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Specimen block counter-staining for localization of GUS expression in transgenic arabidopsis and tobacco

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Abstract A simple counter-staining procedure has been developed for comparative β-glucuronidase (GUS) expression and anatomical localization in transgenic herbaceous arabidopsis and tobacco. This protocol provides good anatomical visualization for monitoring chimeric gene expression at both the organ and tissue levels. It can be used with different histochemical stains and can be extended to the study of woody species. The specimens are paraffin-embedded, the block is trimmed to reveal internal structure, safranin-O staining solution is briefly applied to the surface of the block, then washed off and, after drying, a drop of immersion oil is placed on the stained surface for subsequent photographic work. This gives tissue counter-staining with good structural preservation without loss of GUS staining product; moreover, sample observation is rapid and efficient compared to existing procedures.

Keywords Dissecting microscope · Safranin-O · Specimen block staining · GUS localization · Transgenic arabidopsis and tobacco

Introduction

The β-glucuronidase (GUS) gene fusion system was developed to monitor chimeric gene expression in transgenic plant specimens (Jefferson et al. 1987), and the histochemical localization of GUS expression has been important in studying gene expression in, for example, tobacco, petunia, potato, *Brassica*, maize, soybean, wheat, rice, barley, and arabidopsis. Various methods have also been applied to examine the distribution of GUS expression (Gallagher 1992), including freehand sectioning, whole mounting, and thin sectioning followed by photographic analysis (Craig 1992).

Each approach, however, has advantages and disadvantages. For example, while simple free-hand sectioning is useful for analyzing GUS expression in cross-sections containing vascular tissues, tissue compression and damage during sample preparation can compromise the results obtained. Furthermore, while whole mounting is considered the easiest and simplest method for analyzing GUS expression, at either the organ or tissue level, the resolution obtained is not good. A more sophisticated procedure giving better resolution involves the thin sectioning of samples embedded in paraffin or resin, and this is useful for examining GUS expression at both cellular and sub-cellular levels (Craig 1992). On the other hand, in sections of embedded material, microscope slide preparation and the paraffin clearing procedure (Johansen 1940; Ruzin 1999) require great care and significant effort. Furthermore, the quality of the results obtained can vary with the quality of fixation, infiltration, embedding, and sectioning, as well as from loss of the GUS product during either sectioning or clearing of the paraffin and rehydration.

A major problem in GUS localization is how to obtain clear counter-staining that provides identification of the surrounding anatomical features and, thus, the cellular and tissue localizations of the GUS product. In some cases, this can be achieved by optical manipulation of the image with the microscope (phase contrast, dark field, etc) to create a contrast that provides for improved visualization of both the anatomy and GUS staining product (Craig 1992). In practice, however, this is limited to relatively thick microscopic slide sections with strong GUS staining. In another approach, counter-staining with toluidine blue O was reported (Craig 1992), but this can also mask weak GUS staining.

As an alternative to the techniques and potential problems mentioned above, a rapid and simple counter-

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staining procedure was developed to more clearly monitor the anatomical localization of expression of the *DIRIGENT-GUS* fusion product in transgenic arabidopsis and tobacco. [The dirigent genes encode proteins involved in stipulating the outcome of phenoxy radical-radical coupling (Davin et al. 1997; Kim et al. 2002a).] In this procedure, the target tissue is embedded in paraffin, and the infiltrated block is trimmed. The resulting exposed tissue at the surface of the block is then stained and imaged directly without further sectioning. This procedure thus avoids potential problems related to microscopic slide preparation and paraffin clearing and simplifies specimen preparation and counter-staining protocols while preserving both anatomical detail and the level of GUS staining. Furthermore, large numbers of samples can be readily processed in the time that it typically takes to prepare individual sections.

Materials and methods

Plant material

Transgenic arabidopsis plants expressing western red cedar *DIRIGENT* promoter::*GUS* fusion products were prepared as described by Kim et al. (2002b), whereas the transgenic tobacco expressing the *Forsythia DIRIGENT* promoter::*GUS* fusion products were generated through an *Agrobacterium*-mediated transformation following the leaf-disc method as described by Horsch et al. (1985).

Histochemical staining, fixation, infiltration, and block casting

GUS histochemical staining of transgenic arabidopsis and tobacco was conducted following the procedures described by Jefferson et al. (1987). Fresh plant organs were immersed in GUS staining solution containing 0.25 m*M* X-glu (X-glu = 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt; GBT, St. Louis, Mo.) in 50 m*M* sodium phosphate buffer (pH 7.0) and incubated for 5–24 h at 37°C. The plant tissues were then fixed in FAA (4% formaldehyde, 50% ethanol, 5% acetic acid), dehydrated in an ethanol series, and infiltrated with xylene followed by paraffin. Blocks were cast following a general embedding procedure (Ruzin 1999).

