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Endoplasmic reticulum targeting of active modified β-glucuronidase (GUS) in transgenic tobacco plants

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We have investigated targeting to the endoplasmic reticulum (ER) of wild-type GUS and a modified form (GUS S358) by making an N-terminal fusion of the β-glucuronidase (GUS) enzyme with the wheat α-amylase signal peptide. *In vitro* studies demonstrated that the modified GUS (S358) lacked the glycosylation site present within the wild-type enzyme. Analysis of transgenic tobacco plants revealed that the modified GUS enzyme retained activity upon passage to the ER. When further experiments were carried out to determine the cellular location of the modified GUS enzyme, it was found that (contrary to expectation) the majority of GUS activity was retained within the cell and was not secreted to the cell surface via the default pathway. The data indicated that the modified GUS enzyme is an unsuitable reporter enzyme for studying protein secretion.

Keywords: GUS; endoplasmic reticulum; protein targeting; signal peptide

Introduction

The *E. coli* β-glucuronidase (GUS) enzyme has proven to be a highly versatile and extremely useful reporter system for the study of gene expression in transgenic plants (Jefferson *et al.*, 1987). The ability to withstand both N-terminal and C-terminal fusions with foreign proteins and still retain enzyme activity has also allowed GUS to be used as a reporter enzyme in targeting studies. To date, it has been demonstrated that, given the correct protein targeting information, GUS can be imported into the chloroplast, mitochondrion and the nucleus of plant cells (Kavanagh *et al.*, 1988; Schmitz and Lonsdale, 1989; Restrepo *et al.*, 1990). In all these cases, GUS retains enzyme activity. The one exception to this rule is when GUS has been used in endoplasmic reticulum (ER) targeting studies.

It has been demonstrated that β-glucuronidase (GUS) when fused to the endoplasmic reticulum (ER) targeting signal from the potato storage protein patatin is co-translationally glycosylated during translocation to the ER (Iturriaga *et al.*, 1989). This single N-linked glycosylation event at asparagine residue 358 results in the loss of GUS enzyme activity (Iturriaga *et al.*, 1989). Subsequent studies using protoplast transient expression assays demonstrated that following treatment with the N-glycosylation inhibitor tunicamycin, GUS activity was detected predominantly in the medium, thus apparently demonstrating secretion of active enzyme (Denecke *et al.*, 1990). It was later found that substitution of the asparagine at residue 358 for serine removes the glycosylation site and results in the retention of 64% of GUS enzyme activity (Farrell and Beachy, 1990). It has been suggested that this modified form of the enzyme (GUS S358) could be used as a reporter in secretion and vacuolar targeting studies. The use of *in situ* histochemical staining techniques to monitor GUS secretion from tissues would potentially also allow the development of facile techniques for recognising germinating *Arabidopsis* seedlings mutated in secretory pathways.

We have investigated the targeting to the ER of wild-type GUS and the modified form (GUS S358) by making an N-terminal fusion of the GUS enzyme with the wheat α-amylase signal peptide. This particular signal sequence has been shown to direct efficient secretion in yeast cells (Rothstein *et al.*, 1984). It was found that the modified GUS enzyme retained activity upon passage to the ER. However, contrary to expectation, the majority of detectable GUS activity remained within the cell and was not secreted via the default pathway.

Materials and methods

Plasmid construction and site-directed mutagenesis

The wheat α-amylase signal sequence was produced by synthesizing two complementary oligonucleotides which

