

6 Chapter VI: Investigation of some applications based on GUS^{Ssp}

6.1 Introduction

The results from previous chapters have clearly indicated that GUS^{Ssp} can most likely be used as an improved reporter and effector. In this chapter, we report various experiments carried out to confirm and demonstrate those expectations. This includes the use of GUS^{Ssp} as an improved conventional GUS reporter, as a non-destructive GUS reporter, or as an effector in positive selection and tissue-specific manipulation schemes. A brief introduction on these experiments is presented below.

6.1.1 GUS^{Ssp} as an improved reporter

With its superior biochemical characteristics, such as lower K_m , better thermal stability, and more resistant to commonly used fixatives and chemicals (P. Wenzl and T. Nguyen, unpublished), GUS^{Ssp} could become a significantly better reporter enzyme.

For example, GUS^{Ssp} is expected to offer better conventional histochemical analysis. Histochemical GUS analysis is the most popular technique for the analysis of plant gene expression due to its simplicity as compared to immunocytochemistry or other types of *in situ* hybridizations. The technique, however, often requires chemical and thermal treatments of tissues before enzymatic detection. Although GUS^{Eco} is a robust enzyme that normally survives commonly used chemicals and moderate heating, it is not very tolerant to fixatives, especially to glutaraldehyde (Jefferson 1987). For these reasons, there have been various works aimed to improve the enzyme's tolerance to fixatives (Matsumura et al. 1999), and thermal stability (Flores & Ellington 2002).

A good histochemical protocol should allow precise histochemical localization of the reporter in structurally well-preserved samples. With GUS^{Eco}, good structure

preservation has often been sacrificed for enzymatic activity maintenance: most authors use very mild, or even no, tissue fixation before enzymatic detection. Elaborate protocols (De Block & Debrouwer 1992; De Block & Van Lijsebettens 1998; Kopen et al. 1999), are often needed to obtain fine histological GUS localization. It is expected that improved protocols and much better results can be achieved for histochemical analysis using GUS^{Ssp}.

In general, the sensitivity and robustness of GUS^{Ssp} would also benefit other types of GUS reporter analyses. For example, it could offer more sensitive and reliable quantitative assays to be conducted in a wide range of conditions. As another example, GUS^{Ssp} could offer better heat inactivation of purported plant endogenous glucuronide-hydrolysing activities. These activities, when observed, have been commonly eliminated by heat inactivation (Alwen et al. 1992; Hodal et al. 1992). With GUS^{Ssp}, heat inactivation of plant intrinsic GUS-like activity could be done with almost no compromise of GUS^{Ssp} activity.

6.1.2 GUS^{Ssp} as non-destructive reporter

Unlike GUS^{Eco}, the new enzyme can be secreted in rice cells when provided with a signal peptide (chapter V). Therefore, it could become a non-destructive reporter in plant. Non-destructive assay using a secreted GUS has long been envisaged (Jefferson et al. 1987; Jefferson 1989). A secreted GUS is clearly the key component of such assay. However, for a true non-destructive assay, many other factors have to be taken into consideration. For example, efficient substrate delivery may not be an important issue in systems such as cell suspension or protoplast culture, but could be very important for *in vivo* analysis at the whole plant level. Similarly, other potential interferences, caused by by-products of the enzymatic reactions, or by diffusion and subsequent long distant transport of the secreted enzyme, could have adverse effects on the assay.

6.1.3 GUS^{Ssp} in positive selection schemes

GUS^{Ssp} can act not only as a reporter but also as an effector, when used with the right pro-bioactive substrates. For example, if the active component of the pro-active substrate is the sole sugar source, then a positive selection scheme could be developed such that only GUS-positive cells can use this sugar source for further growth.

Positive selection allows transgenic cells to proliferate while non-transgenic cells are starved but still survive. In contrast, negative (antibiotic or herbicide) selection allows transgenic cells to survive on a selective medium, while non-transgenic cells are killed. In principle, positive selection would eliminate unwanted effects of negative selection, such as the toxic surroundings caused by the death of non-transgenic cells. It also addresses public concerns related to antibiotic or herbicide resistance of transgenic materials.

A number of positive selection systems have been developed. Examples are the xylose isomerase system which enables the transgenic cells to utilize xylose as a carbohydrate source (Haldrup et al. 1998), or the mannose system which enables the transgenic cells to convert growth-inhibiting mannose-6-phosphate to metabolizable fructose-6-phosphate (Joersbo et al. 1998; Zhang et al. 2000).

In a GUS-based positive selection scheme, cytokinin-glucuronides and cellobiouronic acid (CBA) are the most promising substrates, although many other “conceptual” substrates could be used. Positive selection based on GUS^{Eco} and cytokinin-glucuronides has been demonstrated with moderate success (Joersbo & Okkels 1996; Okkels et al. 1997). It is expected that with secreted GUS^{Ssp}, such a scheme will be significantly improved. CBA could be the ideal substrate for positive selection. However, this substrate is not efficiently cleaved by GUS^{Eco} or GUS^{Ssp} (P. Wenzl and R. Jefferson, unpublished). A CBA-cleaving GUS has recently been isolated (J. Mayer, and R. Jefferson, unpublished). Current work is focusing on *in vitro* evolution by DNA

shuffling to obtain GUS(es) that cleaves CBA efficiently while maintains other desirable characteristics.

If successful, a GUS-based positive selection scheme will be a key enabling technology for crop transformation, bypassing existing methods, which are usually not accessible due to exclusive intellectual property licensing provisions. In addition, transgenic crops without antibiotic or herbicide resistance genes will be more readily accepted by the general public and by the regulatory agencies that purport to represent them.

6.1.4 GUS^{Ssp} in tissue-specific manipulation systems

In principle, specific expression of secreted GUS^{Ssp}, and subsequent broad application of a pro-bioactive GUS^{Ssp} substrate, will result in selective manipulation of the cell or tissue expressing GUS^{Ssp}. Depending on the nature of the pro-bioactive compounds, positive or negative effects can be envisioned.

