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Protein Secretion in Plant Cells Can Occur via a Default Pathway

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To study protein secretion in plant cells, we established and evaluated a model system based on transient synthesis of heterologous proteins in tobacco protoplasts. We show that the nonsecretory enzymes phosphinothricin acetyl transferase, neomycin phosphotransferase II, and β-glucuronidase are secreted when targeted to the lumen of the endoplasmic reticulum by signal peptide-mediated translocation. These data are consistent with the view that secretion can occur independent of active sorting mechanisms by nonspecific migration through the exocytic pathway. However, the rate of secretion differs significantly among these enzymes. Furthermore, the presence of signal sequences was found to be correlated with a reduction of the levels of the encoded gene products. This is the result of post-transcriptional events that limit either synthesis or stability of the proteins in vivo.

INTRODUCTION

The requirements for protein secretion in eukaryotic cells are still poorly understood. Signal peptide-mediated translocation across the endoplasmic reticulum (ER) membrane (Walter and Lingappa, 1986) is the first step in the transport route common to vacuolar, ER, or Golgi resident proteins and those that are secreted. Elaborate post- and cotranslational mechanisms maintain precursor proteins in a translocation-competent conformation (Sanz and Meyer, 1988; Zimmermann et al., 1988). Cytosolic proteins, modified by the addition of signal sequences, have already been successfully targeted to the lumen of the ER of plants in vivo (Dore et al., 1989; Iturriaga, Jefferson, and Bevan, 1989).

Once in the lumen of the ER, all further information for specific transport must be present in domains of the protein remaining after cleavage of the signal peptide and other post-translational modifications (Pfeffer and Rothman, 1987). Such signals have been identified for proteins that are targeted to different subcellular compartments in yeast (Johnson, Bankaitis, and Emr, 1987; Valls et al., 1987; Pelham, Hardwick, and Lewis, 1988) or animal cells (von Figura and Hasilik, 1986; Munro and Pelham, 1987). It was also shown that defective secretion and accumulation in the ER of a mutant secretory protein with a single residue substitution is not the result of a major change in protein conformation (McCracken, Kruse, and Brown, 1989). This suggests that discrete signals are also required for selective secretion of proteins. However, the observation that mutations of known targeting signals for subcellular compartments can result in mistargeting of the mutant protein to the cell surface (Machamer and Rose, 1987; Munro and Pelham, 1987) suggests that secretion can also occur via a "default pathway." According to this view, proteins migrate nonspecifically from the lumen of the ER via the Golgi complex toward the cell surface unless they contain signals for directed transport to other cell compartments. This concept is further supported by the observation that overproduction of a vacuolar protein can lead to its partial secretion (Stevens et al., 1986). High concentrations of this vacuolar protein would probably cause saturation of a receptor and secretion of the excess protein via the default pathway. Measurements of the bulk flow rate in the exocytic pathway using glycosylated tripeptides as markers indicated that the bulk flow is not limiting for protein secretion (Wieland et al., 1987). However, the inert nature of these glycosylpeptides, which would exclude any interaction with active sorting mechanisms, has not been proven. On the other hand, nonspecific interactions with stationary components in the exocytic pathway could interfere with the mobility of larger proteins (Rose and Doms, 1988). Results obtained with small peptides cannot, therefore, be extrapolated to all secreted proteins.

In this work, we established and evaluated a model system for the study of protein transport in plant cells. The obtained data are consistent with the view that secretion occurs via a default pathway. It was also observed that the presence of signal sequences correlates with a reduc-

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tion of the level of the encoded gene products and that protein synthesis or stability imposes limitations on secretory protein levels.

RESULTS

Secretion of Neomycin Phosphotransferase II in Electroporated Protoplasts

A suitable model system to test the default hypothesis in plant cells would consist of an inert "passenger molecule" that is translocated into the lumen of the ER. We chose the cytosolic procaryotic enzyme neomycin phosphotransferase II (NPTII) to study protein transport in tobacco leaf protoplasts. We assumed that NPTII does not contain specific sorting signals recognized by the eucaryotic transport machinery and, because putative glycosylation sites are absent, minimal interactions with components in the exocytic pathway were expected. To target NPTII to the lumen of the ER, chimeric genes were designed containing eucaryotic signal sequences fused to the *neo* coding region, as shown in Figure 1. Gene fragments encoding the signal peptide of the pathogenesis-related protein 1b (sPR1), a protein secreted in tobacco mosaic virus-infected tobacco leaves (Cornelissen et al., 1986), and the prepropeptide of cecropin B (sCEC), a peptide secreted into the hemolymph of the insect *Hyalophora cecropia* (Von Hofsten et al., 1985), were made by chemical DNA synthesis (Figure 1A). In the constructions 210 and 211, these signal sequences replace the first codon of the *neo* coding region in construction 212, the latter of which was used as control (Figure 1B). Expression of hybrid genes in plant cells was directed by the TR2′ promoter of the divergent octopine TR promoter fragment (Velten et al., 1984), and in vitro transcripts were obtained using the SP6 promoter (Figure 1B). The pDE plasmids used for electroporation also contained a reference gene that was used as an internal standard for transient expression experiments. The reference gene was composed of the open reading frame of the *bar* gene encoding phosphinothricin acetyl transferase (PAT) (Thompson et al., 1987) under control of the TR1′ promoter.

