

5 Chapter V: Expression and secretion studies of GUS^{Eco} and GUS^{Ssp} in plants

5.1 Introduction

The biggest limitation of the current GUS reporter system is its destructive sampling nature. This limitation could be overcome, if the enzyme is secreted to the outside of the cell, where substrates could be non-destructively delivered without compromising the integrity of the plasma membrane. As mentioned in chapter I, a secreted GUS would act not only as a powerful non-destructive reporter, but also as a very powerful and versatile effector, depending on which of its many proactive substrates one might use.

Various authors have experimented with engineering GUS^{Eco} to be secreted in plant systems. Iturriaga et al. (1989) first demonstrated that the enzyme can be efficiently targeted into the ER using the signal peptide of the potato storage protein patatin. The enzyme, however, is N-glycosylated at the N358 amino acid, causing its loss of enzymatic activity. Subsequently, Denecke et al. (1990) demonstrated that, in protoplast system, GUS^{Eco} activity was detected in the medium, following treatment with tunicamycin, an N-glycosylation inhibitor. However, interpretation of such results in a protoplast system should be approached with caution, as the frequent cell death and aberrant cell surface geometries could affect the measurements' validity. Using an N-glycosylation-free (N358S) variant, Farrell & Beachy (1990) showed that about 64 % of enzyme activity was retained in the cell. Further reports by Pang et al. (1992), Diaz et al. (1992), Firek et al. (1994) and Yan et al. (1997) also confirmed the results of earlier authors.

It should be noted that GUS^{Eco} has been successfully engineered to be targeted to chloroplast, mitochondria, and nucleus of plant cells, and therefore can be used as a

reporter to study protein targeting and trafficking in those pathways (e.g. Kavanagh et al. 1988; Schmitz & Lonsdale 1989; Restrepo et al. 1990). This leaves the *secretory pathway* as the only exception that GUS^{Eco} has failed to adapt. Ironically, all mammalian GUSes, which obviously share many common characteristics with GUS^{Eco}, naturally utilize part of the secretion pathway (from ER to TGN – see chapter I) to reach their lysosomal destination. In fact, these enzymes are rapidly secreted if their interactions with the ER-resident egasyn is disrupted (Satoh 1991; Satoh et al. 1999). More interestingly, Neuhaus et al. (1997) reported that, when expressed in tobacco, the rat β -glucuronidase without its C-terminal lysosomal targeting propeptide is secreted into the medium. With these results, it is reasonable to assume that the failure to secrete is rather specific to GUS^{Eco}, and that other secretory-competent GUSes can be found.

Our current hypothesis for the failure to secrete of GUS^{Eco} is that the enzyme (which has nine cysteine residues naturally in their sulfhydryl state in the reducing environment of the bacterial cytosol) could be retained in the more oxidative environment of the eukaryotic ER, because their many cysteines form inappropriate disulfide bonds with elements of the protein matrix of the ER, preventing the enzyme's secretion. This so-called thiol-mediated retention mechanism has been extensively studied in the retention of unassembled immunoglobulin (Ig) chains, and unassembled subunits of acetylcholinesterase (Sitia et al. 1990; Kerem et al. 1993; Fra et al. 1993; Reddy et al. 1996; Isidoro et al. 1996; Reddy & Corley 1998; Ellgaard et al. 1999).

In plants, there have been a few reports about the effects of thionins (small plant proteins known to form disulfide bonds with other proteins), and reducing agent such as DTT, on expression and secretion of GUS^{Eco} (Diaz et al. 1992; Pineiro et al. 1994; Pineiro et al. 1995). Although these reports do not discuss the clear mechanisms of such effects, they certainly provide some good circumstantial support for the hypothesis above. At CAMBIA, GUS^{Eco} variants, with various numbers and combinations of cysteines eliminated, were developed. But due to the large number of

mutants, and their compromised activities, testing of these materials has been of a low priority and remained unfinished (R. Jefferson et al., unpublished).