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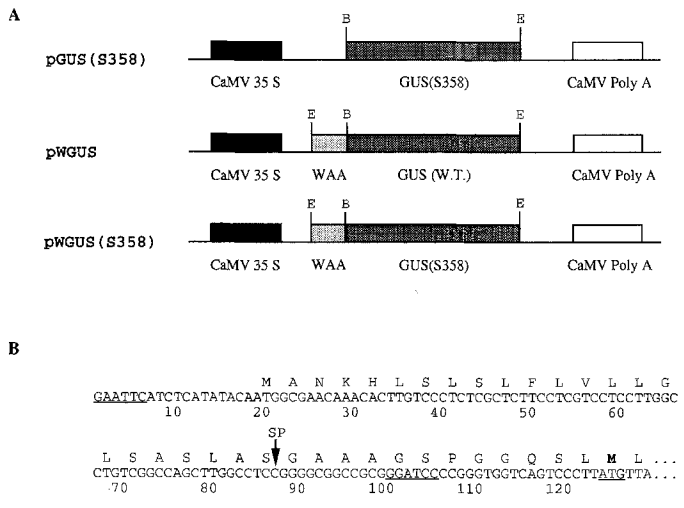

Fig. 1. Schematic representation of promoter-reporter gene constructs. (A) Arrangement of the various *gus* fusions within the T-DNA of the plant transformation vector pBIN19. CaMV 35S = cauliflower mosaic virus 35S promoter with duplicated enhancer element, CaMV polyA = cauliflower mosaic virus polyadenylation signal, WAA = wheat α-amylase signal sequence, GUS (W.T.) = wild-type *gus*, GUS (S358) = modified GUS gene containing an asparagine to serine amino-acid substitution at residue 358, B = *Bam* HI, E = *Eco* RI. (B) Sequence of wheat α-amylase signal peptide-GUS fusion. Underlined is the GUS initiator methionine and restriction enzyme sites used in the construction. SP = signal peptidase cleavage site.

encoded the entire signal sequence and contained *Eco* RI and *Bam* HI complimentary overhangs to facilitate easy cloning. The oligonucleotides were cloned into *Eco* RI-*Bam* HI cut pBluescript SK− and one of the resultant clones, pSK WAA, was sequenced and found to be correctly integrated. The 2.5-kb *Bam* HI-*Eco* RI fragment from pBI 101.1 (Jefferson *et al.*, 1987) containing the GUS-coding region and nos polyA site was isolated and cloned into *Eco* RI-*Bam* HI cut pJIT 30 (Guerineau *et al.*, 1992) to produce pGUS. A translational fusion was then made between the signal sequence and *gus* by ligating the 128 bp *Hin* dIII-*Bam* HI from pSK WAA into pGUS (see Fig. 1B).

The 2.5 kb *Eco* RI fragment from pGUS containing the wheat α-amylase GUS fusion/nos polyA was cloned into *Eco* RI-cut pBluescript pSK− to produce pSK WGUS. This allowed the production of single-stranded DNA template. A mutagenic oligo, 5′-GCTGCAGCTGGCTT-TAGCCTCTCTTTAGGC-3′, was synthesized and site-directed mutagenesis was carried out using the *dut-*, *ung-* system essentially as described by Kunkel (1985). The clone pSKWGUS(S358), which contained the desired mutation, was identified by DNA sequence analysis. pSKWGUS(S358) was then digested with *Bam* HI to excise the 150 bp wheat α-amylase signal *Bam* HI fragment and religated to produce pSK GUS(S358). pSK GUS(S358) therefore contained a mutant *gus* which lacked a signal sequence.

The 2.5 kb *Eco* RI fragments from pSK WGUS and pSK WGUS(S358) were cloned into *Eco* RI cut pJIT 60 (Guerineau *et al.*, 1992) to place *gus* expression under the control of a CaMV 35S promoter with a duplicated enhancer element. An almost identical construct was produced by cloning the 2.4 kb *Bam* HI-*Eco* RI fragment from pSK GUS(S358) into *Bam* HI-*Eco* RI cut pJIT 60. The resultant vectors were digested with *Kpn* I and *Xho* I and the entire expression cassette consisting of CaMV promoter-GUS gene-nos polyA was then isolated and cloned into *Kpn* I-*Sal* I cut pBIN 19 (Bevan, 1984). This resulted in the production of three binary vectors pWGUS, pWGUS(S358) and pGUS(S358) (Fig. 1A).