A very good example for such tissue-specific manipulation systems would be the ablation of pollen, leading to the production of male-sterile plants. A pollen-specific promoter with appropriate strength and specificity is essential for the success of the system. A number of pollen-specific promoters have been described in the literature. Some well-known examples are the tapetum-specific promoter Osg6B from rice, or the anther-specific promoter TA29 from tobacco (Tsuchiya et al. 1994; Tsuchiya et al. 1995; Kriete et al. 1996; Cho et al. 2001; Sa et al. 2002). In the CAMBIA labs, testing was previously carried out for a variety of pollen-specific promoters, such as the apg promoter from *Arabidopsis* (Roberts et al. 1993), zmg13 promoter from maize (Guerrero et al. 1990), and ntp303 promoter from tobacco (Weterings et al. 1992; Weterings et al. 1995; Hulzink et al. 2002). Among them, ntp303 has been the most promising promoter, giving highly specific GUS expression in rice pollen (V. Ramaiah, unpublished). This promoter was therefore chosen in our experiments to demonstrate the GUS^{Ssp} tissue-specific manipulation concept.

6.2 Materials and Methods

6.2.1 DNA constructs

The castor bean catalase intron was amplified by PCR from pCAMBIA1301 using CAT-T-c and CAT-B-mod primers (appendix 2). The PCR product was then cloned into BglII/SpeI sites of pTANG86.1. The resulting vector was pTANH114, which was subsequently named as pCAMBIA1305.1. The intron was then cloned into BglII/SpeI sites of other GUS^{SSP} constructs with signal peptides.

Among several pollen-specific promoters previously tested in the lab: *apg*, *zmg13*, and *ntp303*, the *ntp303* has been the most promising promoter, giving highly specific GUS expression in rice pollen (V. Ramaiah, unpublished). The promoter was cloned to the BamHI/NcoI sites of pTANK101.1 (also known as pCAMBIA 1305.2), and pTANK101.2 (figure 6.1).

6.2.2 Transformation protocols for rice, tobacco and *Arabidopsis*

Rice, *Arabidopsis* and tobacco transformations were done using *Agrobacterium* strain EHA105. Rice transformation (*var.* Millin) was done as described in Hiei et al. (1994) and Hiei et al. (1997). *Arabidopsis* transformation was done with the flower-dipping method as described in Clough & Bent (1998). Tobacco transformation (*cv.* Wisconsin 38) was done as described in Svab et al. (1995).

6.2.3 Effects of fixation on histochemical GUS assay

Rice leaf discs (4 mm in diameter) were cut using a cork borer and then vacuum infiltrated with fixing solutions (1-4% formaldehyde or glutaraldehyde in 1x PBS, 0.05% Triton X-100) for 15 min and kept on ice for one hour. The leaf discs were then stained with X-glcA using standard method (Jefferson 1987).

Replicate leaf discs were ground with liquid nitrogen, and resuspended in standard GUS extraction buffer (Jefferson 1987). Quantitative GUS assays were carried out on

the resuspended extract, which contains both soluble extract and cellular debris. In principle, the soluble extract contains large proportion of soluble enzyme that has not been fixed, while cellular debris containing mostly fixed and cross-linked GUS enzymes that remains active after fixation.

6.2.4 Non-destructive histochemical GUS assays

Proliferating rice calli were stained in a sterile non-destructive GUS assay buffer (50-100 ug/mL X-glcA in 20 mM NaPO₄, pH=7.0). Staining was performed at room temperature and without vacuum infiltration. The speed and intensity of the staining was recorded. Positive calli were washed with liquid media, blotted dry, and placed on new regeneration plates containing 50 ug/mL X-glcA. The stained calli were observed for further proliferation.

For non-destructive screening, randomly chosen regenerating calli were transferred to regeneration plates containing 50 ug/mL X-glcA. The plates were kept in normal conditions (25⁰C in the dark). GUS-positive calli can be identified by the blue halo around them. The calli can be subcultured continuously on media containing X-glcA without apparent abnormality.

For non-destructive staining in rice roots, germinating seedlings were submerged in staining solution (50-100 uL/mL X-glcA in water). Staining was performed at room temperature and without vacuum infiltration.

6.2.5 DNA southern blot

DNA southern blot was performed following standard methods. Genomic DNA was digested with EcoRI or BamHI. The blots were probed with either *gusA*^{Eco} (BstBI/BstEII fragment of pCAMBIA1301) or *gusA*^{Ssp} (MluI/SnaBI fragment of pCAMBIA1305.2) DNA fragment as appropriate.

6.3 Results and Discussions

6.3.1 An intron introduced to the synthetic *gusA*^{Ssp} gene

The introduction of an intron can increase expression levels of *gusA*^{Eco} in monocots and prevent background expression from *Agrobacterium* during plant transformation (Vancanneyt et al. 1990; Tanaka et al. 1990). For similar reasons, we have introduced the castor bean catalase intron to the synthetic *gusA*^{Ssp} gene (figure 6.1). As expected, no apparent *Agrobacterium*-derived GUS^{Ssp} expression was recorded (data not shown). We have used this intron-containing version of the gene in many transformation experiments of rice, *Arabidopsis* and tobacco, and obtained very strong GUS histochemical staining of transgenic materials (see various results in this chapter). The intron-containing *gusA*^{Ssp} gene has been released to the research community in two plant transformation plasmids, pCAMBIA1305.1 (no signal peptide), and pCAMBIA1305.2 (GRP signal peptide).

Further studies on direct expression comparison between the intron and intron-less versions of the gene were intentionally omitted due to time constraints.