Transient expression and secretion of NPTII were studied in tobacco leaf protoplasts after electroporation with plasmid DNA of pDE212, 210, and 211. Intra- and extracellular NPTII and PAT activity levels were measured in the cell suspensions 36 hr after electroporation. The levels of PAT activity allowed us to estimate the cytosolic leakage due to cell death. When pDE210 and 211 containing the hybrid genes with signal sequences were used, approximately 50% of the total measured NPTII activity was present in the culture medium of the protoplast suspensions, as shown in Figure 2A, top. In the case of the control, pDE212, only 2% of the activity was detected

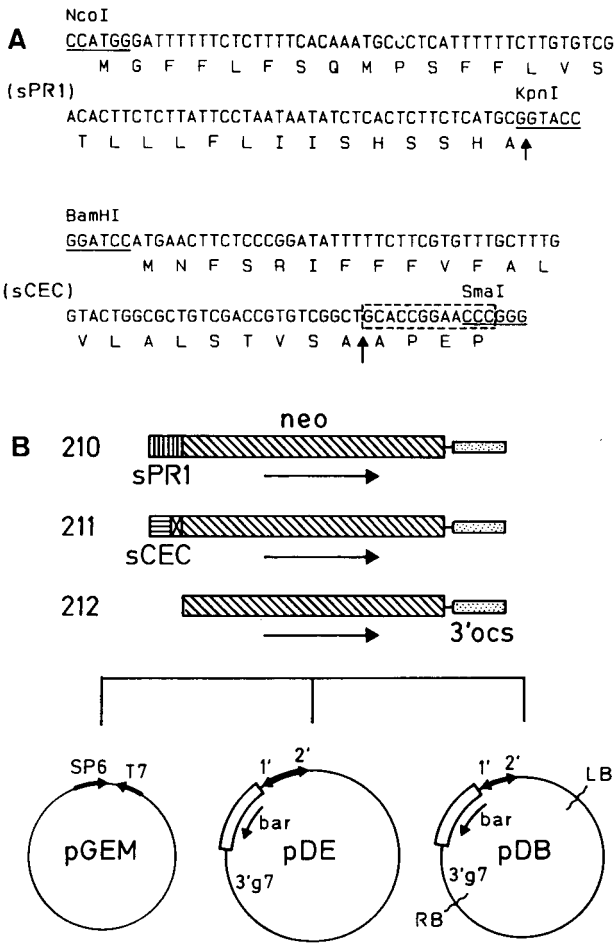

Figure 1. Signal Sequences and Schematic Representation of Plasmid Constructions.

(A) Synthetic DNA fragments encoding the signal sequences of the pathogenesis-related protein 1b (sPR1) and the prepro-fragment of cecropin B (sCEC). Restriction sites are underlined and the processing sites are indicated by arrows. The pro-fragment of the cecropin B coding sequence is boxed.

(B) Chimeric *neo* constructs. Hybrid genes containing the *neo* coding sequence fused in frame with both signal sequences (sPR1 in 210 and sCEC in 211) and *neo* itself (212) are placed under control of the SP6 promoter (pGEM) and the octopine TR-DNA TR2′ promoter (indicated as 2′ in pDE and pDB plasmids). The T-DNA border repeats are indicated as LB and RB. The *bar* coding sequence is under control of the TR1′ promoter (indicated as 1′). 3′-Untranslated ends containing a polyadenylation signal are indicated by 3′ ocs and 3′ g7.

extracellularly. The latter value was comparable with the fraction of total PAT activity detected in the culture medium of all three cell suspensions (Figure 2A, bottom). These results demonstrate that the extracellular NPTII activities measured for constructions 210 and 211 result from a

