Histochemical localization of ß-glucuronidase (GUS) reporter activity in plant tissues

Stanislav Vitha
Microscopy and Imaging Center, Texas A&M University, BSBW 119, College Station, TX 77843-2257.
Email: vitha@mic.tamu.edu

This text is an abbreviated version of my published protocol *(35)*, and also includes some results from my dissertation *(36)*. There are numerous variations of staining protocols that have been successfully used for histochemical GUS localization. The protocol given below is a guideline that should be compatible with many plant specimens. Those wanting to learn about enzyme histochemistry in depth should consult the literature *(1-5)*.

1. Introduction

1.1 General Remarks

Jefferson (*6-9*) very properly exerted the possibilities existing for the localization of ß-glucuronidase (GUS) in transgenic plants bearing the *uidA (gusA)* reporter gene from *Escherichia coli*. The starting point of his work was the fact that in plant tissues GUS is present only very rarely, and even in such cases intrinsic GUS activity can be usually distinguished from the introduced one (*10,11*). The procedures proposed by Jefferson have been used by many authors to study various aspects of gene regulation and cell biology.

It should be stressed that any *in situ* assay of enzymes - including GUS - must follow appropriate steps to avoid false positive or false negative results, and false localization as well (*12,13*). Therefore, procedures for the study of GUS localization, which proved competent, will be provided and possible sources of errors highlighted here.

1.2. Characteristics of the Procedure

When assaying enzyme activity, three stages can be identified:

1. Processing of the object under study.
2. The incubation with the substrate („staining“) itself
3. Post-incubation handling.

Special attention should be paid to possible sources of error and their elimination.

1.2.1. Pre-incubation Processing of the Objects

For histochemical detection of enzymes, sections from fixed material are usually preferable (*see* Note 1). Fixation decreases the enzyme activity, but it minimizes the problems caused on the one hand by diffusion of the enzyme out of the tissue and on the other hand by capricious penetration of the substrate and/or other ingredients of the medium into the object (Fig. 1). In the case of GUS assays in sections from transgenic plants only faint or no staining is sometimes obtained. Moreover, in order to assess tissue- and organ-specific expression patterns, it is desirable to view the whole seedling or even the whole plant. That is why the *in toto* incubation is preferred, very often in combination with the addition of detergents into the medium and with vacuum infiltration (*see* Note 2). The objects are either live or, preferably, gently fixed (for a short time, in an ice-bath). Which procedure has been used must always be precisely stated. This concerns various combinations - to fix vs. not to fix, to section vs. not to section - in various orders. The fixation employed must always be explicitly stated as well. Most often, a buffered formaldehyde

or glutaraldehyde solution is used, which can be prepared according to different prescriptions (*4*).

1.2.2. Incubation (Histochemical Staining)

In the incubation itself it is necessary to distinguish the enzymatic reaction on one hand, and the visualization reaction on the other hand. In the enzymatic reaction the substrate is hydrolyzed and the soluble, colorless primary product is formed, which is then processed in the visualization reaction. Finally a colored product is obtained, the localization of which should correspond to the site within the tissue, where the enzyme is present. Various types of reactions can be distinguished.

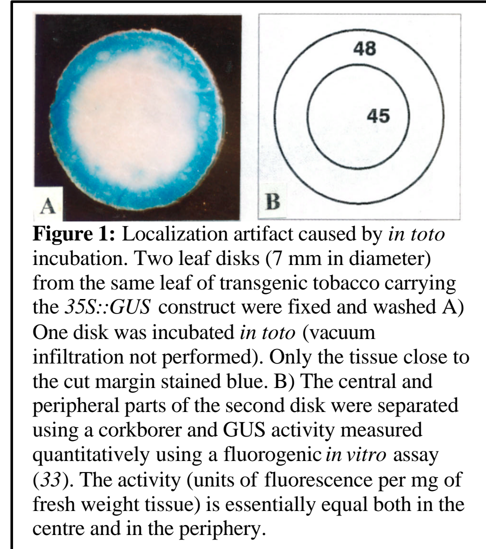

Figure 1: Localization artifact caused by *in toto* incubation. Two leaf disks (7 mm in diameter) from the same leaf of transgenic tobacco carrying the *35S::GUS* construct were fixed and washed A) One disk was incubated *in toto* (vacuum infiltration not performed). Only the tissue close to the cut margin stained blue. B) The central and peripheral parts of the second disk were separated using a corkborer and GUS activity measured quantitatively using a fluorogenic *in vitro* assay (*33*). The activity (units of fluorescence per mg of fresh weight tissue) is essentially equal both in the centre and in the periphery.