Instead of engineering GUS^{Eco}, recent work has been focused on finding new GUSes that could be efficiently secreted. Although mammalian GUSes could be engineered to secret (Neuhaus et al. 1997), they lack characteristics of a desirable reporter (robustness, acidic pH optimum, public and regulatory acceptability etc). Therefore, the work has been focusing on new GUSes of microbial origin. With this approach, a novel GUS with superior enzymatic characteristics from a soil *Staphylococcus* sp. (GUS^{Ssp}), was isolated. Beside its biochemical characteristics, the new enzyme, although sharing about 47% identity with that of *E. coli*, has only one cysteine in contrast to nine cysteines from its *E. coli* counterpart. It is therefore an excellent candidate for a better and secretable GUS. However, the native gene is very AT-rich, and consequently has low levels of expression in heterologous systems of interest to us. Therefore, a synthetic codon-optimized version of the gene was designed and constructed (chapter II). The enzyme has been shown to be secreted in *E. coli* (A. Kilian, unpublished), and in yeast (chapter IV).

To target the GUS^{Eco} and GUS^{Ssp} into the plant secretory pathway, we have constructed modular vectors containing the two signal peptides of extensin from tobacco (dicot), and glycine rich protein (GRP) from rice (monocot). Extensin and GRP are two secreted proteins that make up most of the plant cell wall structure (Cassab & Varner 1988; Lei & Wu 1991; Fang et al. 1991; Memelink et al. 1993). The extensin signal peptide has been used to target *nptII* marker gene into the plant secretory pathway (De Loose et al. 1991).

5.2 Materials and Methods

5.2.1 DNA constructs

GUS^{Ssp} and GUS^{Eco}, with or without a signal peptide, all driven by the 35SCaMV promoter, were introduced to pCAMBIA1301 backbone (GenBank acc. nr. AF234297). The pCAMBIA series is a set of modular vectors based on the pPZP family (Hajdukiewicz et al. 1994).

For GUS^{Ssp}, the native protein, and three of its variants, N11Q, C499A, and N118Q-C499A, were used. GUS^{Eco} was an N-glycosylation deficient N358Q mutant, which is now used as standard GUS^{Eco} reporter in all pCAMBIA vectors.

The signal peptides from rice (*Oryza sativa*) glycine-rich protein (Fang et al. 1991), and tobacco (*Nicotiana plumbaginifolia*) extensin were introduced to the NcoI/BglII sites (A. Kilian, unpublished), while GUS^{Ssp} and GUS^{Eco} were introduced into the BglII/BstEII sites of pCAMBIA1301.

5.2.2 Rice apoplastic protein extraction

The apoplastic protein extraction protocol was modified from that of Reimers et al. (1992). Rice leaf was cut into 2-cm long pieces. About 10 pieces (0.2-0.3 g) were held vertically together in a 1.5 mL Eppendorf-type microfuge tube with the bottom cut off. The samples were submerged in extraction buffer (20 mM Tris, pH=7.5, 1 mM EDTA, pH=8.0, 1 M NaCl), and vacuum infiltrated at 20 inch Hg until bubbles were no longer seen. The leaf samples were then blotted dry, reweighed, and kept vertically in an eppendorf with the bottom pierced with a needle. Another eppendorf was used as an apoplastic fluid collector. Both tubes were placed in a 15-mL tube for centrifugation. The samples were spun at 30,000xg for 20 min, and the apoplastic fluid collected (typically about 100-200 ul) and assayed for GUS activity.

5.2.3 GUS quantitative and histochemical assays

GUS quantitative assays were done according to Jefferson (1987). For histochemical assay, rice leaf and root samples were fixed with 1% formaldehyde (1% formaldehyde in 1x PBS buffer) for 1-2 hrs on ice following vacuum infiltration. Fixed samples were sectioned (20 μ m) with a vibratome. The sections were then stained with ELF-97-glcA (10 μ M ELF-97-glcA, 0.1% DMSO, 0.01% Triton X-100 in 1xPBS buffer). This substrate produces an insoluble green fluorescent signal at the site of GUS activity (Zhou et al. 1996). Note that simultaneous staining with other dyes that have maximal fluorescence in acidic pH (like the popular nuclei counterstain DAPI) is less than optimal, because ELF-97 fluorescence is quenched in an acidic environment. Sections were observed on a Zeiss fluorescent microscope with a UV-longpass filter, and recorded on Fuji Sensia II 100ASA color reversal film.

5.2.4 Detection of post-translational modifications of GUS^{Eco} and GUS^{Ssp}

The protocols were modified from Dixit et al. (2000). Rice calli were vacuum infiltrated with 100 mM iodoacetamide in an extraction buffer (100 mM Tris, pH=8.0, 1 mM EDTA, pH=8.0, 1 mM PMSF, 1 μ g/mL pepstatin A) and then incubated in the dark for 20 min. This step blocks existing free cysteines, and prevents new or rearranged disulfide bonds formation. Note that the extraction buffer contains no DTT.