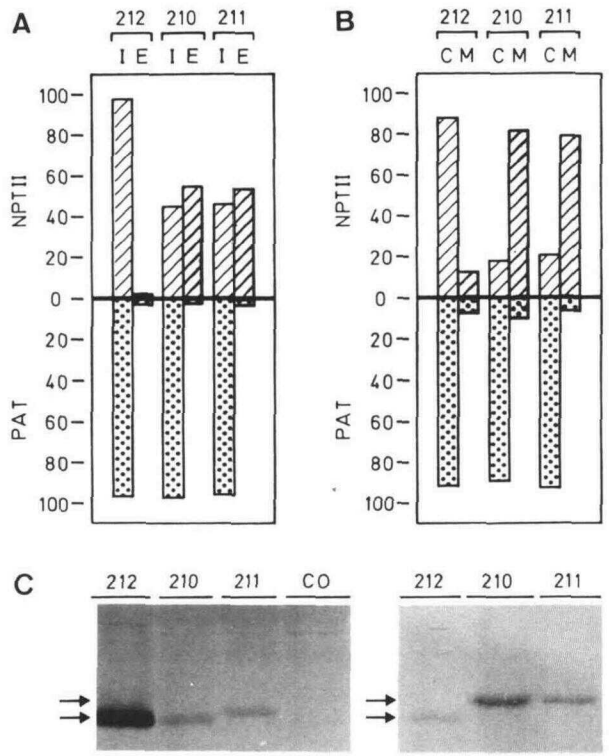

Figure 2. Transient Gene Expression Levels Directed by pDE210, 211, and 212 in Electroporated Tobacco Leaf Protoplasts.

(A) NPTII and PAT activities measured in the intra- (I) and extracellular (E) fraction after protoplast harvest. Values are the average of three experiments and are shown as a percentage of the total measured activity in each cell suspension. The numbers above the lanes refer to the different constructions.

(B) Distribution of the intracellular NPTII and PAT activities in soluble (C) and cell membrane (M) fractions after gentle osmotic shock. Values are shown as a percentage of the total measured intracellular activity.

(C) Protein gel blot analysis (left) and in vitro translation products (right). Total cell extracts and labeled in vitro translation products were simultaneously separated by SDS-12.5% PAGE and blotted on nitrocellulose membrane. The NPTII products were either visualized with a polyclonal NPTII antiserum (left) or by autoradiography of the nitrocellulose membrane (right). The sizes of the NPTII and its precursors are indicated by arrows. CO refers to the negative control and consists of an extract prepared from untransformed protoplasts.

cellular transport process rather than from leakage.

Partial lysis of protoplasts was used to separate the cytoplasm from a membrane-enriched fraction that also contained intact microsomes (Figure 2B). In cells secreting NPTII (pDE210 and 211), the majority of the intracellular NPTII activity was detected in the membrane-enriched fraction, in contrast to the control (pDE212). On the other hand, PAT was found predominantly in the cytoplasm in all three cases. To determine whether NPTII was present in a processed form or as a precursor, the mobilities of the NPTII derivatives in total cell extracts were compared with labeled in vitro translation products of the chimeric precursors after separation on denaturing polyacrylamide gels. As shown in Figure 2C, the apparent molecular weight of the intracellular NPTII proteins corresponds to processed forms of the precursors. A slightly different mobility was observed for the NPTII derivative obtained with pDE211 containing the cecropin B prepro-sequence. Most likely, only the pre-signal is processed in tobacco, perhaps because plants lack a dipeptidyl peptidase activity, which is suggested to be responsible for processing of the pro-fragment in *Hyalophora* (Lidholm et al., 1987). The association with the membrane fraction and the predominance of a processed form indicate that NPTII was translocated to the lumen of the ER. We concluded that both the plant and the insect signal peptide can target NPTII to the ER and that this transport event results in the secretion of the protein.

Addition of Signal Sequences Correlates with Reduced Expression Levels

Comparison of the total intra- and extracellular NPTII activities, which were measured in the different cell suspensions, revealed remarkable differences in the expression levels directed by the chimeric genes. The secretory constructions yielded fivefold to 10-fold lower NPTII activities relative to the control construction, as seen in Figure 3A, top. In contrast, the internal reference gene (*bar*) present on each plasmid yielded comparable expression levels in all three cases (Figure 3A, bottom). The constant level of expression of the chimeric *bar* gene allowed us to exclude that the reduction of NPTII activity was caused by differences in the efficiency of electric field-mediated DNA uptake (Denecke et al., 1989). Furthermore, the observed reduction in enzymatic activity was unlikely to be caused by an intracellular accumulation of inactive precursor molecules because no chimeric precursors were detected in the cellular extracts (Figure 2C). To determine whether the chimeric genes show transcriptional differences, the transient steady-state levels of *neo* and *bar* transcripts were quantified after electroporation with the three plasmids (Figure 3B). As expected from measurements of the PAT activities, only minor differences in *bar* mRNA levels were observed. Interestingly, the *neo* transcript of the control gene (212) was present at the same levels as the larger chimeras (210, 211). Quantification by slot blot analysis revealed differences of less than 20% among the three constructions. We concluded that the observed discrepancies between *neo* mRNA levels and NPTII protein levels are the result of translational or post-translational events. A decrease in the efficiency of translation or lower protein