In vitro *transcription/translation*

An Amersham *in vitro* transcription kit was used according to the manufacturer's instructions to produce RNA *in vitro* from pSK WGUS, pSK WGUS(S358) and pSK(S358) linear templates using T7 RNA polymerase. *In vitro* translation reactions using Promega wheat germ lysate were then performed using the *in-vitro*-produced RNA, according to the manufacturer's instructions. Canine pancreatic microsomal vesicles were obtained from Promega and added to *in vitro* translation reactions, according to the manufacturer's instructions.

Plant transformation

The binary transformation vectors were mobilized into *Agrobacterium tumefaciens* strain GV2260 by triparental mating with the aid of the pRK2013 helper plasmid (Deblaere *et al.*, 1985). Tobacco leaf disc transformation and regeneration were performed as described in Draper *et al.* (1988). Briefly, young leaves from three- to six-week-old tobacco plants (SR1) raised in greenhouse conditions were sterilized in 10% commercial bleach. Leaf explants were inoculated with an overnight culture of bacteria diluted to 1×10^8 cell/ml and then co-cultivated on MSD4 × 2 (MS medium, 0.1 mg l^{-1} naphthaleneacetic acid (NAA), 1.0 mg l^{-1} 6-benzylaminopurine (BAP) and 3% sucrose) medium for two days. Leaf disc explants were then transferred to selection medium consisting of MSD4 × 2 containing 100 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin. Regenerated shoots were rooted on MS0 (MS medium and 3% sucrose) medium with 100 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin.

Protein extraction and GUS activity assays

Fluorometric GUS assays were based on methods described by Jefferson (1987) with the following modifications. Leaf tissue was homogenized in GUS extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM EDTA, 0.1%

Triton X-100, 0.1% Sarkosyl and 10 mM β-mercaptoethanol) and the soluble extractable protein isolated. Protein concentration was measured by the Bradford assay (Bradford, 1976) and GUS activity was determined by measuring the rate of increase in fluorescence produced by the conversion of 4-methylumbelliferyl-β-D-glucuronide (MUG) to 4-methylumbelliferone (MU) (Jefferson, 1987). The assay reaction was sampled at 0, 5, 15, 35 and 75 min and the fluorescent MU product was assayed using a Perkin Elmer LS-50 luminescence spectrometer, with an excitation wavelength of 365 nm and an emission wavelength of 455 nm.

Extraction and analysis of apoplastic proteins

Apoplastic proteins were extracted using well-established techniques by cutting tobacco leaves into approximately 3 cm × 3 cm pieces and vacuum infiltrating with ice-cold 50 mM $CaCl_2$. The apoplastic protein fraction was isolated by placing the infiltrated leaf pieces into the barrel of a 20 ml syringe and spinning at 2000 **g** at 4° C. Proteins contained within the remaining leaf tissue minus the apoplastic fraction were extracted for GUS assays as described above.

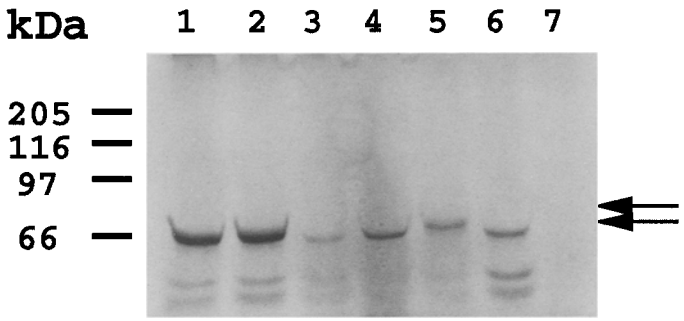

Fig. 2. *In vitro* translation and glycosylation of the various GUS fusions. Autoradiograph of SDS gel containing *in vitro* translation products produced from *in vitro*-produced RNA complementary to the three GUS fusion proteins. The wheat germ extract was supplemented with canine microsomal membranes where indicated. Lane 1, GUS (S358); lane 2, WGUS; lane 3, WGUS(S358); lane 4, GUS (S358) + microsomes; lane 5, WGUS + microsomes; lane 6, WGUS(S358) + microsomes. Lane 7 is a control lane consisting of an *in vitro* translation mixture without mRNA. Arrows indicate the non-glycosylated (lower band) and glycosylated (upper band) GUS protein.