Total soluble protein was then extracted from the calli using the same extraction buffer. The protein (10 μ g/lane) was separated on an SDS-PAGE using loading buffer with or without 100 mM DTT as appropriate.

Protein gels were blotted onto either nitrocellulose or PVDF membrane using the Trans-Blot cell apparatus (Bio-rad, CA, USA) following recommended protocols. Blocking was done overnight with 5% skim milk powder or 1% BSA in TBST. The primary antibodies were affinity-purified rabbit polyclonal antibodies raised against purified hexa-histidine-tagged GUS^{Eco}. The secondary antibody was mouse monoclonal

anti-rabbit antibody coupled with horseradish peroxidase, clone AG-16 (Sigma Cat. No. A2074). Chemiluminescent detection was done with Supersignal West Dura substrate (Pierce, IL, USA). Signal was detected on X-ray film.

5.3 Results and Discussions

5.3.1 SP-GUS^{Ssp} plants have strong histochemical GUS staining despite their relatively low total GUS activity (*)

A total of 141 lines of transgenic rice, expressing GUS^{Ssp} or GUS^{Eco}, with or without glycine-rich protein (GRP) and extensin (Ext) signal peptide, were regenerated (figure 5.1).

Leaf segments of all transgenic plants were stained with X-glcA overnight, and their staining intensities were recorded as scores: weak, medium, strong, and very strong (figure 5.2). Without signal peptide, GUS^{Eco} and GUS^{Ssp} plants had similar staining score distribution. With signal peptide however, it was clear that most GUS^{Ssp} plants stained strong/very strong, while most GUS^{Eco} plants only stained weak/medium.

Similar results were observed with root samples. With an *in vivo* GUS staining scheme (roots immersed in water containing 50 ug/ml of X-glcA), only roots of many SP-GUS^{Ssp} plants showed clear staining after 30 min, whereas root samples from other constructs gave completely negative results (data not shown).

For each construct, five transgenic plants representing different levels of GUS staining were further analysed for quantitative specific GUS activity in leaf extracts (figure 5.3). The average specific GUS activity from these plants showed a significantly reduced total enzyme activity when both enzymes were fused with signal peptides. Such results are not unexpected, as it has been known that the addition of signal peptides often correlates with reduced expression levels (Denecke et al. 1990). There could be many

(*) SP-GUS^{Ssp} denotes GUS^{Ssp} with either the GRP or Ext signal peptide.

reasons for such an observation. For examples, introduction of a protein into the secretory pathway could lead to saturation of a limiting step in the pathway. In addition, none or improper cleavage of signal peptides could lead to mature proteins with altered (often reduced) activity/stability. Due to time constraints, I have not looked further into these possibilities.

The data from figure 5.2 and 5.3 showed a clear correlation between staining intensity and total GUS activity: strong staining/high activity (no signal peptide constructs), and weak staining/low activity (SP-GUS^{Eco} constructs). The only exception was with the SP-GUS^{Ssp} constructs, where most plants had strong GUS staining, despite their relatively low total enzyme activity. This is compatible with the hypothesis that GUS^{Ssp} is secreted and therefore has better contact with the substrate, resulting in stronger staining.