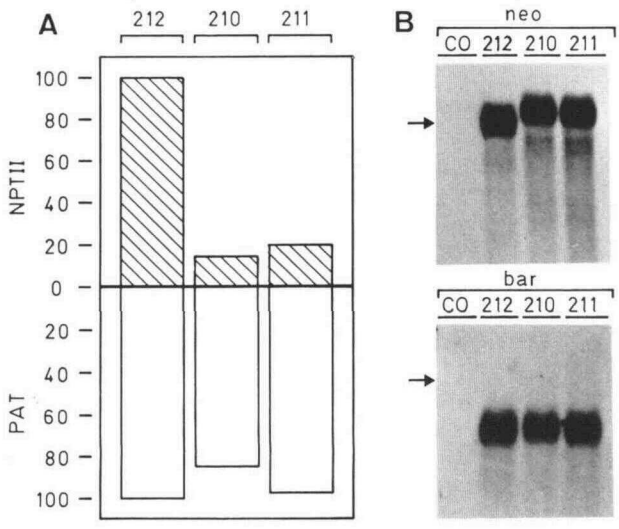

Figure 3. Activity in Total Cell Suspensions and Steady-State mRNA Levels.

(A) Total NPTII and PAT activities (intra- and extracellular) measured in cell suspensions shown relative to those measured for construction 212.

(B) RNA gel blot analysis. Equal amounts of total extracted RNA from protoplasts were loaded, and *bar* and *neo* mRNAs were visualized by hybridization with complementary ^{32}P-labeled riboprobes. The arrow indicates the mobility of the 1.34-kb standard.

stability are two plausible explanations for the observed reduction in expression levels.

Reduction of the Expression Levels Is not Caused by a Saturation of the Transport Route

We wanted to investigate whether the observed reduction in expression level was biologically meaningful or simply related to the transient expression system. A problem inherent in this method is that the majority of the synthesized gene product in a population of electroporated protoplasts is present in a minority of cells containing large amounts of the introduced extrachromosomal genes (Denecke et al., 1989). Overproduction of a protein that is introduced in the secretory pathway could possibly lead to saturation of a limiting step in this pathway. Moreover, the stress imposed on the cells by the electric discharge might partially disrupt membrane structures of the exocytic pathway. To evaluate whether our results obtained with transient gene expression are biased by such potential problems, secretion of NPTII was also studied in protoplast suspensions prepared from transgenic plants that express the described chimeric genes. Large differences in transgene expression in independent transformants (Willmitzer, 1988) would allow us to study whether the efficiency of secretion is influenced by the level of gene expression.

After cocultivation of protoplast suspensions with *Agrobacterium* strains carrying the three pDB plasmids (Figure 1B), transformed calli were selected on medium containing either kanamycin (Km) or phosphinothricin (PPT). The chimeric *bar* gene was present in all constructions as an independent selectable marker. The apparent transformation frequencies given as the ratio between the number of resistant calli and the number of protoplasts initially used for cocultivation are represented in Table 1. Because the chimeric *bar* gene was identical in the three constructions, experimental differences in the efficiency of *Agrobacterium* infection and/or T-DNA transfer are reflected by the frequency of PPT-resistant calli. The ratio between the transformation efficiency on Km-containing and on PPT-containing medium, respectively, was approximately fivefold lower for constructions 210 and 211 compared with 212 (Table 1). This reduction in transformation efficiency correlates well with the reduction in NPTII activity seen during transient gene expression. There may be, however, additional factors contributing to the lower frequency of transformation, such as the inability of lumenal or excreted NPTII to protect the plant ribosomes from the antibiotic. To avoid biased conclusions by using a population of transgenic plants selected for high NPTII levels, only calli selected for PPT resistance were used for plant regeneration.

A set of transgenic plants covering a 10-fold range of NPTII expression levels was analyzed further. In protoplast suspensions prepared from a representative transgenic plant for each construction, intra- and extracellular NPTII activities were measured in a function of time. The results are shown in Figure 4A. In the case of constructions 210 and 211, linear NPTII accumulation was observed in the culture medium during the first 30 hr after protoplast preparation. The cessation in accumulation correlates with the time that cell wall synthesis becomes significant and, thus, can interfere with the release of proteins into the culture medium. No NPTII secretion was observed in suspensions of protoplasts carrying the control gene without

Table 1. Frequencies of Transformants

Plasmid	PPT (%)	Km (%)	Km/PPT
pDB212	2.6	0.6	0.23
pDB210	1.4	0.08	0.05
pDB211	0.9	0.03	0.03

Protoplast suspensions were cocultivated with the *Agrobacterium* strains carrying the respective plasmids. The mixtures were split in two parts, and calli were selected on medium containing either PPT (2 mg/L) or Km (50 mg/L). The percentage of transformed calli was calculated relative to the number of protoplasts used in the cocultivation.

