwise secreted plant protein. Thus, the situation in plants

Table 1. Localization of intracellular markers in preparations of protoplasts and vacuoles from transgenic *N. silvestris* plants overproducing chitinases

N. silvestris plants	Units* per 10^6 protoplasts	Units* per 10^6 vacuoles†	% total in vacuoles
Expressing tobacco chitinase A with its own C-terminal sequence			
α-Mannosidase	89	89	100
Chitinase	66,300	75,100	113
Hexose-6-phosphate isomerase	2,640	170	6
Chlorophyll	27	<4.5	<17
Expressing cucumber chitinase with the C-terminal sequence of tobacco chitinase A			
α-Mannosidase	59	59	100
Cucumber chitinase‡	12,000	12,200	102
Hexose-6-phosphate isomerase	490	90	18
Chlorophyll	62	<2.2	<4

*Picokatals (pkat) for enzymes, μg for chlorophyll.
†Counting data normalized to values for α-mannosidase as 100%.
‡Chitinase activity measured in the presence of antibodies against tobacco chitinase.

may be analogous to yeast, in which a contiguous sequence of only four amino acids within the N-terminal propeptide of carboxypeptidase Y has been identified as being necessary and sufficient for correct targeting (10). It differs in detail since the sequence identified in yeast (10), QRPL, is not present in the C-terminal domains of the two chitinases targeted to the vacuole. In this context, it is interesting that the plant vacuolar phytohemagglutinin A is correctly targeted to the yeast vacuole but that the targeting sequence delineated in yeast is not sufficient for vacuolar targeting of this protein in plants (32). There is no obvious similarity between the C-terminal vacuolar targeting sequence described here and the longer propeptide sequences necessary for correct targeting of barley lectin (14) or sporamin (13), indicating that several different, unrelated sequences may carry out vacuolar targeting in plants (6), a situation also suspected in yeast (10). Indeed, immunolocalization and fractionation studies have shown that the C-terminal propeptide of barley lectin is also sufficient to target cucumber chitinase to the vacuole (33). At this point, it should be noted that cucumber chitinase is an excellent reporter protein for targeting studies in plants since it is efficiently transported through the endomembrane system as a normally secreted protein (unpublished data). However, it cannot be excluded that this protein contains cryptic targeting information activated by the added C termini. Thus, it will be important to study the C-terminal targeting sequences in combination with other, unrelated reporter proteins as well.

We conclude that the specific localization in the vacuole and in the extracellular space of chitinases induced by ethylene and pathogenesis depends on the presence or absence of the C-terminal extension containing vacuolar targeting information and is not the result of either imprecise sorting or relocalization. The fact that the targeting sequence is short and acts in a C-terminal position makes it particularly well suited as a tool for examining the functional significance of differential localization of antifungal hydrolases in pathogenesis, for identifying sorting receptors, and for directing heterologous proteins to the vacuole of transgenic plants.

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