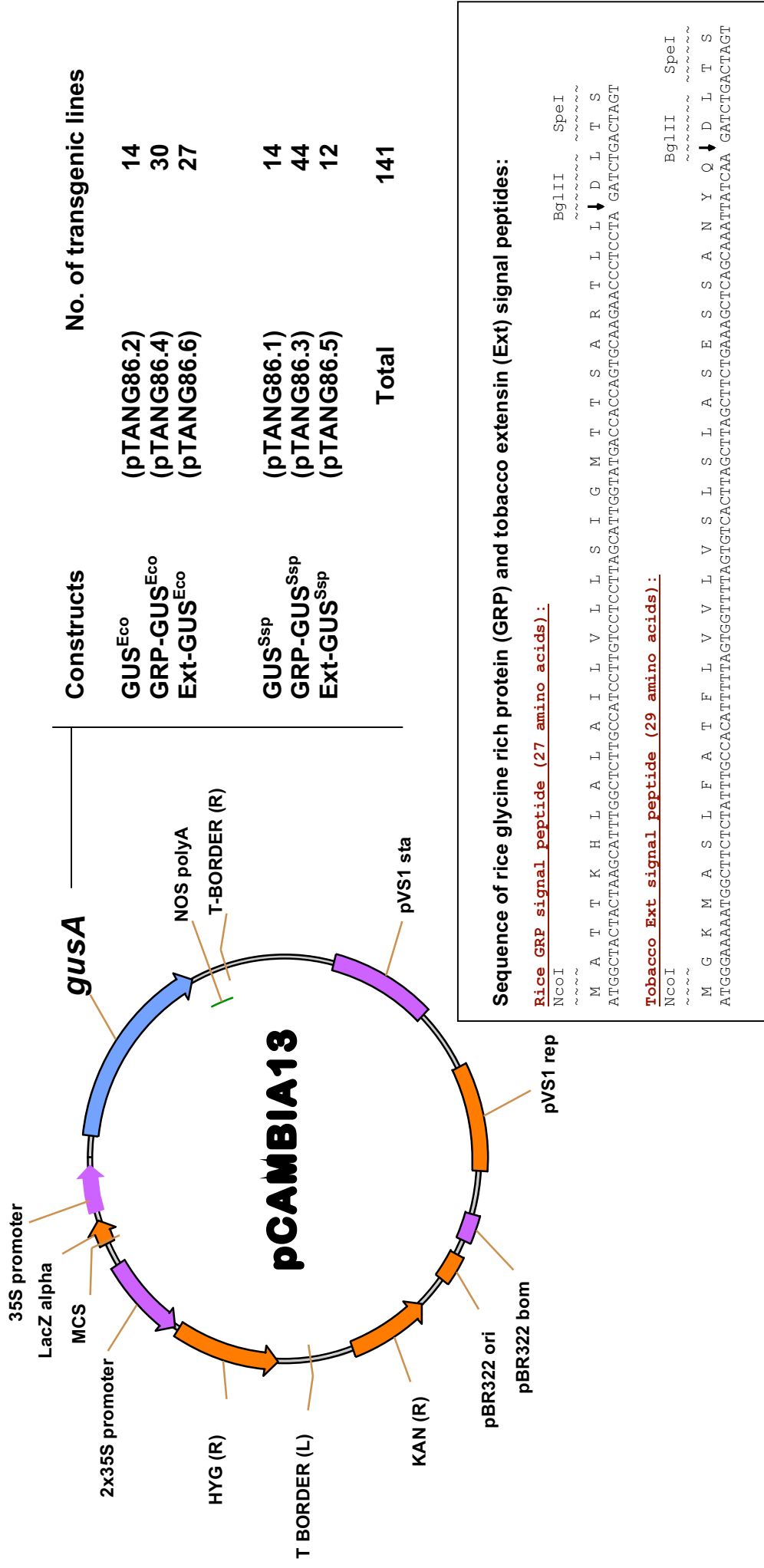


Figure 5.1. DNA constructs used in plant expression and secretion experiments. Both GUS^{Eco} and GUS^{Ssp}, with and without the glycine rich protein (GRP) or extensin (Ext) signal peptide, were constructed. All constructs were based on pCAMBIA1301 backbone, with the corresponding genes driven by CaMV35S promoter. A total of 141 independent transgenic rice lines were regenerated.

Construct	negative	weak	medium	strong	very strong	Total
GUS ^{Ssp}	5	1	2	3	3	14
GUS ^{Eco}	3	1	1	4	5	14
GRP-GUS ^{Ssp}	11	1	1	18	13	44
GRP-GUS ^{Eco}	10	7	9	2	2	30
Ext-GUS ^{Ssp}	4	0	0	2	6	12
Ext-GUS ^{Eco}	6	12	8	0	1	27
Total	39	22	21	29	30	141

Figure 5.2. Distribution of GUS staining scores of 141 transgenic rice plants. Staining was performed on small leaf segments overnight with X-glcA. The scores were based on the blue color intensity: weak, medium, strong, and very strong. Without signal peptide, GUS^{Eco} and GUS^{Ssp} plants had similar staining score distribution. With signal peptide however, most GUS^{Ssp} plants stained strong/very strong, while most GUS^{Eco} plants stained weak/medium.