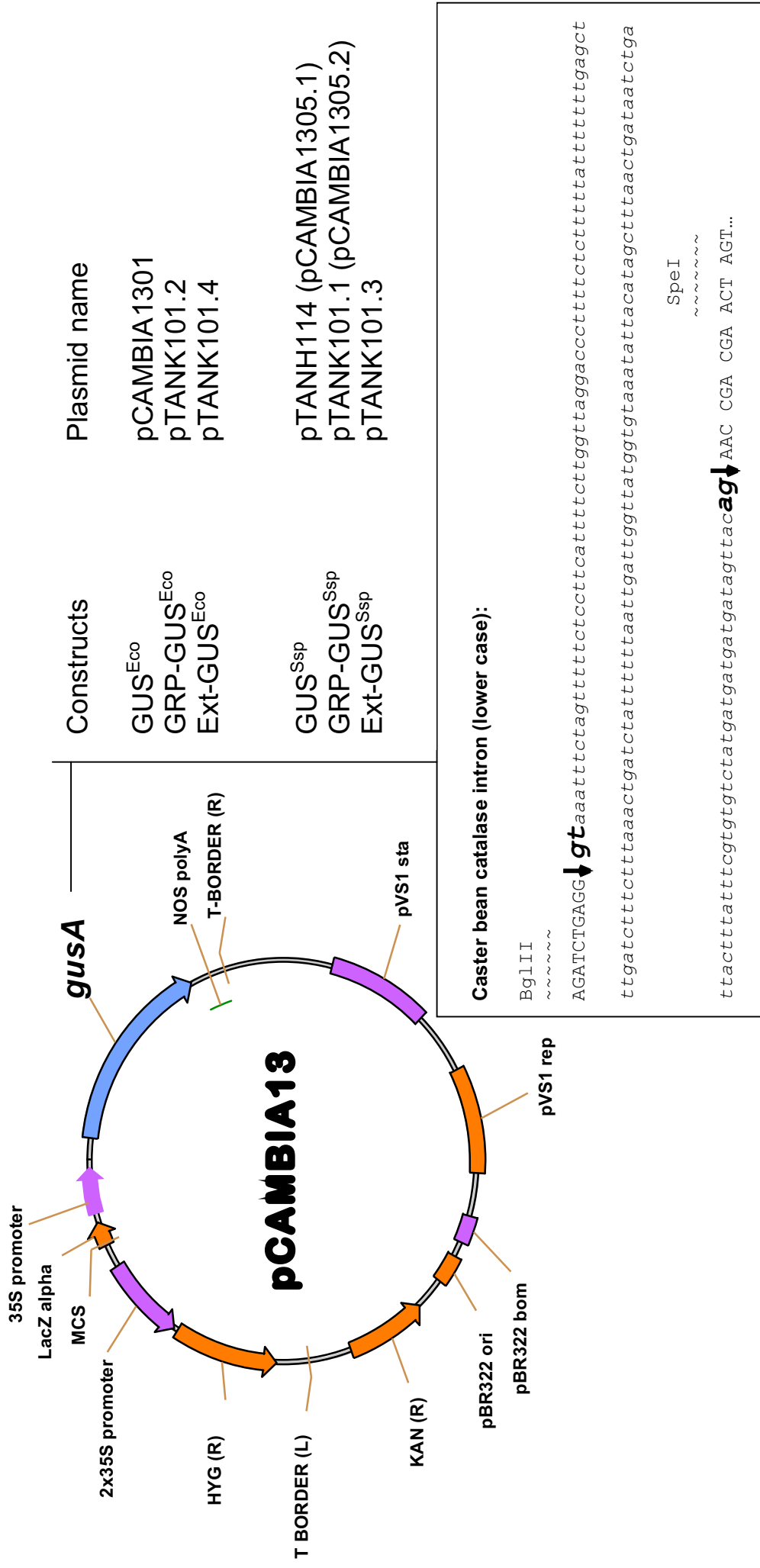


Figure 6.1. Plant transformation vectors with intron-containing *gusa*^{Ssp}. The castor bean catalase intron was taken from pCAMBIA1301. Note that pTANH114 and pTANK101.1 were later released to the research community as pCAMBIA1305.1 and pCAMBIA1305.2, respectively.

6.3.2 Demonstration of improved histochemical assays with GUS^{Ssp}

To validate the use of GUS^{Ssp} as a reporter for plants, we have transformed and regenerated a large number of transgenic rice, *Arabidopsis* and tobacco plants with various constructs containing GUS^{Ssp}, either with or without GRP and extensin signal peptides. Since very similar results were obtained for GRP and extensin signal peptide constructs, the results for the extensin constructs were omitted for clarity of presentation. For comparison, equivalent constructs of GUS^{Eco} were also tested. All genes are with the catalase intron inserted within the coding sequence near the amino terminus, and driven by 35S promoter (figure 6.1).

A series of experiments were carried out on these materials to confirm and demonstrate significant improvements on histochemical analysis using GUS^{Ssp}. Figure 6.2 shows the results of histochemical staining on rice calli or tobacco leaf discs a few days after co-cultivation with *Agrobacterium* – a routine technique to monitor the transformation efficiency of the experiment. It is clear that the new GUS^{Ssp} reporter offers much quicker and stronger histochemical detection. With GUS^{Ssp}, blue spots on rice calli are visible within the first few minutes of staining, whereas with GUS^{Eco}, it normally takes more than 30 minutes (figure 6.2.A). Similar results are recorded with tobacco leaf discs. With GUS^{Ssp}, blue staining can be observed after 1 hour staining, whereas with GUS^{Ssp} signals are normally detected after overnight incubation (figure 6.2.B).

With GUS^{Ssp}, not only was the staining quicker, but the sensitivity threshold also increased significantly, resulting in a much larger number of blue spots on the transformed tissues (figure 6.2.A and 6.2.B). This means that GUS^{Ssp} offers more accurate estimation of the true transformation efficiency, which would have been underestimated by GUS^{Eco}.



GUS^{Eco}



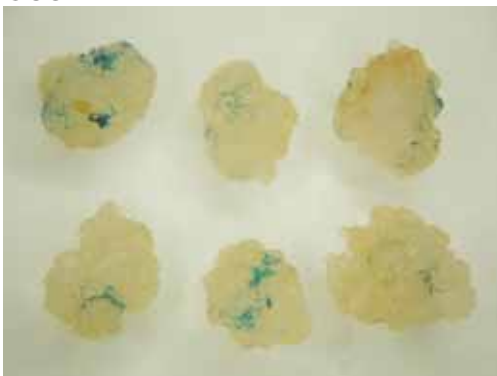
GRP-GUS^{Eco}



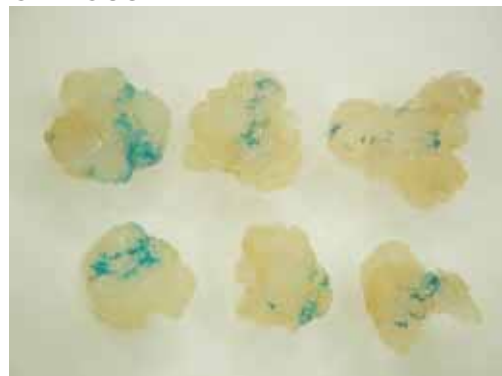
GUS^{Ssp}



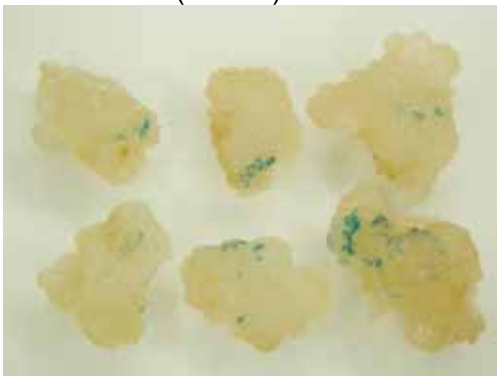
GRP-GUS^{Ssp}



GRP-GUS^{Ssp} (C499A)



GRP-GUS^{Ssp} (N118Q)



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

Figure 6.2.A. GUS^{Ssp} offers improved histochemical analysis.