Results

To investigate ER targeting and protein secretion, three separate GUS constructs were produced (Fig. 1A). The first of these, GUS (S358), is identical to wild-type GUS with the exception of an asparagine to serine substitution at residue 358 which results in the removal of the N-glycosylation site. This construct has no ER targeting signal and is used as a control in these experiments. The second construct, WGUS, consists of a wild-type *gus* fused to the N-terminal signal peptide from wheat α-amylase (Fig. 1B). The third construct, WGUS(S358), is identical to WGUS in respect of the signal sequence, but utilizes the *gus* S358 gene containing the asparagine to serine substitution and therefore lacks a glycosylation site.

Prevention of N-glycosylation in vitro *by the asparagine to serine substitution at GUS residue 358*

To demonstrate that the removal of the N-glycosylation site at residue S358 did actually prevent glycosylation taking place, a series of *in vitro* translation experiments were carried out. RNA was produced *in vitro* from each of the three GUS gene constructs GUS (S358), WGUS and WGUS(S358). This RNA was then translated in wheat germ extracts in the presence or absence of canine pancreatic microsomal membranes. Figure 2 shows the result of such an experiment. It can be seen that in the absence of microsomal membranes, all three GUS constructs produce *in vitro* translation products of approximately the same molecular weight (Fig. 2, lanes 1–3). However, in the presence of microsomal membranes, WGUS shows a marked shift in size relative to GUS (S358), which lacks a signal sequence and would therefore not be sequestered into microsomal vesicles (compare lanes 4 and 5). This increase in apparent molecular weight would be consistent with the expected ER specific co-translational N-glycosylation. In contrast, by comparing lanes 5 and 6 it is clear that WGUS(S358) does not show a shift in size in the presence of microsomal membranes, indicating that, as expected, the asparagine to serine substitution has resulted in the removal of the glycosylation site. It can be concluded that a similar situation should prevail *in planta*, which implies that WGUS(S358) should not be glycosylated on passage to the ER and therefore should retain enzyme activity.

Endoplasmic reticulum-targeted GUS (S358) is active

A range of independent transgenic tobacco plants were produced for each of the GUS constructs. Plants rooting *in vitro* in the presence of kanamycin were established in compost and grown to the eight-leaf stage in contained growthrooms. Ten transformants for each construct exhibiting vigorous growth were chosen for further work and characterized initially by measuring GUS activity in total protein extracts (Fig. 3). In these plants expression of the three GUS genes was from the strong, constitutively expressed CaMV 35S promoter (Fig. 1A). As expected from previous studies (e.g. Ozcan *et al.*, 1993) which have utilised the CaMV35S promoter, the GUS (S358) plants displayed a range of constitutive GUS activities with peak levels greater than 60 pmoles 4MU h^{-1} μg^{-1} of protein