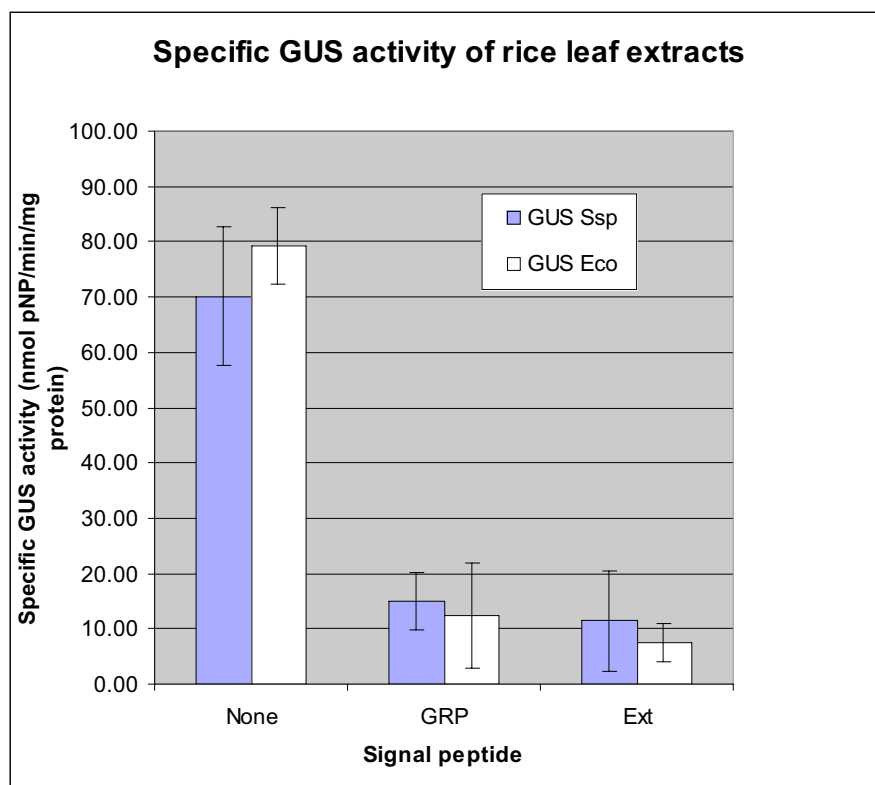


Figure 5.3. Specific GUS activity of rice leaf extracts. For each constructs, five transgenic plants representing different levels of GUS staining were chosen for this experiment. Total GUS activity was significantly reduced when both enzymes were fused with signal peptides.

5.3.2 SP-GUS^{Ssp} plants have GUS activity in the apoplastic fluid extract

Apoplastic fluid extraction methods based on vacuum infiltration and centrifugation are well-established for dicot species (Terry & Bonner 1980). However, little information about such methods is available for monocots. Direct adaptation of the protocols for dicots to monocots did not give satisfactory results, presumably due to their anatomical differences. For example, compared to a tobacco leaf, a rice leaf has parallel veins, thicker cuticles and less air space.

I have adapted the protocol for rice leaf apoplastic extraction from Reimers et al. (1992). This protocol uses more complete vacuum infiltration, and much higher g force (30,000xg instead of 3,000xg which would be adequate for dicot) applied on vertically-arranged leaf fragments, to recover the apoplastic fluid. Preliminary experiments were carried out to assess apoplastic fluid recovery and cytoplasmic contamination with successive steps of increased centrifugation force (10,000-30,000xg). In these experiments, GUS (without signal peptide) was used directly as a cytosolic marker enzyme, instead of other cytosolic markers, such as malate dehydrogenase (Lopez-Millan et al. 2000). Centrifugation at 30,000xg gave highest fluid recovery without detectable cytoplasmic contamination (data not shown).

Using this apoplastic fluid extraction protocol, it was shown that GUS activity was only detected in the apoplast of plants with SP-GUS^{Ssp} constructs (figure 5.4). There was no detectable activity in the apoplast of all plants with other constructs. Note that apoplastic GUS activity of constructs without a signal peptide was also an indicator for cytoplasmic contamination. As shown in figure 5.4, no contamination was found, even in the apoplast of an intron-containing GUS^{Eco} plant with total GUS activity several-fold higher than other plants used in these studies.

	No SP			GRP		Ext	
	Intron-GUS ^{Eco}	GUS ^{Eco}	GUS ^{Ssp}	GUS ^{Eco}	GUS ^{Ssp}	GUS ^{Eco}	GUS ^{Ssp}
Apoplastic fraction	ND (*)	ND	ND	ND	2.2	ND	5.4
Remaining fraction	332.0	35.3	73.3	26.4	30.5	24.3	88.8

(*) ND: not detectable

Figure 5.4. Specific GUS activity (nmol pNP/min/g fresh weight) of apoplastic and cytoplasmic (remaining) fractions of rice leaf. GUS activity was only detected in the apoplast of SP-GUS^{Ssp}. The apparent secretion efficiency, however, is less than 10%.