GUS staining of rice calli a few days after co-cultivation with *Agrobacterium*. Data recorded after 30 min staining.

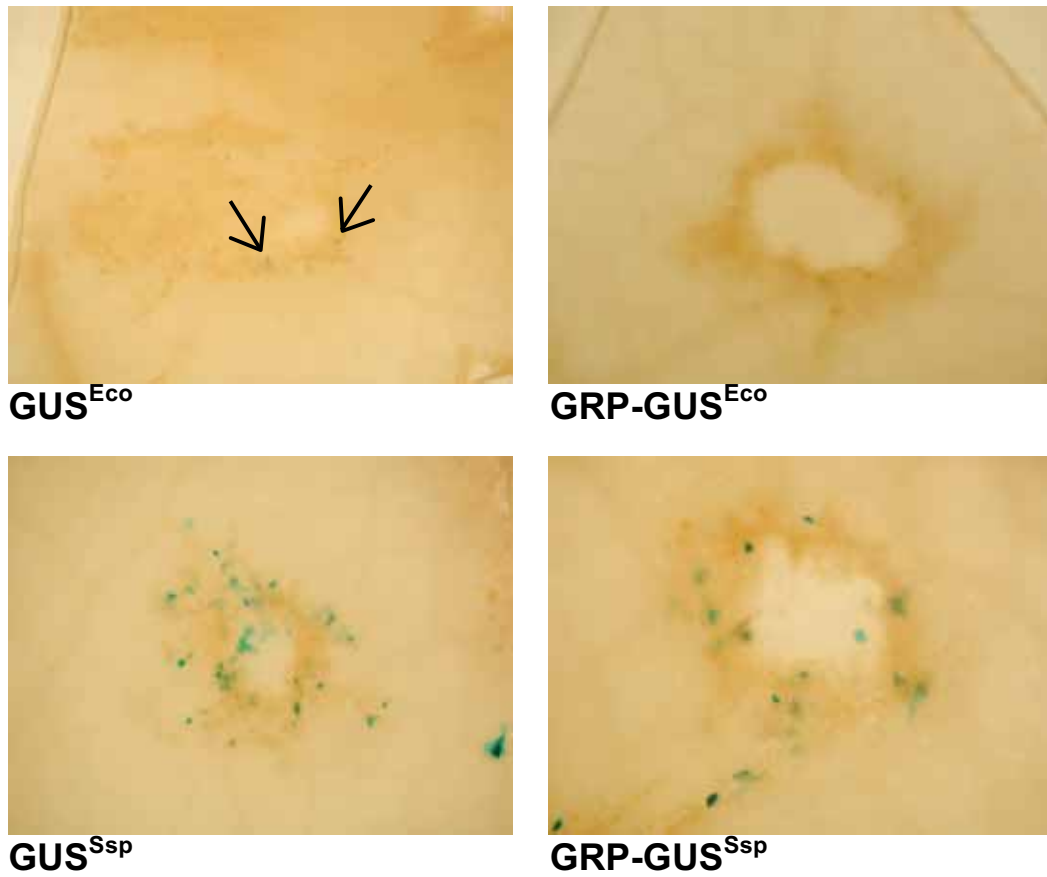


Figure 6.2.B. GUS^{Ssp} offers improved histochemical analysis.

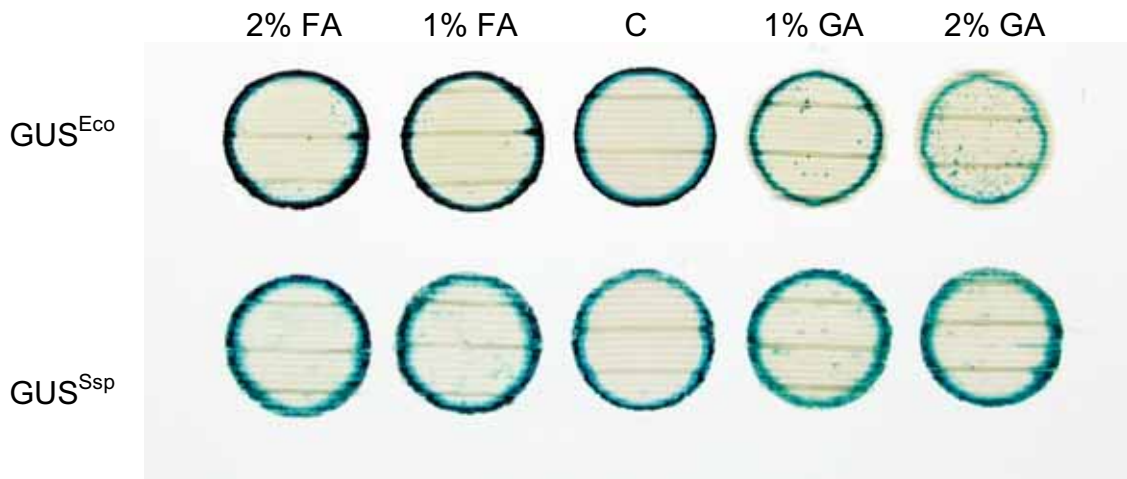
GUS staining of tobacco leaf discs a few days after co-cultivation with *Agrobacterium*. Data recorded after overnight staining.

It should be noted that in these experiments, the three GUS^{Ssp} variants: N118Q, C499A and N118Q&C499A, performed very similar to the wildtype GUS^{Ssp} (figure 6.2.A.). This indicates that their expression and enzymatic activities in plants were not significantly affected, and that their secretion was also similar to that of the wildtype.

Quick and strong staining results were also obtained with various tissues of transgenic rice and *Arabidopsis* plants. For example, in leaf sections of both species, staining signals are normally detected almost instantly for GUS^{Ssp}, and after about 30 min for GUS^{Eco} (data not shown). Several lab members at CAMBIA have used the new reporter gene constructs described in this thesis in their experiments to create libraries of thousands of transactivator-facilitated enhancer trap rice lines. They have found clear improvements in reporter performance using GUS^{Ssp}, especially in detecting more subtle enhancers whose activities would not have been detected using GUS^{Eco} (Koerniati and Fu, unpublished).