For the GUS reporter in plants, the indigogenic reaction is used almost exclusively. Variously halogenated substrates can be utilized, most often the 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc). The primary reaction product, 5-br-4-Cl-3-indolyl is colorless and soluble. This product is then oxidized and dimerized in the visualization reaction to form an insoluble, intensely blue final reaction product (indigo) The presence of ferri- and ferrocyanide in the incubation medium is a critical point for the visualization reaction in this procedure. It accelerates the formation of the final reaction product. It also protects the formed indigo from further oxidation, which would convert it to colorless or yellowish products. As illustrated in Fig. 2, the presence of the ferro- and ferricyanide minimizes the diffusion of primary reaction product and provides more precise localization. On the other hand, it concurrently inhibits the enzyme activity, though in our case the inhibition usually does not exceed 60 % (unpublished results).

Optimal concentration of the ferri-and ferrocyanide should be tested for a given specimen to find the best compromise between the intensity of staining and the precision of localization. Generally, concentrations between 0.5 and 5 mM each are used for most specimens, 1 mM being a good starting point. The principles derived from the works on carboxylesterases (*19,20*) are useful for all indigogenic procedures.

A procedure with a different chromogenic substrate, Sudan II-glucuronide, has been described (*23*), but the availability of the substrate is limited. Detailed comparative experiments are necessary before the usefulness of this technique is evaluated.

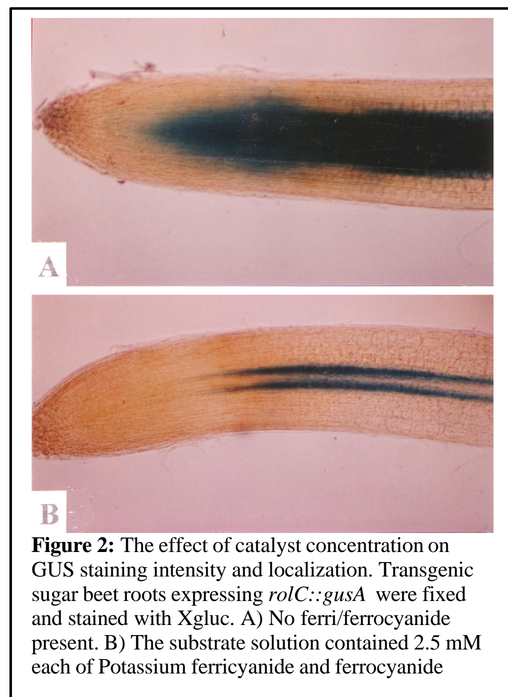

Figure 2: The effect of catalyst concentration on GUS staining intensity and localization. Transgenic sugar beet roots expressing *rolC::gusA* were fixed and stained with Xgluc. A) No ferri/ferrocyanide present. B) The substrate solution contained 2.5 mM each of Potassium ferricyanide and ferrocyanide

1.2.3. Post-incubation handling

For most plant tissues, removing chlorophyll and other pigments is necessary after GUS staining in order to see the blue reaction product. If the specimen is to be examined under the microscope, it may also be necessary to clear the specimen, i.e., to minimize light

scattering in the tissue. In most cases, extracting the chlorophyll in 70% ethanol and subsequent infiltration (clearing) with glycerol is sufficient. However, some specimens are rather difficult to clear. For specimens like seeds, a combined method of ethanol-acetic acid extraction and subsequent clearing in Hoyer's medium has been published (34).

The nature of postincubation handling should be considered in cases where permanent preparations are intended or if the treated material is to be stored for very long. Often the latter is the case, where the crystallization of final product could occur. This usually does not affect the results at the tissue level. In the case of permanent preparations the final reaction product is exposed to the actions of dehydrating, clearing and mounting media that could dissolve it or cause its redistribution. Fortunately, the final reaction product of the indigogenic reaction is insoluble in most solvents and is compatible with paraffin wax embedding and resin embedding. If embedding and sectioning of the stained specimen are to be performed, proper procedures (fixation, dehydration) must be followed to preserve tissue structure.

1.2.4. Possible Errors and Their Elimination

In order to minimize the possibility of false negative results, *in vitro* and *in situ* findings should be compared. For *in vitro* assays the fluorometric technique with 4-methylumbelliferyl ß-D-glucuronide as a substrate is usually used (*7,8,9*). The procedure based on hydrolysis of p-nitrophenyl ß-D-glucuronide is less sensitive.