In this experiment, the apparent secretion efficiency, *i.e.* the proportion of GUS^{Ssp} apoplastic activity over total activity, of GUS^{Ssp} with a signal peptide is less than 10%. However, the extraction efficiency using this method is still unknown. Furthermore, the secreted GUS^{Ssp}, being a tetramer of around 280 KDa, could be trapped between the cell membrane and the cell wall, and if so, would not be extracted efficiently. In this regard, the use of a protoplast culture system would be more accurate in determining the secretion efficiency, but subject to other, perhaps more severe interpretational challenges due to the “non-biological” nature of such artificial cell cultures.

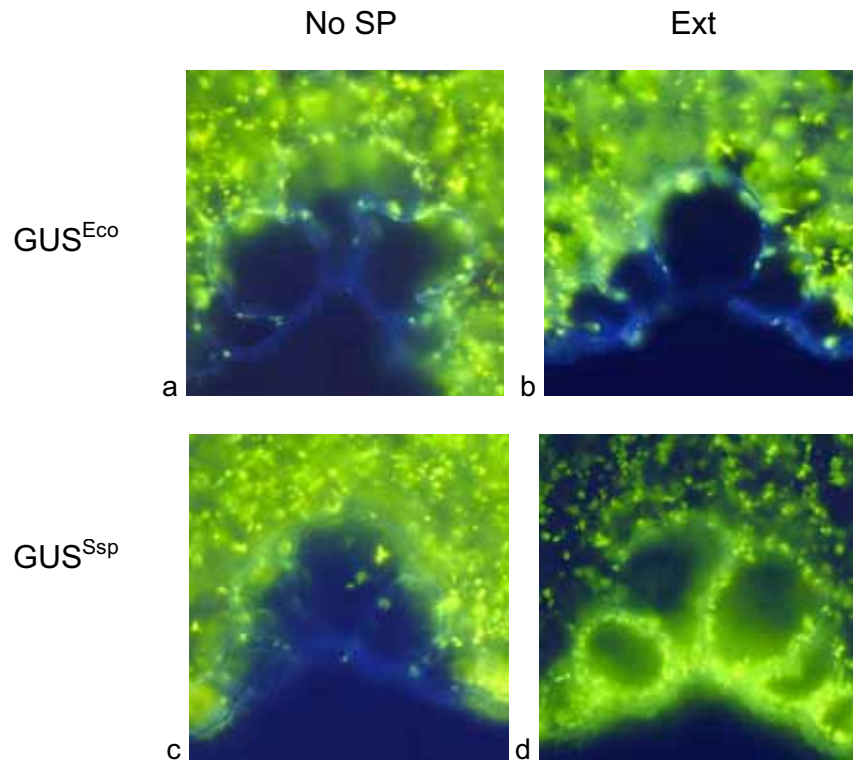
5.3.3 SP-GUS^{Ssp} reveals cell-wall localized GUS activity

Histochemical analysis on rice leaf and root tissues was carried out to confirm the subcellular localization of GUS^{Eco} and GUS^{Ssp}, with and without signal peptides. Formaldehyde-fixed sections were stained with ELF-97-glcA, a GUS substrate that produces an insoluble green fluorescent signal at the site of GUS activity (Zhou et al. 1996). Compared to the commonly used histochemical substrate X-glcA, this substrate offers more accurate localization, and higher signal to noise ratio. However, it is also more expensive and requires fluorescence detection facilities.