GUS localization with the new procedure

Specimen block trimming and counter-staining

The specimen block was trimmed to give a trapezoid shape and then cut with a microtome until the internal anatomy of the specimen was exposed at the region of interest. A droplet of 0.1% (wt/v) aqueous safranin-O staining solution was placed directly on the trimmed specimen block surface for 5–60 s, with the excess stain washed off with distilled water followed by air drying. In some instances, the exposed surface was first imaged without safranin-O staining and then imaged again after safranin-O staining.

Photographic work

Stained surfaces of specimen blocks were treated with immersion oil and analyzed using a dissecting microscope (Wild, Heerbrugg, Switzerland) and epi-illumination, with images captured using a Micro Image Video System (Bartels and Stout, Bellevue, Wa.) interfaced to a MacIntosh computer at the Washington State University Electron Microscopy Center. In some cases, a droplet of Histo-Clear (International Diagnostics, Atlanta, Ga.) or Hemo-D (Hemo-De clearing agent; Fisher, Norcross, Ga.) was momentarily placed on the specimen, this being washed off with ethanol, with the specimen analyzed as before.

GUS localization with a conventional procedure

Section and slide preparation, paraffin clearing, rehydration, and counter-staining

Each specimen block was trimmed to give a trapezoid shape, and sections were cut with a microtome and steel blade to make ribbons. The ribbons were floated on a water bath at 50°C for 5 min to evenly spread out the compressed sections, and then they were placed on gelatin-coated slides. The slides were placed on a warm plate (40°C) for at least overnight. When the slide sections were completely dried, the paraffin was removed with xylene, and the samples were rehydrated in an ethanol series. Rehydrated sections on slides were stained in 0.5% safranin-O (Fisher) solution for 6–12 h. After staining, the sections were dehydrated through an ethanol series to 100% ethanol, placed in xylene, and then covered with mounting medium (Permount, Fisher) and a cover slip.

Photographic work

Slide-mounted sections were analyzed with an Olympus BH-2 compound microscope (Olympus, Tokyo, Japan), with images captured using a Micro Image Video System at the Washington State University Electron Microscopy Center.

Results and discussion

Enbloc counter-staining of GUS localization: specimen block staining versus conventional microscopic section preparation

Microtome sections of paraffin blocks gives specific exposure of the anatomical region(s) of the specimen of interest, with these having flat exposed surfaces with a well-preserved structure. Thus, for controls embedded in paraffin, the safranin-O staining results in red cell walls following short periods of staining (Fig. 1A, C). Then, depending on the intensity of GUS staining, specimen blocks counter-stained for 5–60 s gave a good contrast of red wall staining and blue GUS staining product. Generally, 20–40 s of counter-staining gave an optimal contrast that was intense enough to identify the tissue localization of blue GUS staining without masking GUS expression. However, a longer exposure with safranin-O can, in some cases, stain the cytoplasm and thus eventually mask GUS staining. Yet even over-staining with safranin-O can be used to advantage (Fig. 1C–K): the surface of an over-stained block can be sliced at a thickness of 2–6 µm with a microtome until the required contrast level is achieved – i.e. continuous sectioning gradually reveals weaker and weaker safranin-O staining and thus permits a variety of levels of counter-staining. Accordingly, this method is superior to that of toluidine blue O (blue color) staining, which has been reported to aid in the localization of GUS histochemical staining; in