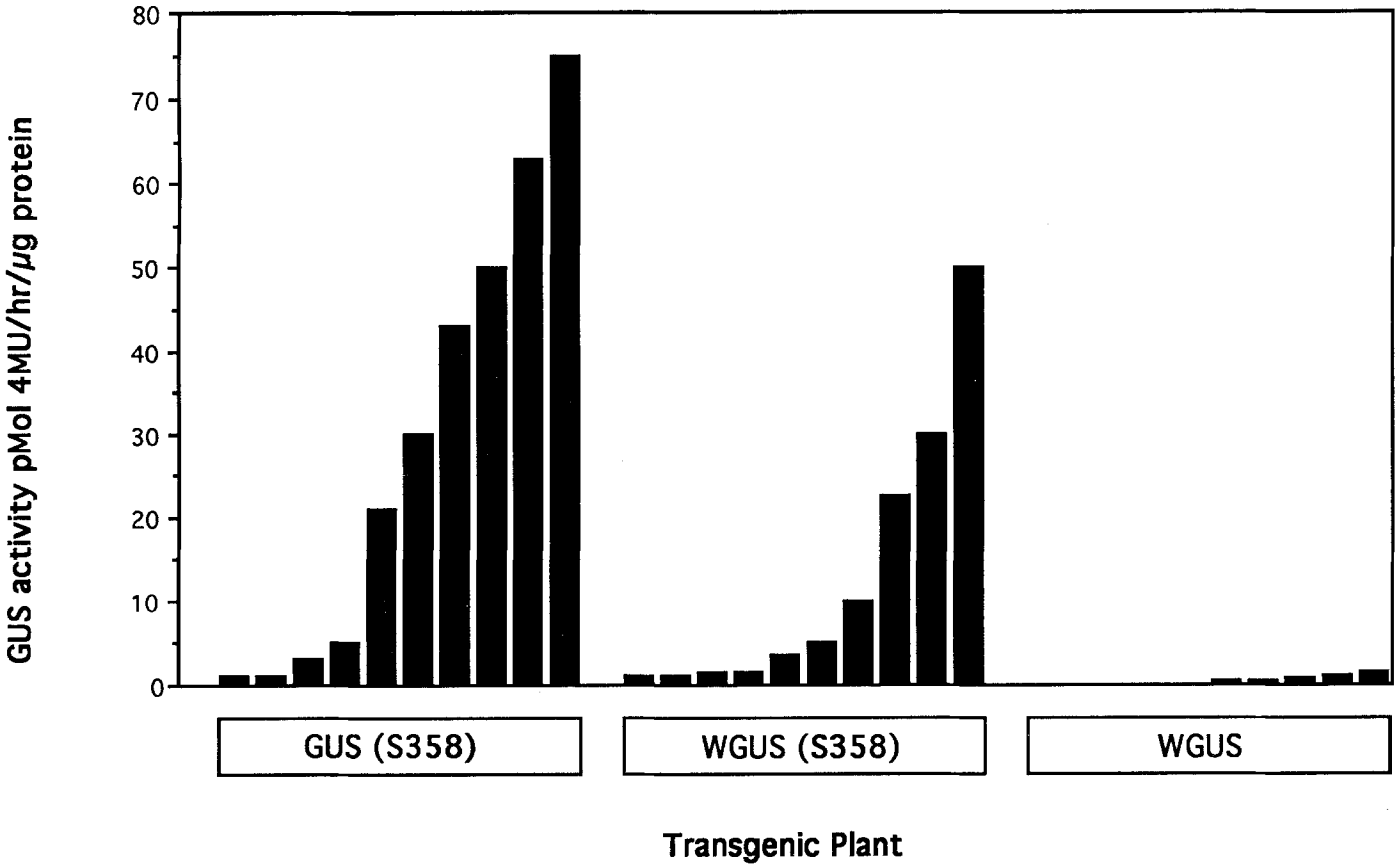

Fig. 3. Range of GUS activity in leaf tissue of the three transgenic plant lines. Ten representative plants expressing each of the three GUS genes GUS (S358), WGUS, and WGUS(S358) were chosen for analysis. GUS activity was measured in extracts produced from total leaf tissue.

(Fig. 3). These high expression levels confirm that the asparagine to serine substitution does not affect GUS activity *in planta.* In contrast, the WGUS plants showed no detectable GUS activity, indicating that the wheat α-amylase signal is targeting GUS to the ER, which has resulted in inactivation of the wild-type GUS enzyme by N-glycosylation (as has been previously reported (Iturriaga *et al.*, 1989)). Analysis of the WGUS(S358) transformants revealed that these plants had a range of GUS activity levels comparable to that achieved with the cytosolically expressed GUS S358. These data demonstrate clearly that activity of the modified GUS enzyme is not significantly altered following translocation to the ER. Transformants exhibiting moderately high expression levels were chosen for further analysis on the compartmentalization of GUS activity.