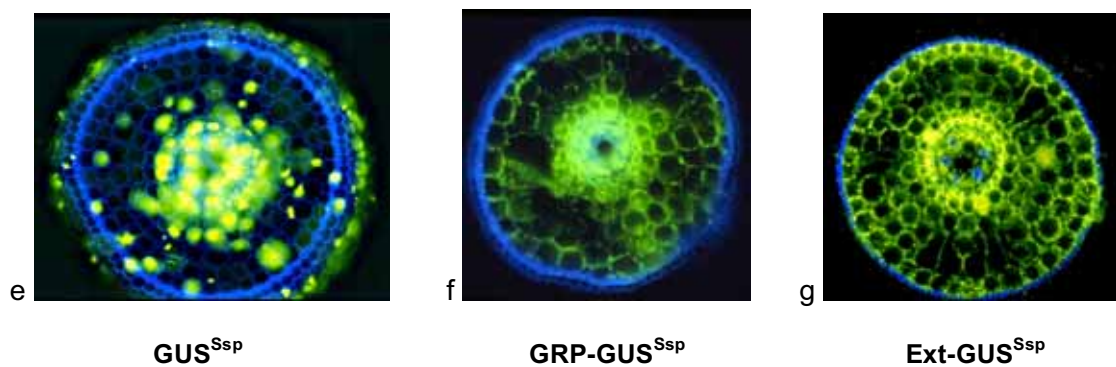
Figure 5.5.A shows histochemical staining of rice leaf *motor* cells. These cells are considerably larger than other epidermal cells, and responsible for rolling of the leaves. Due to their large size and presumably high water content, their cellular contents were probably not well-fixed, and therefore lost during sample processing, leaving the clean cell wall as observed in GUS^{Eco} (a), Ext-GUS^{Eco} (b), and GUS^{Ssp} (c). Only with Ext-GUS^{Ssp} (d) that clear cell-wall-localized GUS activity is observed.

In root sections (figure 5.5.B), only GUS^{Ssp} with signal peptides displayed apparent cell-wall localization of enzymatic activity (f and g). In contrast, GUS^{Ssp} without a signal peptide shows no preferential localization of GUS (e). We could not detect GUS activity in GUS^{Eco} sections, most probably due to its sensitivity to fixation conditions. However, staining of unfixed tissues confirmed that only GUS^{Ssp} with a signal peptide shows GUS activity in the vicinity of cell walls.

The data from histochemical assays are consistent with those of the apoplastic assays, and confirm that unlike GUS^{Eco}, GUS^{Ssp} can be secreted if engineered. The accurate secretion efficiency remains to be determined in future experiments.



A. GUS histochemical staining of formaldehyde-fixed rice leaf (motor) cells. Samples were stained with ELF-97-glucuronide, a GUS substrate that produces an insoluble green fluorescence signal at the site of GUS activity. In this figure, only Ext-GUS^{Ssp} (d) shows clear cell-wall-localized GUS activity.



B. GUS histochemical staining of formaldehyde-fixed rice root. Cell wall of GUS^{Eco} without signal peptide is virtually free of GUS activity (e), while clear cell-wall-localized signal is observed for SP-GUS^{Eco} (f and g).

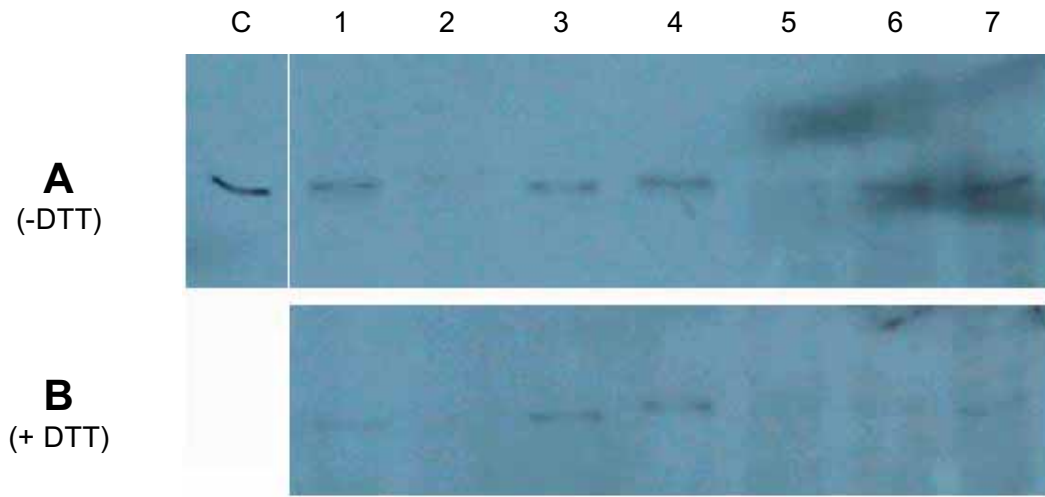
Figure 5.5. Histochemical analysis of GUS^{Eco} and GUS^{Ssp}, with and without signal peptide, in rice leaf and root tissues.