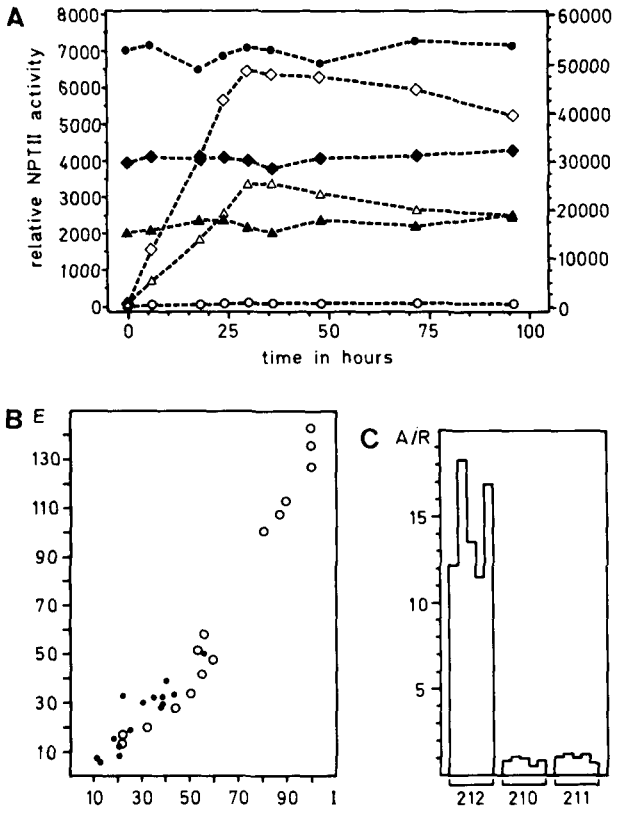

Figure 4. Secretion of NPTII from Protoplasts of Transgenic Plants.

(A) Kinetics of the measured intra- and extracellular NPTII activities at different time points after incubation of protoplasts prepared from a representative plant transformed with either pDB210 (triangles), 211 (diamonds), or 212 (circles). The open and closed symbols represent extra- and intracellular NPTII levels, respectively. Activities for 210 and 211 are shown on the left ordinate and for 212 on the right, and are given in counts per minute per milliliter of cell suspension.

(B) Correlation between the measured NPTII activity in protoplast extracts (I) and in the culture medium (E) of incubated protoplasts prepared from independent plants transformed with pDB210 and 211. NPTII activity was measured in cultivated protoplast suspensions from five independent transgenic plants of pDB210 (●) and 211 (○). Values are from three experiments and are shown as a percentage of the intracellular activity of the cell suspension with the highest expression level.

(C) Ratio between the total relative NPTII activity (A) measured in protoplast extracts and culture medium and the relative *neo* mRNA levels (R) of five independent transformants for each construct.

a signal sequence (212). This reinforces the conclusion that little of the extracellular activity detected is due to lysis of cells. In all cases, intracellular NPTII levels remained approximately constant in a function of time (Figure 4A).

To analyze the relationship between the level of *neo* gene expression and the efficiency of NPTII secretion, protoplast suspensions prepared from independent transformants were analyzed 20 hr after protoplast isolation. This was still within the period of linear NPTII accumulation in the culture medium (Figure 4A). In a control experiment, no significant NPTII degradation in the culture medium was observed within 20 hr (data not shown). Hence, extracellular NPTII levels measured at this time point reflect mainly de novo synthesis and are directly proportional to the average rate of NPTII secretion during this time interval. As shown in Figure 4B, a positive correlation was found between the measured extra- and intracellular NPTII levels for both constructions. A least-squares fit analysis for a linear relationship yielded a correlation coefficient of 0.94 for 210 and 0.98 for 211. This indicates that the rate of NPTII secretion is proportional to the intracellular steady-state NPTII levels. No saturation of the transport route occurs within the range of this experiment because the curve does not reach a plateau. Moreover, the efficiency of NPTII secretion observed in transgenic cells was comparable with those obtained with transiently transformed cells.

Furthermore, we wanted to determine whether the secretory genes show a similar reduction of the expression levels as observed in the transient expression system. To this end, we compared the steady-state mRNA levels and the total intra- and extracellular NPTII levels in cell suspensions prepared from five independent transformants for each construction after 20 hr incubation. Cells transformed with constructions 210 or 211 showed a 10-fold lower NPTII level than cells transformed with the control gene when normalized with respect to the *neo* mRNA levels (Figure 4C). This agrees with the values obtained with the transient expression system (Figure 3).

The results demonstrate that neither the transient expression system nor the stably transformed cells are significantly biased by saturation effects.

The Rate of Secretion Depends on the Passenger Molecule

To evaluate whether the results observed with NPTII as a model enzyme can be reproduced with other proteins as markers, the transient expression system was used to analyze chimeric gene constructs with *bar* and *gus*. Simultaneously, we wanted to investigate whether the reduced levels of expression obtained with chimeric *neo* genes carrying a signal sequence could be related to differences in the efficiency of translation initiation. Sequence configurations flanking the translation initiation codon in the different pDE plasmids might influence translation. To this end, a second series was made of chimeric genes that contain the *neo*, *bar*, and *gus* open reading frames fused to the cauliflower mosaic virus (CaMV) 35S

promoter. In the case of the secretory genes, the translation initiation codon was replaced by the PR1b signal sequence, as shown in Figure 5A. These constructions were used for electroporation of tobacco leaf protoplasts, and PAT, NPTII, or β-glucuronidase (GUS) activities were measured in cellular extracts and in the culture medium at different time points after electroporation. Since *N*-linked glycosylation of GUS within the exocytic pathway is known to interfere with its enzymatic activity (Iturriaga et al., 1989), tunicamycin was added to the culture medium to inhibit glycosylation.