ER-targeted GUS activity is found predominantly in the intracellular fraction

It was assumed that, following translocation to the ER, GUS would be secreted via the default pathway (Denecke *et al.*, 1990) and would therefore be found predominantly in the apoplast. To determine the cellular location of the GUS enzyme, we employed a standard technique frequently used to isolate extracellular proteins. Leaf tissue from representative plants from each of the transgenic lines was vacuum infiltrated with 50 mM $CaCl_2$ and the extracellular proteins extracted by centrifugation. The remaining soluble proteins (which correspond to the intracellular fraction) were also extracted from the residual tissue. In parallel studies on secretion of antibody fragments in transgenic tobacco, we have used these techniques to demonstrate successful secretion of scFv proteins (Firek *et al.*, 1993). In this previous work (data not shown) it was found that up to 98% of the ER-targeted scFv was located in the extracellular fraction, thus demonstrating that vacuum infiltration is a very effective technique for extracting apoplastic protein.

When GUS assays were performed on the separate cellular fractions, it was clear that the enzyme activity in GUS (S358) transgenic lines was found almost exclusively in the intracellular protein fraction (Fig. 4). When a similar experiment was carried out on the WGUS(S358) transformants, unexpectedly, the GUS activity was again found to be predominantly in the intracellular fraction (Fig. 4). However, in this case the ratio of GUS activity between intra- and extracellular fractions was much lower compared to the ten-fold difference seen for the GUS

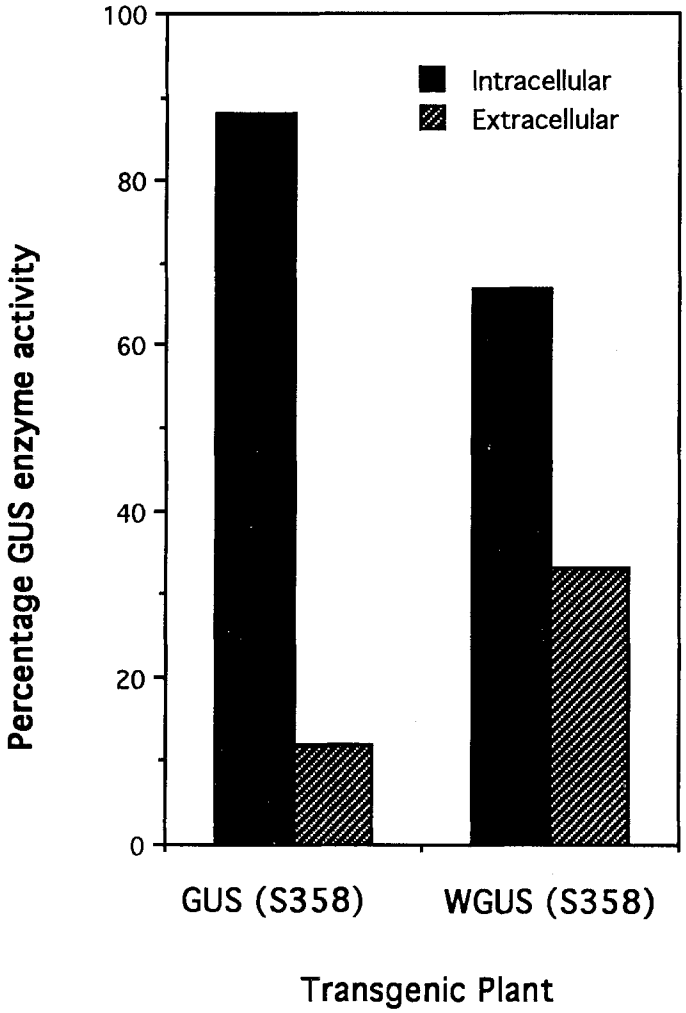

Fig. 4. Location of GUS activity in transgenic tobacco plants expressing a modified GUS enzyme lacking an N-glycosylation site, with and without an α-amylase signal peptide.

(S358) plants in which GUS expression is cytosolic. It therefore seems that although active GUS is being targeted to the ER it does not appear to accumulate predominantly in the apoplast, which is in contrast to our previous studies on scFv protein secretion.