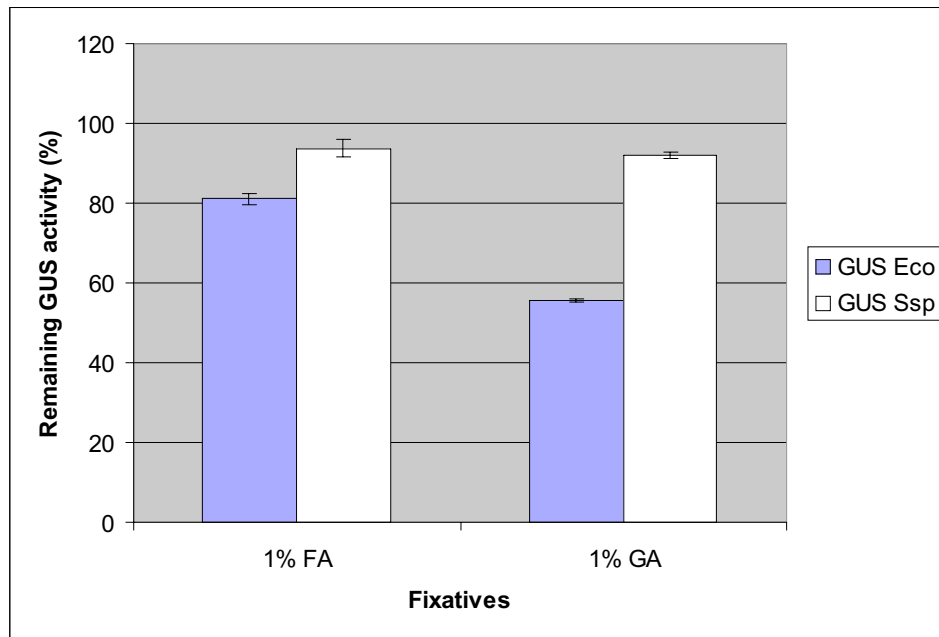
With higher detection sensitivity, we anticipate that in some applications such as promoter analysis, one might find that the promoter become “stronger” or more “leaky” (driving expression in some non-specific timing/tissues) than previously observed with GUS^{Eco} reporter. Such observation should not be regarded as “noise”, but rather a more sensitive reflection of the promoter activity.

Experiments were carried out to confirm and demonstrate better histochemical GUS detection after tissue fixation using GUS^{Ssp}. Small leaf discs of transgenic rice expressing GUS^{Ssp} and GUS^{Eco} were fixed with either formaldehyde or glutaraldehyde, and the remaining GUS activity was visually and quantitatively assayed (figure 6.3).



FA: formaldehyde
 GA: glutaraldehyde
 C: control

A. Staining of leaf discs after fixation with formaldehyde or glutaraldehyde.



B. Percentage of GUS activity remaining in rice leaf discs after one hour of fixation.

Figure 6.3. GUS^{Ssp} offers better histochemical detection after tissue fixation

As seen in figure 6.3.A, the most noticeable loss of staining signal is seen with GUS^{Eco} fixed in glutaraldehyde. No signal was seen at the edges of the GUS^{Eco} leaf discs, indicating that the enzyme activity was completely abolished in those areas. The high sensitivity of GUS^{Eco} to glutaraldehyde has been described in earlier research (Jefferson 1987). Biochemical analysis using purified GUS^{Eco} and GUS^{Ssp} enzymes has shown that, in 1% formaldehyde, GUS^{Ssp} retains more than 80% of its activity, while GUS^{Eco} activity drops very quickly to less than 20%. In 1% glutaraldehyde, GUS^{Eco} activity is completely abolished very quickly, while GUS^{Ssp} can retain about 20-40% of its activity (P. Wenzl and T. Nguyen, unpublished).

Quantitative measurement of the remaining GUS activities on the fixed leaf discs revealed the same trends for both enzymes (figure 6.3.B). There was no significant loss of GUS^{Ssp} activity after one-hour fixation in either 1% formaldehyde or 1% glutaraldehyde. For GUS^{Eco}, there was no significant loss of activity with formaldehyde, but with glutaraldehyde, only about 50% of activity was retained. It should be noted that in these quantitative assays, remaining activity was measured as a sum of activity in soluble extract (containing large proportion of soluble enzyme that has not been fixed) and in cellular debris (containing mostly fixed and cross-linked enzyme that remains active after fixation). If the experiment is repeated in the future, separate assays of these two fractions should be performed, to provide additional information.

It should also be pointed out that, as seen in figure 6.3.A, even penetration of the fixatives was not, and will likely never be, achieved. As a result, the centers of the leaf discs would presumably have retained full GUS activity. Therefore, the percentages of remaining activities obtained with the quantitative measurement above (figure 6.3.B) have obviously been overestimated, and should be normalized with the completeness of the fixation process.

The results of these experiments demonstrate that by using GUS^{Ssp}, better histochemical GUS detection after tissue fixation can be obtained. In general, the

robustness of GUS^{Ssp} would also be beneficial to other applications that require rough thermal and chemical treatments of tissues before enzymatic detection.

6.3.3 Demonstration of non-destructive GUS staining in various plant tissues

To demonstrate the possibility of non-destructive GUS staining, experiments were carried out to determine suitable non-destructive staining conditions, and cell viability after exposure to such conditions.

Regenerating rice calli harbouring GUS^{Ssp} and GUS^{Eco}, with and without GRP signal peptide were tested. The assays were carried out using X-glcA as substrate. With proliferating rice calli, staining with low X-glcA concentration (50-100 ug/mL) was as good as high concentration (1 mg/mL, as normally used in standard GUS staining protocols for plant tissues), in terms of staining speed and signal intensity. Furthermore, low X-glcA concentration effectively avoids overstaining, which could severely affect viability of the stained calli (data not shown). The non-destructive assay buffer consists only of X-glcA (50-100 uL/mL) in 20 mM NaPO₄, pH=7.0. Other components of the standard GUS staining buffer (Jefferson 1987) were omitted due to their potential interferences with cell viability.

With the non-destructive staining conditions mentioned-above, staining of GRP-GUS^{Ssp} calli were almost instant (within 1-2 mins), followed by GUS^{Ssp} (about 10 mins), and GUS^{Eco} (about 30 mins). With GRP-GUS^{Eco}, blue signals were only detected after overnight incubation.