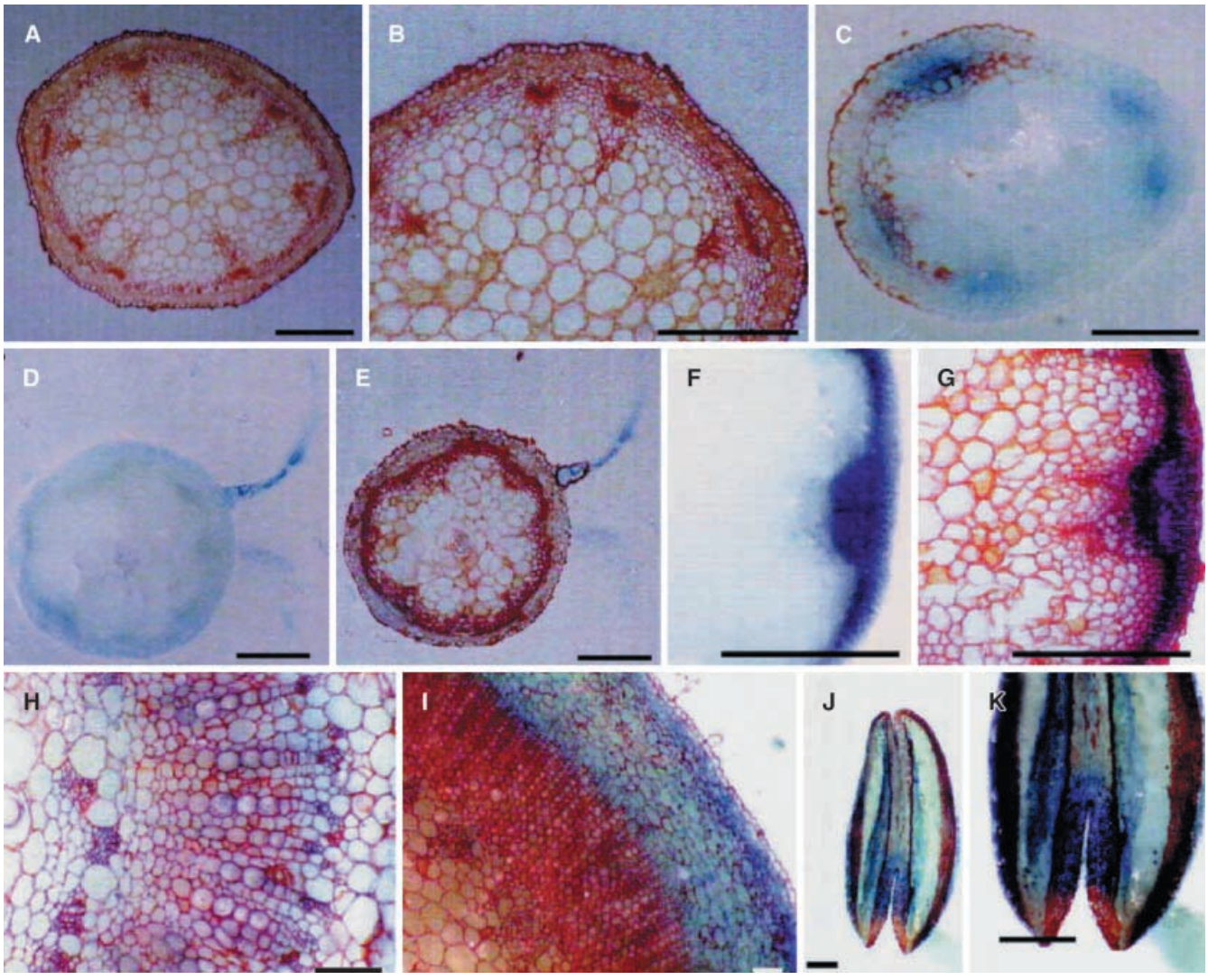

Fig. 1A–K Specimen enbloc staining with safranin-O for localization of histochemical GUS staining, with staining carried out directly on the paraffin-embedded specimen block surface. **A, B** Safranin-O staining reveals the anatomy of wild-type arabidopsis stem sections, **C** safranin-O counter-staining showing histochemical GUS localization in one-half of an arabidopsis stem section, **D** histochemical GUS staining of a trichome in an arabidopsis stem section, **E** safranin-O counter-staining showing histochemical GUS localization in the trichome of an arabidopsis stem section, **F** histochemical GUS staining of an arabidopsis stem section, **G** safranin-O counter-staining showing histochemical GUS localization in arabidopsis stem section, **H, I** safranin-O counter-staining showing histochemical GUS localization in a tobacco petiole and stem sections, respectively, **J, K** safranin-O counter-staining showing histochemical GUS localization in transgenic tobacco anther tissues. *Bars* 300 µm

those cases, both stains are blue and thus are difficult to differentiate (Craig 1992).

Various permutations of this method can also be applied. One example is enbloc staining of one-half of an arabidopsis stem surface with safranin-O, leaving the other half unstained (Fig. 1C); this is achieved by simply placing a droplet of staining solution on one-half of the block surface. Others stains, such as protein-selective dyes and phloroglucinol for lignin staining, can be successfully applied to the surface of interest in the same way (data not shown).

In an intact block, GUS staining was observed in a specific area 2–3 µm deep. For example, in a trichome (Fig. 1D, E), subsequent counterstaining revealed the surrounding cell structures, yet the GUS expression localized in the trichome still remained clearly (Fig. 1E). This is in contrast to typical microscope section preparation, whereby several serial sections need to be cut and mounted. In such cases, it is more difficult to obtain an image of a trichome region through the sample tissue, since sections can either be lost or damaged during paraffin removal with xylene and/or in the subsequent rehydration, staining, dehydration, and infiltration steps. In addition, sections through small or thin objects, such as trichomes, may only contain a portion of the object in any one section (data not shown).

