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ORIGINAL RESEARCH

Fake news blues: A GUS staining protocol to reduce false-negative data

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Abstract

The β-glucuronidase gene, *uidA* (*GUS*), has remained a favorite reporter gene in plants since its introduction in 1987 for its stability and versatility in a variety of fluorometric, spectrophotometric, and histochemical techniques. One of the most popular uses is as a reporter gene for visualizing endogenous promoter activities within plant tissues. Despite this popularity, specific protocols for minimizing nonrepresentative staining patterns, including false negatives, in challenging tissue types are not common. This became a large issue during our work on dark-grown *Arabidopsis* hypocotyls, and we set out to develop a protocol that would ensure accurate staining in a tissue that is biologically resistant to reagent penetration. Through extensive testing using a variety of constitutive and endogenous promoter::GUS fusion lines, we have developed an optimized GUS staining protocol that combines the use of acetone as a fixative, deliberate physical damage, and proper positive and negative controls to help ensure accurate staining along the hypocotyl while minimizing false negatives. Hopefully, our recommendations will allow for improved staining that more accurately reflects the true activity of cloned endogenous promoters and thus facilitate a more accurate understanding of promoter activity in *Arabidopsis* hypocotyls and other hard-to-stain tissues.

KEYWORDS
dark-grown hypocotyls, GUS stain, marker gene, β-glucuronidase

1 | INTRODUCTION

The use of the *Escherichia coli* β-glucuronidase gene *uidA* (*GUS*) as a reporter gene in plants dates back to 1987 with its introduction by Jefferson et al. (1987) and is still used extensively today. It remains a favorite reporter gene due to its high sensitivity, enzyme stability, and simplicity of detection via fluorometric, spectrophotometric, or histochemical techniques.

GUS is particularly popular as a reporter gene for assaying endogenous promoter activity via histochemical staining, wherein plants expressing the transgenic *uidA* gene under the control of a cloned promoter sequence are treated with a buffer containing the substrate 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-Gluc). X-Gluc is cleaved by GUS into two indoxyl derivatives that oxidatively dimerize to form the compound 5,5′-dibromo-4,4′-dichloro-indigo, the blue pigment that is a characteristic of GUS staining. In plants where *uidA* expression is driven by an endogenous promoter sequence, this blue pigment will theoretically only appear in tissues and cells in which the promoter is currently active, making for an easy and quick way to determine or verify the location of promoter activity.

Although the use of fluorescent reporter proteins such as green fluorescent protein (GFP) may also be used for promoter activity

investigation, GUS still retains some advantages. First, GUS staining does not require the use of expensive confocal microscopy equipment and involves a protocol that is accessible to all skill levels. Second, GUS is a very stable protein with a protein half-life of multiple days, compared to that of GFP (~18 h), allowing for increased flexibility for experiments (Ruijter et al., 2003). GUS is also much more sensitive than GFP; GFP signal is constrained by the total amount of protein in a cell, whereas a single GUS protein can process multiple X-Gluc molecules, thus amplifying the signal and allowing for very lowly expressed protein abundances to be visualized. Using GFP in plants is also constrained by the overlap of the excitation of GFP and chlorophyll; the auto-fluorescence of chlorophyll can obscure the GFP signal when the protein is lowly abundant (Ruijter et al., 2003). Finally, there are hundreds of GUS promoter-fusion lines available from stock centers, such as the Arabidopsis Biological Resource Center (ABRC) and the Nottingham Arabidopsis Stock Centre (NASC), providing a large resource pool for ongoing Arabidopsis research without requiring de novo cloning and transgenic plant generation.