Stained calli were then transferred back to regeneration plates, containing 50 ug/mL X-glcA, and observed for further proliferation. Figure 6.4 shows the results of proliferation two weeks after staining. It was clear that only GUS^{Ssp}, with or without GRP signal peptide, had good proliferation. There was no apparent proliferation with stained GUS^{Eco} calli.



Figure 6.4. Proliferation of rice calli after non-destructive staining.

Regenerating rice calli were stained with a non-destructive GUS staining solution. GUS-positive calli were subsequently transferred to new regeneration plates containing 50 ug/mL X-glcA. Only calli of GUS^{Ssp}, especially with the GRP signal peptide, show good proliferation. Data recorded two weeks after non-destructive staining.

Screening for GUS-positive regenerating rice calli is also possible by directly plating the calli on media containing 50 ug/mL X-glcA (figure 6.5). These calli were regenerating on hygromycin-containing media, so they should be hygromycin-resistant and GUS-positive. Almost all GRP-GUS^{Ssp} calli show very strong blue 'halo' in the media. Most calli of GUS^{Ssp} without signal peptide also show clear blue staining, but not as strong as that of the GRP signal peptide. In contrast, GUS^{Eco} calli, both with or without signal peptide, mostly show no staining.

Among the four constructs tested, GRP-GUS^{Ssp} was, as expected, the most suitable for use in non-destructive assays. GUS^{Ssp} without signal peptide (showing no apparent secretion – chapter V) performed surprisingly well. One possible explanation is that with the rice calli at regeneration stage (a stage when a large amount of cells in the calli are GUS-positive), there would be a significant amount of GUS^{Ssp} released to the extracellular space, due to cell death rather than secretion. Similarly, calli of GUS^{Eco} without signal peptide would also have a significant amount of GUS^{Eco} in the extracellular space. However, the robustness of GUS^{Ssp} would account for the better performance of GUS^{Ssp} over GUS^{Eco} in the assay. GRP-GUS^{Eco} was expectedly the least suitable for non-destructive assay on proliferating rice calli.

Taken together, these experiments (figure 6.4 and 6.5) demonstrate that with GUS^{Ssp}, especially the signal peptide construct, non-destructive screening for GUS-positive regenerating rice calli is possible, and the assayed calli are able to further proliferate. These non-destructive screening are also semi-quantitative assays for secretion of GUS^{Ssp} in rice calli, confirming the various results presented in chapter V.

It should be noted that our demonstration of non-destructive GUS staining was only done at the organ levels (rice calli). Although it may be argued that the results at the organ level reflect the sum of those at the single-cell level, confirmation of staining, survival and proliferation of GUS-positive individual cells (e.g. cell suspension or protoplast culture, with proper cell viability staining...) would be worthwhile.

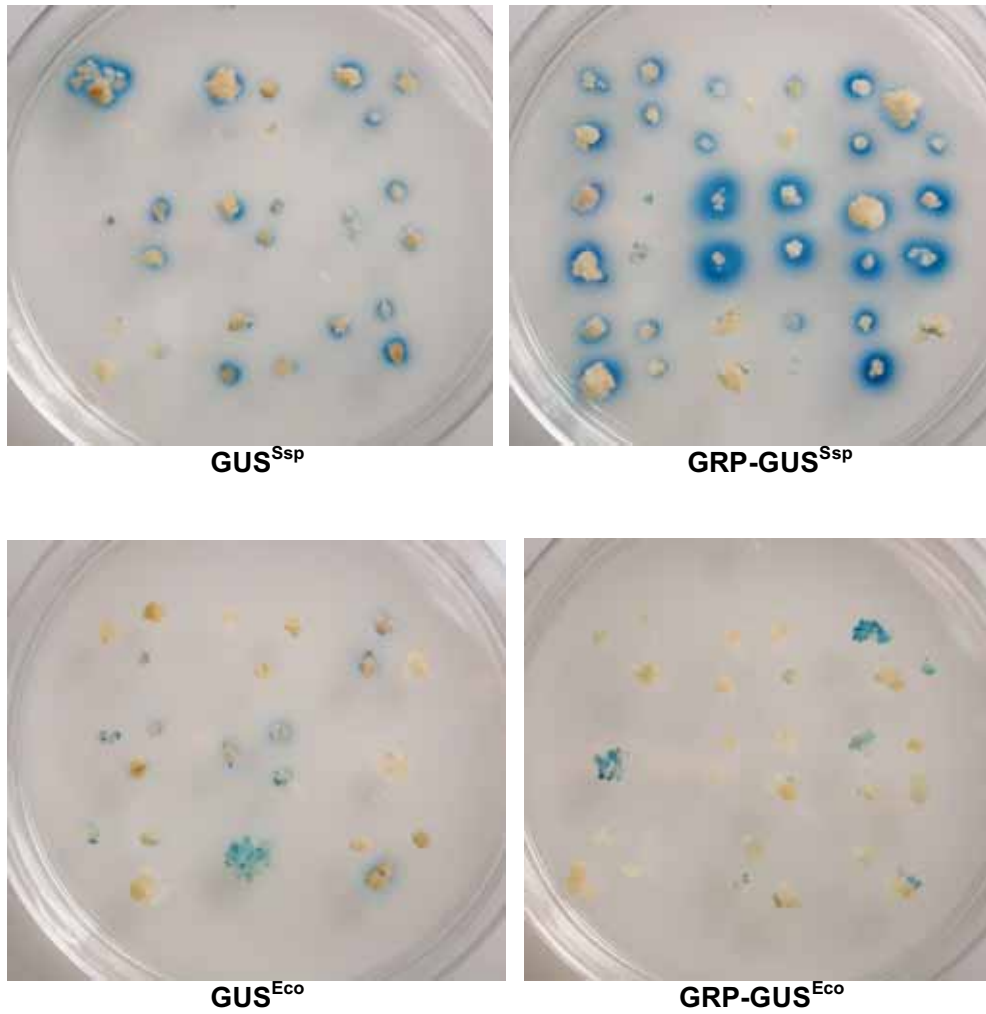


Figure 6.5. Screening for GUS-positive regenerating rice calli. Rice calli were transferred to new plates containing 50 ug/mL X-glcA for screening purpose. Data recorded after 2 hours.