Discussion

A series of experiments were conducted to investigate whether the modified GUS enzyme (GUS S358) in which the N-glycosylation site has been removed was suitable for use as a secretion reporter enzyme in transgenic plants. If the modified GUS enzyme could be secreted in an active form then it would allow us to transfer the construct to *Arabidopsis* and develop facile large-scale screening techniques to look for plants mutated in secretory pathways. *In vitro* translation studies indicated that the removal of the N-glycosylation site prevented glycosylation of the GUS protein. Analysis of transgenic plant lines expressing the modified GUS S358 enzyme fused to the α-amylase signal sequence demonstrated that the modified GUS enzyme retained activity after passage to the lumen of the ER.

Since GUS is of bacterial origin, it was assumed that it would possess no further protein-targeting information and would, after entering the lumen of the ER, be secreted via the default pathway (Denecke *et al.*, 1990). However, in the present experiments GUS activity was found to be localized predominantly in the intracellular fraction. In control experiments in which other polypeptides were fused to a plant signal sequence, the majority of the targeted protein was found in the extracellular fraction (Firek *et al.*, 1993). Similar data were obtained using a protoplast secretion assay described by Denecke *et al.* (1990), in that a large proportion of the GUS activity in protoplasts prepared from WGUS(S358) transgenics did not enter the incubation medium. To ensure that this result was not due to the origin of the signal sequence, an identical construct was made in which GUS (S358) was fused to the tobacco PR1a signal sequence. This signal sequence has been shown by us to direct highly efficient secretion of a mouse single-chain Fv protein in transgenic tobacco (Firek *et al.*, 1993). Studies on transgenic plants expressing the PR1a-GUS (S358) fusion yielded identical results to those obtained using the wheat α-amylase signal (data not shown). It can therefore be concluded that the retention of a large proportion of the GUS enzyme activity within the cell (or lack of accumulation in the apoplast) is due to an inherent property of the GUS protein.

Three possible explanations exist as to why, after passage to the ER, active GUS enzyme is apparently retained within the cell. Firstly, GUS may have some form of cryptic targeting signal which results in the retention of the protein or incorrect targeting. This seems unlikely, since analysis of the primary protein sequence does not reveal any similarities to any known sequences of this type, e.g. ER retention or vacuolar targeting signals. However, it is known that some proteins which fail to adopt a native structure or do not assemble into their correct multimeric form are retained and often degraded within the ER. Also mutations that perturb native folding are often found to have a detrimental effect on the 'transport competence' of the protein (Vitale *et al.*, 1993). It is not known what effect the single asparagine to serine alteration has on GUS enzyme folding or assembly, but the fact that enzyme activity is detected would suggest that correct assembly has taken place.

A second possibility is that GUS is actually physically prevented from being secreted in an active form. The active GUS enzyme is a tetramer made up of four 70 kDa GUS monomers, giving a total size of 280 kDa. However, mammalian antibodies of 160 kDa have been shown to be assembled and secreted in an active form from plant cells

(Hein *et al.*, 1991). Also, in the protoplast assays the absence of a cell wall would mean no size exclusion limit is imposed, yet GUS activity was again detected primarily within the intracellular fraction.

The third and perhaps most likely possibility is that GUS is secreted via the default mechanism but is rapidly inactivated or degraded after passage through the endomembrane system. The measured intracellular GUS activity would then be the active GUS enzyme 'in transit' through the secretion pathway. The fact that some GUS activity is always found in the extracellular fraction (see Fig. 4) may add some support to this proposal. Further work involving GUS activity measurements on subcellular fractions from WGUS (S358) plants may provide further support for one of the above suggestions.

In conclusion, our experiments would suggest that although the modified GUS enzyme (GUS S358) does remain active after passage to the lumen of the ER it may not be the most suitable reporter enzyme for use in secretion and vacuolar targeting studies. This study also suggests that, once targeted to the ER, export via the default pathway is not necessarily automatic and may require proteins to possess certain characteristics which are necessary to allow efficient secretion.

Acknowledgements

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