There are several possibilities for obtaining false positive results. Although the intrinsic GUS activity is negligible in many plant materials, it can be significant in some objects. It applies namely to pollen grains (*10,11,24*). That is why it is important to perform a parallel assay with either non-transgenic material or material transformed in the same way but by a construct lacking the *uidA* gene. Various methods (*24-27*) of eliminating the intrinsic GUS activity (addition of methanol, elevated pH, increased incubation temperature; *see* Note 3) have been described.

Besides the intrinsic activity, false positive results can be caused by the presence of *uidA*-bearing *Agrobacteria* that survived the action of antibiotics. This danger is avoided by the use of constructs with an intron inserted in the *uidA* gene (*28,29*). Tör (*30*) has reported false positive results caused by endogenous *Corynebacterium*, possessing GUS activity. In the indigogenic reaction, false positive results could also appear due to the formation of colored product from ferri/ferrocyanide. The use of without-substrate controls is therefore recommended.

The danger of false localization has already been mentioned, namely when tissue processing was discussed. It is always necessary to consider the limits of the technique used. If the *in toto* incubation of large tissue blocks is performed, the results often cannot be assessed at the cellular or even subcellular levels. It is very difficult to disprove an objection that the enzyme pattern revealed after the *in toto* incubation is rather a consequence of uneven penetration of the medium into the tissue than the result of differential gene expression (*31, see also Fig. 1*).

2. Materials

1. 2x Buffer: phosphate buffer pH 7.0 (made of 0.1 *M* NaH_2PO_4 and 0.1 *M* Na_2HPO_4) or citrate-HCl buffer pH 7.0 (made of 0.1 *M* sodium citrate and 0.2 *N* hydrochloric acid).
2. Fixative: 4% formaldehyde, prepared fresh from paraformaldehyde, in 1x buffer. Prepare the fixative fresh on the day it is going to be used. Do not store for later use.
3. X-gluc substrate solution: Dissolve 1 mg 5-bromo-4-chloro-3-indolyl β-D-Glucuronide (X-Gluc) in 0.1 mL methanol (*see* Note 3), add 1 mL 2x buffer (*see* Note 4), 20 μL 0.1 *M*

potassium ferrocyanide, 20 μL 0.1 *M* potassium ferricyanide (see Note 5), 10 μL 10 % (w/v) solution of Triton X-100, 0.85 mL.water.

4. 70 % (v/v) ethanol.
5. 50 % (v/v) and 100 % glycerol.
6. Vacuum pump and dessicator

3. Staining Procedure

1. Fix for 30 minutes in ice cold fixative, shaking occasionally.
2. Wash for 30-60 minutes in several changes of ice cold 1x buffer.
3. Vacuum infiltrate the X-gluc substrate medium into the objects.
6. Incubate in darkness at room temperature or at 37 °C (see Note 6) several hours or overnight or until distinct blue staining appears (no longer than 24h).
5. Rinse in distilled water.
7. Incubate green objects in 70% ethanol until the chlorophyll is removed, then transfer to distilled water again. For seeds and other difficult objects, use alternative methods (34).
8. <u>Optional:</u> place specimens in 50 % glycerol (*see* Note 7), for 1 h, then transfer to pure glycerol, again leave for 1 h or more. Mount objects in 100% glycerol on microscope slides, examine under microscope.

4. Notes

1. The objects should be as small as possible to avoid localization artifacts caused by limited penetration of the substrate.
2. If problems with intrinsic GUS activity are encountered, it is recommended to use incubation medium with an increased concentration of methanol (20 % v/v), to elevate pH to 7.5 or to raise temperature to 60 °C (*24,25,26,27*).
3. In the original indigogenic procedure of Jefferson (*7,8,9*) N,N-dimethylformamide is used to dissolve the substrate. As dimethylformamide inhibits GUS activity (*25*) the use of methanol provides about 25% higher GUS activity and is threfore preferable (*32*).
4. In the citrate buffer, the GUS activity is about 20% higher than in the phosphate one. This was confirmed by in vitro experiments with X-gluc (unpublished results) and with the fluorogenic substrate (*33*).
5. The optimal concentration of the ferri- and ferrocyanide should be tested. A good starting point is 1 mM each.
6. With most specimens, no differences were found between results after incubation at the room temperature or at 37°C.
7. Mounting in glycerol after staining is not necessary; the objects can be examined mounted in water. Glycerol infiltration and mounting, however, provides better optical quality for microscopy.
8. The stained tissue can also be embedded in wax or resin and sectioned. Care should be taken interpreting the results.

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