At the whole plant level, efficient and non-destructive delivering of substrate, into the extracellular space of relevant cells or tissues, would be a key issue that needs further examination. In our preliminary observations, for example, substrate delivery into roots (rice and *Arabidopsis*) is possible simply by submerging the roots into an X-glcA containing solution. Uptake and transport of the substrate would require more extensive studies beyond this thesis.

The usefulness of these non-destructive staining in many current transformation protocols may be rather limited. Since transformed materials are typically selected according to their antibiotic resistance, there is little practical need for non-destructive GUS screening apart from monitoring purposes. The ultimate goal is to replace antibiotic selection altogether, by using secreted GUS^{Ssp} as a screenable marker as well as a selectable marker. In principal, it would be possible to physically isolate stained (transformed) cells and then propagate them. Such a scheme should be easily applicable with, for example, protoplast or suspension culture, in combination with a microscope, or a fluorescence-activated cell sorter (FACS). However, for transformation with rice calli or tobacco leaf discs, for example, physical isolation of few transformed cells among a large number of untransformed ones would be quite challenging. Here, a positive selection scheme, based on certain pro-active GUS substrates that give transformed cell an advantage to outgrow their neighbors, would be essential. That said, in transformation experiments where the efficiency of gene transfer is very high, and chimerism is readily dealt with, no selection at all could be a very effective tool, albeit perhaps not very efficient.

Positive selection based on GUS^{Eco} and cytokinin-glucuronides has been demonstrated with moderate success (Joersbo & Okkels 1996; Okkels et al. 1997). I was unable to carry similar experiments using secreted GUS^{Ssp}, due to the unavailability of the substrate. Synthesis of GUS^{Ssp}-cleavable cytokinin-glucuronides, and testing of the system will be of high priority. It should also be noted that, cellobiouronic acid could be

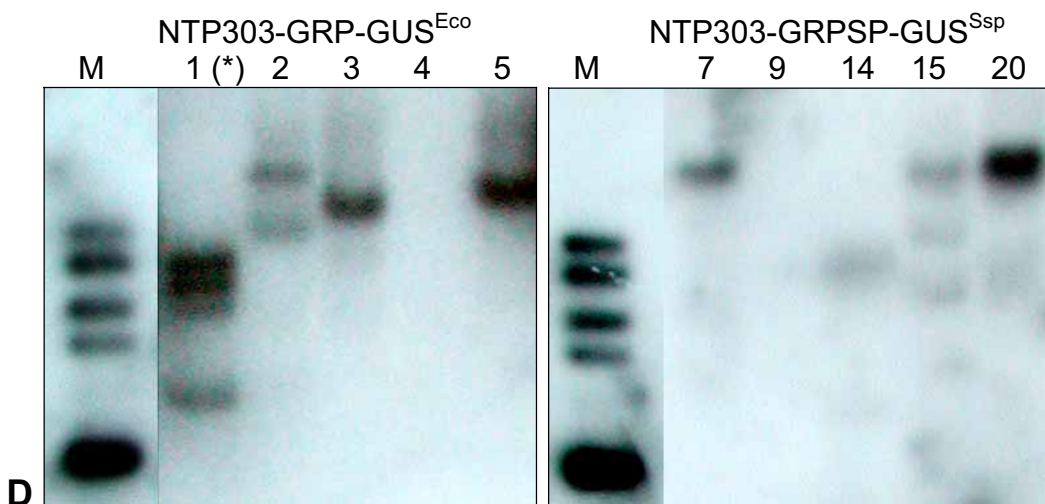
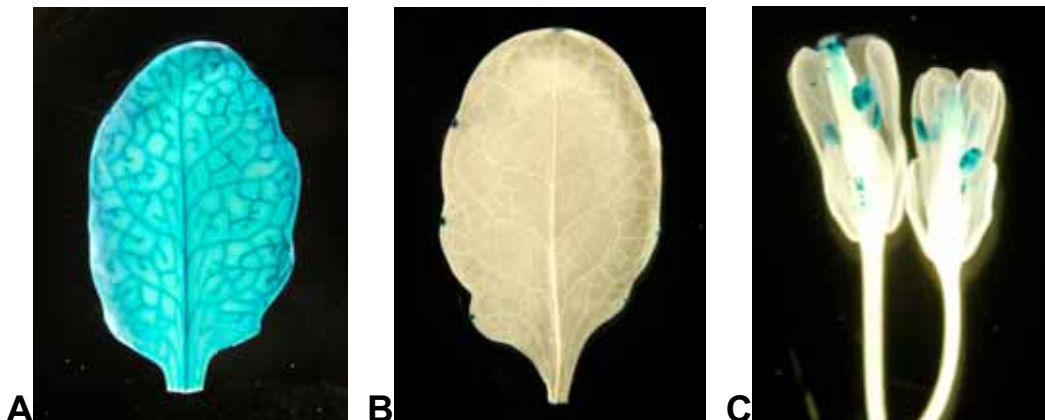
the ultimate substrate to be used in a positive selection scheme for crop transformation, and is a strong focus of future work.

6.3.4 Demonstration of potential use of GUS^{Ssp} as a tool for the manipulation of specific cells and tissues

Experiments were carried out to demonstrate the potential use of secreted GUS^{Ssp} in the manipulation of *Arabidopsis* pollen. In this experiment, the GRP-GUS^{Ssp} and GRP-GUS^{Eco} were under the control of the ntp303 pollen-specific promoter.

Twenty one transgenic *Arabidopsis* carrying the GRP-GUS^{Ssp} were regenerated. Histochemical GUS staining showed that all transgenic plants had very strong GUS expression in the mature pollens. However, only five plants showed GUS^{Ssp} expression exclusive in pollens. The other 14 plants had additional GUS expression in various other tissues (figure 6.6). It is probable that much of the “non-specific” GUS expression is a consequence of the strong enhancer activity of the CaMV35S promoter present in the same T-DNA (driving hygromycin-resistance). DNA constructs for co-transformation experiments to eliminate possible effects of the CaMV35S have been constructed, but not yet studied due to time constraints. In principle, this approach employs co-transformation of two independent T-DNAs (one carrying the ntp303-GRP-GUS^{Ssp}, and the other carrying CaMV35S-Hygromycin), to regenerate transgenic plants in which the two T-DNAs can be later separated via segregation.

Seven transgenic plants carrying the GRP-GUS^{Eco} were regenerated. No GUS staining was detected in any tissue, including in pollens, of these plants. The plants were later confirmed, by southern hybridization, to be truly transgenic (figure 6.6). This result is consistent with previous results presented in this thesis (chapter V and VI), that there is very little or no GUS activity detected in transgenic materials expressing SP-GUS^{Eco}.