A suitable criterion for evaluating the efficiency of secretion is provided by the ratio between extra- and intracellular enzymatic activity at discrete time points after electroporation. We designated this parameter the "secretion index" (SI). As shown in Figure 5B, SI values for each passenger protein increased almost linearly with time after electroporation. In the case of the control genes, no secretion above that due to cell death was observed (see Figure 5B legend). At discrete time points, significant differences were observed in SI values for the three proteins. SI values show an inverse correlation with the molecular mass of the passenger proteins: the highest SI values were found with PAT (21 kD), the lowest with GUS (68 kD).

We also compared total enzyme levels directed by each secretory construct with those directed by the unmodified genes. As observed in the first set of experiments (Figures 3 and 4C), a clear reduction of the expression levels was observed for the three chimeric genes that contain the signal sequence. This reduction correlated positively with the molecular weight of the gene products and was approximately fivefold for PAT, 10-fold for NPTII, and 30-fold for GUS (data not shown).

These experiments show that the three marker proteins can be secreted in tobacco protoplasts upon signal sequence-mediated translocation. However, the rate of secretion is strongly dependent on the gene product. Moreover, reduced expression levels were found to be correlated with the presence of a signal sequence in the case of all three chimeric genes.

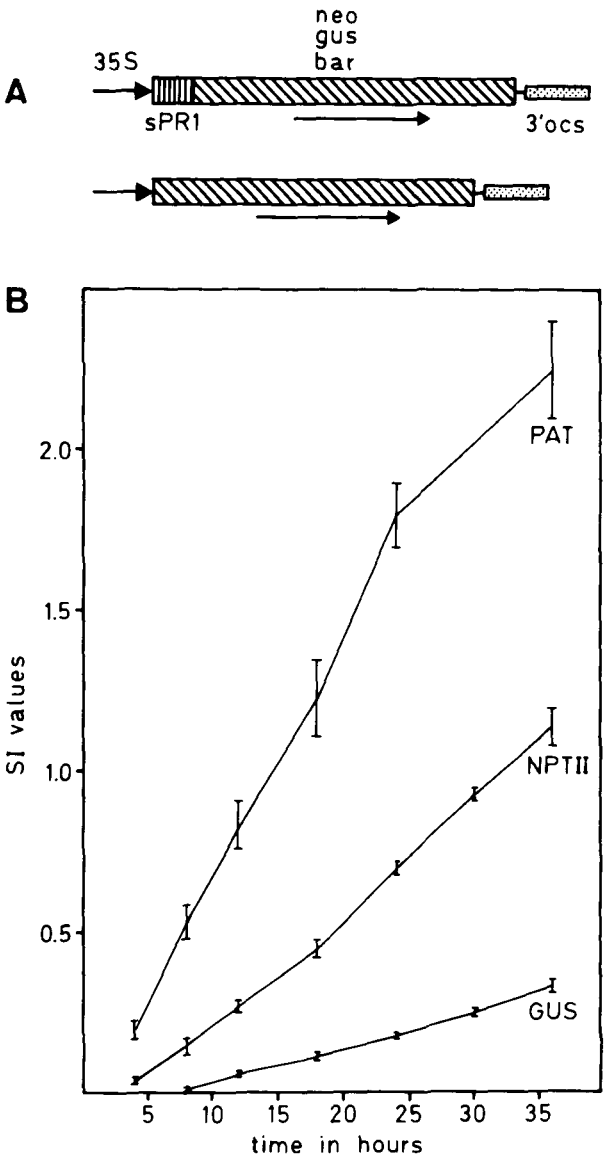

Figure 5. Efficiency of Secretion for NPTII, PAT, and GUS.

(A) Schematic representation of the chimeric constructions. The *neo*, *bar*, and *gus* coding regions and hybrid genes fused in frame to the sPR1 signal sequence were placed under control of the CaMV 35S promoter and are followed by the 3′ ocs fragment (top). The control genes are identical except for the lack of the sequence coding for the sPR1 signal (bottom).

(B) Secretion index of transiently expressed *bar*, *neo*, and *gus* gene products in a function of time. Highest and lowest values observed in three independent experiments are indicated with bars. The secretion index for plasmids carrying the respective coding sequences directly fused to the CaMV 35S promoter was varying for *neo* from 0.01 to 0.03, for *bar* from 0.02 to 0.03, and for *gus* from 0.02 to 0.04 during the analyzed time period.