5.3.4 Detection of post-translational modifications of GUS^{Eco} and GUS^{Ssp}

Experiments were carried out to detect possible post-translational modifications of GUS^{Eco} and GUS^{Ssp} when they are targeted to the plant secretory pathway. Total soluble protein was extracted from rice calli in the presence of iodoacetamide (to block existing free cysteines, preventing new or rearranged disulfide bonds formation), and absence of the reducing agent DTT. The proteins were separated on SDS-PAGE in reduced (+DTT) or non-reduced (-DTT) condition, and subsequently detected in a western blot using GUS^{Eco} antibody. In principle, if GUS^{Eco} or GUS^{Ssp} is cross-linked with other proteins via disulfide bonds, then extra higher molecular weight bands should be detected in non-reduced condition. These extra bands should disappear upon treatment with the reducing agent DTT.

As seen in figure 5.6, no signal was detected for GRP-GUS^{Eco} (lane No. 2 of panel B). It is likely that the protein was retained in ER (as confirmed by numerous authors – section 4.1) and targeted for degradation, therefore, was present in much smaller amount compared to that of other constructs (panel B).

Because the signal for GRP-GUS^{Eco} was not detectable in the presence of DTT (lane No. 2 of panel B), the detection of possible cysteine cross-linking was certainly not possible (lane No. 2 of panel A), because cross-linking would spread the principal signal (reduced state) into smaller quantities (cross-linked state).



Lane 1: GUS^{Eco}

Lane 2: GRP-GUS^{Eco}

Lane 3: GUS^{Ssp}

Lane 4: GRP-GUS^{Ssp}

Lane 5: GRP-GUS^{Ssp} (C499A)

Lane 6: GRP-GUS^{Ssp} (N118Q)

Lane 7: GRP-GUS^{Ssp} (C499A&N118Q)

C: 10 ug of purified GUS^{Eco} (pLADF48) expressed in *E. coli* KW1 strain.

Figure 5.6. Detection of possible posttranslational modifications of GUS^{Eco} and GUS^{Ssp}.

Total soluble proteins were separated on SDS-PAGE in the presence or absence of reducing agent DTT, and subsequently detected in a western blot using GUS^{Eco} antibody. See text for discussion.

It is currently hypothesized that GUS^{Eco} is retained in the ER because it forms disulfide bonds with other proteins in the ER matrix. As illustrated in this experiment, verification of the hypothesis could be challenging if the protein is targeted for degradation, and therefore present in small amounts below the detection limit. Clearly, optimization of the immunodetection scheme to further increase the detection sensitivity (section 3.3.5) is vital before this experiment can be repeated to confirm the validity of the hypothesis.

Because GUS^{Ssp} is secreted when provided with a signal peptide, its cross-linking with other proteins in the ER is unlikely. In this experiment, there was expectedly no apparent indication for GUS^{Ssp} cysteine cross-linking (lane No. 4, 5, and 7, on both panel A and B). However, due to the sub-optimal quality of the blot in terms of signal strength, the results should be treated as “indicative”, and need to be reconfirmed.

The apparent slight increase in molecular weight of GRP-GUS^{Ssp} compared to that of GUS^{Ssp} (lane No. 3 and 4 on both panel A and B) is an indication that, either 1) the GRP signal peptide may not be cleaved, or 2) the protein is N-glycosylated at its single potential N-glycosylation site.

Confirmation of signal peptide cleavage is very important because none or improper cleavage of signal peptides could lead to mature proteins with altered (often reduced) activity or stability. N-terminal sequencing should be carried out in the near future to confirm proper cleavage of the GRP signal peptide.

In principle, whether GUS^{Ssp} is N-glycosylated when it is targeted in the ER can be confirmed by comparing the native GUS^{Ssp} and its N-glycosylation-free variants (lane No. 4, 6, and 7 on both panel A and B), However, due to the sub-optimal quality of the blot in terms of signal strength and resolving power (i.e. mini gel), a clear answer was not obtained.

It should be noted that this experiment is only preliminary, therefore, its results should be interpreted with cautions. However, the experiment clearly indicates the power of

the biochemical approach in unraveling important post-translational modifications when GUS^{Eco} and GUS^{Ssp} are targeted to the secretory pathway.

In future experiments, various improvements should be included. For example, analysis on the insoluble fraction is necessary, since there have been indications that GUS^{Eco} could be associated with the cell membrane when targeted to the ER (Yan et al. 1997). The experiments should ideally be carried out on enriched microsomes fraction, instead of on total cellular extract. Other technical details, such as better separation resolution (i.e. bigger gels) and improved immunodetection scheme, are also important to the success of future experiments that will provide answers for the long-standing question of why GUS^{Eco} is not secreted in plant cells.