Southern analysis of transgenic *Arabidopsis*

(*) The number depicts the transgenic plant ID

Figure 6.6. Analysis of ntp303 pollen-specific promoter in *Arabidopsis*.

Some GUS^{Ssp} plants had strong staining in other tissues such as leaf (A). Other plants had no staining in vegetative tissues, or very weak staining in the tips of the leaf (B). All plants showed very strong staining for pollens. The pollens can be stained by spraying the plants with a solution containing 50 ug/mL X-glcA (C).

All GUS^{Eco} plants had no staining in any part of the plants, including pollens. The plants were confirmed by southern analysis as true transformants (D).

GRP-GUS^{Ssp} plants were either sprayed with, or submerged briefly in, a staining solution (100 ug/mL X-glcA in 20 mM NaPO₄, pH=7.0). With these methods of broad substrate application, the pollens were always instantly stained upon contact with the X-glcA solution (figure 6.6). No staining was detected in other parts of the plants, even though they were stained with conventional GUS histochemical assays. It is assumed that the X-glcA substrate cannot penetrate the thick protective cuticle layers of the plants, nor can it be efficiently taken up and/or transported via the root system.

This experiment provides the proof-of-concept for pollen ablation methods. Synthesis and testing of pro-active substrates with cytotoxic aglycones is clearly necessary to further demonstrate the potential use of the method. In addition, uptake and systemic transport of different GUS substrates in plants is another line for future research.



7 Chapter VII: Conclusion and prospects

The codon-optimized *gusA*^{Ssp} gene, encoding a novel β -glucuronidase from *Staphylococcus* sp., was successfully constructed. Non-silent errors in the coding sequence were corrected. Several variants of the gene with different 5' ends, as well as N-glycosylation free and cysteine-free variants were created. Hexahis-tagged GUS^{Ssp} and GUS^{Eco} were expressed in *E. coli* and purified to high homogeneity. Polyclonal antibodies against these antigens were produced and affinity purified.

In yeast, both GUS^{Eco} and GUS^{Ssp} proteins, when provided with the invertase signal peptide, were very efficiently secreted, and mostly localized in the periplasmic space. This is the first report of efficient secretion of GUS^{Eco} in yeast. All GUS^{Ssp} variants (wildtype, N118Q, and C499A mutants) were secreted with high efficiency.

In plants, only GUS^{Ssp} was secreted when provided with either the GRP or extensin signal peptide. In contrast, GUS^{Eco} was not secreted, consistent with earlier reports. Secretion of GUS^{Ssp} resulted in the accumulation of the enzyme in the extracellular space, which was detected by a variety of methods, such as *in situ* enzyme histochemistry, and apoplastic fluid extraction.

An intron-containing version of the synthetic *gusA*^{Ssp} gene was constructed and used in many later experiments aimed at validating the use of GUS^{Ssp} as an improved reporter in plants. GUS^{Ssp} offers faster and more sensitive histochemical detection. Its activity can also be better detected after tissue fixation, allowing more precise histochemical localization of the enzyme. With secreted GUS^{Ssp}, non-destructive GUS assays were possible with tissues such as calli and roots. Tissue specific manipulation system using secreted GUS^{Ssp} was also demonstrated, with the example of secreted GUS^{Ssp} driven by a pollen-specific promoter ntp303.

Although the project has achieved many of its main goals, some important experiments remain to be done. For example, a comprehensive study on GUS^{Ssp} expression in *E. coli* needs to be carried out using proper expression cassettes in inducible systems, and a *recA*⁻ version of KW1 host, such as JEMA99.9 strain (chapter II).

Proper cleavage of signal peptides needs to be confirmed for many yeast and plant constructs. Such confirmation will provide important information for many aspects of expression and secretion in yeast and plants (chapter IV and V).

A very important remaining question is why GUS^{Eco} is not secreted in plants. The work in this thesis has provided some new and important information to what previously known (section 5.2). For example, we now know that GUS^{Eco} (N358Q) is secreted in yeast. Therefore, in yeast, the hypothesis that GUS^{Eco} is retained in the ER via cysteine-mediated retention is not valid. Whether the hypothesis is valid in plants can be addressed shortly, with the availability of materials and methods established in this project.

An important outcome of this thesis is the establishment of an improved reporter system based on GUS^{Ssp}. This system offers improved conventional GUS analysis, possible *in vivo* GUS analysis, and a variety of novel applications based on secreted GUS^{Ssp} and its pro-active substrates.

The amount of future work in developing this system is substantial. Extensive testings of the system (i.e. in different plant species and types of experiment) need to be performed in parallel, following the broad release of GUS^{Ssp} to the research community. It will be a long while until this new system can, if ever, match the maturity of the current GUS^{Eco} system.

The secretion efficiency and possible diffusion of secreted GUS^{Ssp} need to be established for various plants, and, especially, types of tissue or cell. In some cases,

further engineering with a membrane-anchoring signal, to retain the secreted enzyme within the secreting cells, may be necessary to prevent unwanted “cross-talk” effects.

Non-destructive GUS^{Ssp} analysis needs to be fine-tuned for different plants and types of tissue or cell. Transport and effective delivery of substrates to the appropriate extracellular space will be a topic for future research.

Novel applications utilizing secreted GUS and pro-active substrates will clearly be the focus of future work, which will be both challenging and rewarding. An effective GUS^{Ssp}-based positive selection scheme for the regeneration of transgenic crops will have profound and intertwined technological, intellectual property, and social impacts. Similarly, the manipulation of specific tissues using the combination of secreted GUS^{Ssp} expressed in specific tissue and pro-active substrates could lead to interesting applications, such as specific cell ablation methods (e.g. the production of male sterile plants).

The success of all these novel applications depends on the availability of proactive substrates. Testing of new and existing pro-active substrates (e.g. their cleavage efficiency, possible side effects, systemic transport etc) will certainly become more important. So will further engineering of the enzyme to obtain a desirable substrate specificity profile. It is anticipated that there will be several versions of GUS to fit a variety of specific applications. Many of these versions will be the results of advanced engineering, such as directed molecular evolution technique, to combine desired characteristics of different GUSes isolated from various sources.

The work in this thesis is clearly only the beginning of an expanded body of work aimed at developing a dual reporter/manipulator system using GUS^{Ssp}. The prospects of the new system are significant, and so is the amount of future work.