DISCUSSION

We established and evaluated an in vivo model system to study protein transport in plant cells. It is based on the transient synthesis and translocation of nonspecific passenger molecules to the lumen of the ER of tobacco leaf protoplasts. We demonstrate that the procaryotic cytosolic enzymes NPTII, PAT, and GUS can be secreted if they are first targeted to the lumen of the ER, irrespective of the signal peptide used. This supports the view that secretion occurs via a default pathway, although it cannot be excluded that the three proteins coincidently contain signals

that are specifically recognized by the sorting system of plant cells.

Pronounced differences in the rate of secretion were observed for the three transport markers. Lower rates of secretion correlated with higher molecular weight of the passenger proteins. At present, limiting steps and discrete factors influencing the efficiency of protein secretion in eucaryotes are poorly understood. The importance of protein conformation in the ER is stressed by the observation that mutants of secretory proteins blocked at different stages of the folding pathway fail to be transported (Gething, McCammon, and Sambrook, 1986). Proteins similar to the highly conserved glucose-regulated protein GRP78, which may assist the folding of proteins and prevent transport of malfolded proteins in animals (Munro and Pelham, 1986; Kassenbrock et al., 1988; Pelham, 1988), are expected to be present in plants as well. Heterologous proteins might not meet the structural requirements for efficient transport in the plant host cell, in spite of their correct folding. Different affinities between those proteins and GRP78-like proteins could explain variations in their rate of secretion. On the other hand, it is possible that nonspecific interactions with components of the exocytic pathway largely influence mobility and, thus, transport of proteins by bulk migration. In this case, composition and structure of the heterologous proteins would influence their "stickiness" and, hence, the efficiency of secretion. Therefore, our experiments do not exclude the possibility that active sorting signals might be required to assure the secretion of some endogenous secretory proteins. It is possible that rapidly secreted proteins or proteins whose structure imposes restrictions on their mobility depend on such signals.

A lower protein/mRNA ratio was observed for chimeric genes containing a signal sequence compared with the control genes that lack such a sequence. Discrepancies increased with increasing molecular weight of the gene products. The data are consistent with those of an earlier report in which deletion of the signal sequence of wheat α-amylase, synthesized in yeast, led to a 10-fold increase of the protein level (Rothstein et al., 1984). It is tempting to assume that certain steps inherent in secretory protein synthesis, such as the coupling of translation elongation on membrane-bound polysomes with protein translocation across the ER membrane, limit the rate at which these proteins can be synthesized. However, rapid degradation of the proteins in the ER could also provide a possible explanation (Lippincott-Schwartz et al., 1988).

In conclusion, our work demonstrated the suitability of transient expression assays as a rapid in vivo system to study protein transport in plant cells. Passenger molecules that are transported via a default pathway can be useful as substrates for the functional analysis of discrete targeting signals present on other proteins. Moreover, enzymes that can be assayed enzymatically and histochemically may provide interesting tools to follow secretion of proteins in intact plant tissues.

METHODS

Plasmid Constructs

The signal sequences were obtained by cloning overlapping synthetic oligonucleotides in the polylinker of a pLK vector (Botterman and Zabeau, 1987). The *neo* gene is obtained from pKM109/9 (Reiss, Sprengel, and Schaller, 1984) and ligated with the filled BamHI site to the trimmed KpnI site at the 3′ end of the sPR1 signal sequence (210) or to the SmaI site at the 3′ end of the sCEC prepro-sequence (211). These hybrid genes contain 10 linker-derived codons between the fusion point and the fifth codon of *neo*. The chimeric TR2′ constructs are obtained by ligating the filled NcoI or BamHI site at the translation initiation codon of the signal sequences to the filled ClaI site of the TR2′ promoter fragment in pGSH160 (Deblaere et al., 1987). In 212, the *neo* coding sequence from pKM109/9 is ligated at the BamHI site of the TR2′ promoter of pGSH160. The *bar* gene is obtained as a BamHI cassette from pGSFR1 (De Block et al., 1987) and cloned under control of the TR1′ promoter. The chimeric TR promoter constructs were cloned in the polylinker of pUC18 for electroporation and in pGSC1700 (Cornelissen and Vandewiele, 1989) for transformation. Restriction fragments containing the 3′ end formation and polyadenylation signals of the octopine synthase (3′ ocs) and gene 7 (3′g7) from the T-DNA were placed 3′ end to the *neo* and *bar* coding sequences, respectively (De Block et al., 1987). The pGEM plasmids contain the chimeric *neo* coding regions in the polylinker of pGem1 (Promega Biotec) in sense orientation downstream of the SP6 promoter.

The *gus*, *bar*, and *neo* coding sequences were fused at the second codon to the trimmed KpnI site at the 3′ end of the sPR1 signal sequence. These hybrid coding sequences in parallel with the unmodified coding regions themselves were ligated at the NcoI site of a CaMV 35S promoter fragment (Denecke et al., 1989), provided with the 3′ ocs fragment, and cloned in the polylinker of pUC19. *bar* was obtained from pGSFR2, which differs from pGSFR1 by the presence of an NcoI site overlapping with the translation initiation codon. *gus* was obtained from pRAJ275 (Jefferson, Kavanagh, and Bevan, 1987).