It was also found that when either a deep counterstaining was required or when counter-staining was not substantial (e.g., with a specimen fixed for improved

structural preservation with either a high ratio of formaldehyde or glutaraldehyde), then a droplet of Histo-Clear (International Diagnostics) or Hemo-D (Fisher) could be briefly placed on the specimen (Fig. 1F, G) followed by washing with ethanol. Histo-Clear or Hemo-D treatment dissolved either the etched surface and/or air bubble-trapped rough surface, which resulted in a smoothening of the surface. Accordingly, the subsequent counter-staining was significantly facilitated with deep staining clearly showing anatomical features (Fig. 1G): this treatment thus improved specimens having air bubbles, such as those obtained during incomplete infiltration, presumably since it results in some etching of the paraffin from the surface (specimens presented in Fig. 1G–I were generated via this treatment).

In contrast, for procedures involving conventional microscope slide preparation, there are in general several factors that cannot be determined until the final specimen section is analyzed in the microscope; these can include quality of fixation, infiltration and sectioning, knife condition, microtome operation, and specimen cleanliness. Each of these can have a critical impact on the final specimen image. Other limitations of conventional microscope slide preparation that are commonly experienced include: (1) in tissue collections, small tissue samples are preferred to avoid a hard material, otherwise a tissue-softening procedure is required to facilitate microtomy (Johansen 1940; Peterson et al. 1989); (2) during dehydration and infiltration, transferring the tissue through a series of liquids can cause loss of material that affects ultimate quality of the section (Feder and O'Brien 1968); (3) embedding with either trapped gas bubbles or poorly infiltrated tissues can interfere with sectioning (Ruzin 1999); (4) during microtomy for sectioning and section mounting on the slide, section and ribbon defects, such as block chatter, compression, vertical marks, curling, ribbon wrinkle, ribbon crooking, and bubbles under paraffin sections, are common (Ruzin 1999). However, these limitations are overcome using this specimen block counterstaining protocol.

Additionally, making large paraffin sections from older tobacco petioles and stem tissues has historically been difficult due to both the lignified tissues present and the sample size (approximate diameter of stem: 0.5 cm). In contrast, specimen block staining provides a high-quality surface with minimal damage, good contrast with the counter-stain technique, and easily detectable GUS product at the tissue and cellular levels in both the petiole (Fig. 1H) and even with the tougher stem tissues (Fig. 1I). As generally recognized, microscope slide preparation can be a major limitation for the examination of hard, woody, tissues by molecular techniques such as immunolocalization and in situ hybridization methods (Jackson 1991). Thus, the specimen block staining procedure described is potentially useful and applicable to both in situ hybridization and immunolocalization of woody plant tissues. Another potential application is with in situ polymerase chain reaction (PCR) for detection of foreign gene infection (Hasse et al. 1990) using a resin specimen block in the PCR reaction solution.

Retention and localization of product generated by GUS activity in conventional paraffin and resin embedded material is also very time-intensive and requires many processing steps that can lead to loss of the GUS product. Hence, in summary, this specimen block staining procedure can save considerable time, chemicals, equipment, space, and use of facilities when compared with typical microscope slide preparations**.**

Photographic work

Photographic documentation is also a critical step in a histochemical GUS assay (Craig 1992). However, in the case of tobacco anther tissue sampled at a late anthesis stage, thin sections only gave a faint GUS localization with compound microscope imaging (data not shown). In contrast, the whole block procedure provided a vivid image of localized GUS product (Fig. 1J, K). Additionally, whereas sections conventionally prepared are analyzed with a compound microscope, stained specimen blocks are analyzed with a dissecting microscope with greater depth of field and more flexible overview of the sample. This was demonstrated with the clear color contrasts between GUS product and tissue anatomy (see Fig. 1C, E, G–K). By contrast, observing sections with a compound microscope and adjusting the optics to generate cell-boundary contrast can lead to significant dispersion and diffraction which interfere with the color contrast, resulting in either diminished GUS product detectability or more artificial color output in the final photograph (data not shown).

In conclusion, with respect to microscopic resolution and the capacity for blue GUS product detection, specimen enbloc staining is suitable for generating good contrast between tissues and cells of interest and the GUS product. The magnification that can be achieved with a dissecting microscope is optimal for detecting GUS localization at the tissue and organ levels. It is also suitable for the rapid examination of a large number of samples. In addition to GUS localization, the specimen enbloc staining procedure may be useful for other types of histochemical staining as well as for the molecular and anatomical studies of historically difficult sample types, such as those in woody species and bone-containing animal tissues.

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