All DNA manipulations were done according to established procedures. The *Escherichia coli* strain used for plasmid growth was MC1061 (Casadaban and Cohen, 1980).

Protoplast Preparation and Electroporation

Protoplasts were prepared from leaves derived from in vitro grown shoot cultures of *Nicotiana tabacum* cv Petite Havana SR1 (Maliga, Breznowitz, and Marton, 1973) and electroporated with the pDE plasmids as described (Denecke et al., 1989). As a control, protoplasts were electroporated with plasmids containing only coding sequences without promoter. Protoplasts of transgenic plants were prepared from leaves and incubated in culture medium as described for electroporated cells.

Cell Culture and Preparation of Samples

Different batches of electroporated protoplasts were pooled, washed, and cultivated at high concentrations (5×10^5 cells/mL culture medium) at 24°C in the dark. Tunicamycin (20 μg/mL) was added after the washing step to inhibit glycosylation of GUS. Samples of 2-mL cell suspension were taken at different time points after electroporation. After floating the protoplasts, the culture medium was separated, and secreted proteins were concentrated by ammonium sulfate precipitation (60%) after addition of protein extract from nontransformed plants as carrier to a final concentration of 100 μg/mL. Protoplasts were washed once in culture medium and concentrated to 10^7 protoplasts/mL prior to extraction. Crude cell fractionation into cytoplasm- and membrane-enriched fractions was carried out by gentle osmotic shock. Two volumes of extraction buffer (Denecke et al., 1989) were added to the concentrated protoplast suspension and mixed gently by pipetting several times. Cell membranes containing the microsome fraction were pelleted 5 min at 16,000*g* at 4°C. The pellet was resuspended in extraction buffer, sonicated, and treated similarly as described earlier (Denecke et al., 1989) to obtain complete extraction of soluble proteins trapped in closed cell compartments.

In Vitro Transcription and Translation

DNA from the appropriate plasmids was isolated by CsCl centrifugation and was transcribed in vitro using the SP6 RNA polymerase and the cap analog 7mGpppG following established procedures described in the Promega Biotec manual. Cell-free translation of in vitro SP6 transcripts was performed using a wheat germ extract and ^{35}S-labeled methionine according to Morch et al. (1986).

Enzymatic Assays

NPTII and GUS enzymatic activities were measured as described (Denecke et al., 1989). All measured enzymatic activities were at least 10-fold higher than the background levels present in the negative controls.

PAT activities were determined by thin-layer chromatography as described by De Block et al. (1987) with the following modifications: cell extracts were partially purified by differential ammonium sulfate precipitation (30% to 60%), and secreted proteins were precipitated as described. Protein pellets were resuspended in 200 μL of 100 mM Tris-HCl, pH 7.5, and kept on ice. Reaction mixtures [100 mM Tris-HCl, pH 7.5; 1:10 dilution of ^{14}C acetylCoA, 58.1 mCi/mmol, (Du Pont-New England Nuclear); 10-fold molar excess of cold acetylCoA] containing appropriate dilutions of the protein samples were preincubated at 37°C for 2 min. Reactions were started by the addition of PPT to a final concentration of 1 mM, and samples of 5 μL were taken after 2 min, 5 min, 10 min, and 20 min by spotting directly on the silica gel plate. After separation, reaction product (acetylated PPT) and acetylCoA were quantified by scintillation counting. Dilutions were such that less than 10% of the acetylCoA reacted with PPT after 20 min for all samples, and the slope of the curve was taken as a measure for the relative activity.

RNA Gel Blot Analysis and RNA Quantification

Total RNA was extracted from protoplasts after 20 hr cultivation as described (Denecke et al., 1989). 2.5 μg of total RNA was used for visualization by RNA gel blottings. For quantification by slot blot analysis, 1 μg of each RNA preparation was blotted together with a dilution series of cold SP6-derived sense RNA ranging from 1 pg up to 200 pg. 1 μg of total RNA prepared from untransformed protoplasts was added to all dilutions to equalize the final amount of RNA blotted. Hybridizations with riboprobes were done according to Amersham protocols, and hybridization signals were quantified by liquid scintillation counting. Relative levels of mRNA were calculated after normalization using the dilution series.

Plant Transformation

The pDB plasmids were mobilized to *Agrobacterium tumefaciens* C58C1Rif^R (pGV2260) (Deblaere et al., 1987). Transformed plants were obtained by cocultivation of protoplasts from *N. tabacum* SR1 with the respective *Agrobacterium* strains. Transformants were selected either on phosphinothricin (2 mg/L)- or kanamycin (50 mg/L)-containing medium according to De Block et al. (1987). For each construct, randomly chosen plants were analyzed for NPTII expression, and a subpopulation covering a 10-fold range of expression levels was kept.

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