Despite the popularity of promoter-GUS fusions, there remain some caveats, the largest of which is that GUS staining relies on the penetration of the X-Gluc substrate and other reagents into the tissue. During our work with dark-grown *Arabidopsis* hypocotyls, we noticed a tendency for GUS staining to vary in intensity and accuracy in a way that did not reflect the predicted activity of promoters under study (based on RNA-based expression experiments in the lab). In our experience, GUS staining in dark-grown hypocotyls was often patchy and did not match the patterns seen in plants expressing other reporter genes, such as GFP; we also noted that such patchy staining was common in the literature. Thus, to help improve our interpretation of GUS staining data in the lab and provide guidance for other scientists, we set out to optimize our staining protocol to enhance the staining accuracy in dark-grown hypocotyls. After testing both fixation and agitation techniques, we recommend a combination of acetone fixation and deliberate tissue damage, maximizing the penetration of the staining solution, to improve staining accuracy.

2 | RESULTS

2.1 | Acetone fixation improves GUS staining intensity

During our work, we noticed that GUS staining in dark-grown hypocotyls oftentimes produces patchy staining patterns that did not correspond with the localization patterns seen when using other reporter proteins. Hypocotyls in particular have several physiological features that may be causing suboptimal GUS staining. The cells of dark-grown hypocotyls are not uniform along the length of the organ; they vary in length, cell wall composition (Bou Daher et al., 2018), cell wall thickness (Refrégier et al., 2004), and cuticle composition and structure (Narukawa et al., 2015; Refrégier et al., 2004), all factors that may be affecting penetration of the GUS staining solution. Due to the high prevalence of GUS as a promoter–reporter fusion in plants, we set out to develop a protocol that would allow for confidence in our interpretation of staining patterns.

To initially assess the efficiency of GUS staining in dark-grown hypocotyls, we tested whether the use of acetone as a fixative could improve staining. The use of a fixing agent before staining offers several advantages, including preservation of the tissue, prevention of transcriptional and translational changes after harvest, potentially improved penetration of the staining buffer, and a more flexible time frame for staining. Although several fixatives may be used with GUS staining, including chloroform and various alcohols, acetone provides several attractive features: It penetrates tissues quickly (Talbot & White, 2013), is a more efficient preservative for DNA, RNA, and proteins at a variety of temperatures and for longer periods (Fukatsu, 1999), and can double as a pigment clearing agent (Su et al., 2010; Sudhakar et al., 2016). Most importantly, it is more effective at disrupting the wax cuticle of plants (Myung et al., 2013), which we hypothesized was a major obstacle for achieving uniform GUS staining in dark-grown hypocotyls.

Dark-grown seedlings carrying a transgene for "constitutive" GUS expression (*p35S::GUS*) were harvested 48-h post-germination (HPG) and treated either with or without an overnight incubation in ice-cold 90% acetone (Figure 1). In nonacetone-treated hypocotyls, we observed little staining (Figure 1a) or patchy staining (Figure 1b) along the hypocotyl organ, and when quantified, GUS signal was extremely low at all points along the hypocotyl (Figure S2); we hypothesize that patches of localized staining were caused by accidental damage during processing, suggesting that a lack of staining in the hypocotyl may be due to dye access and not lack of promoter activity. Although the *p35S* promoter is known to exhibit some differences in expression based on developmental stage and tissue type (Sunilkumar et al., 2002), we do not believe this is an issue in the dark-grown *Arabidopsis* hypocotyl based on examination of transgenic lines that exhibited "constitutive" patterns with other reporter genes (e.g., p35S::H2B, Figure S1). The use of acetone improved three aspects of GUS staining in dark-grown seedlings (Figure 1c): (1) As expected, it cleared the pigments from the cotyledons to allow for better visualization of GUS in the tissue, (2) it increased the intensity of the staining overall both visually and quantitatively (Figure S2), and (3) it improved staining within the base and middle regions of the hypocotyl, with intense vascular staining. In addition, acetone fixation allows for easier processing of time-series samples. Despite these improvements, we still observed a lack of staining within the hypocotyl, particularly in the apical region and hook, as well as large areas of the cotyledons. Given the increased staining in the hypocotyl by both acetone and accidental damage, we next undertook a hybrid approach to further improve staining solution penetration in the dark-grown hypocotyl.

2.2 | GUS staining is improved by deliberate physical damage

Although 90% acetone can solubilize components of the cuticle, thus allowing for enhanced penetration of staining solution, it does not

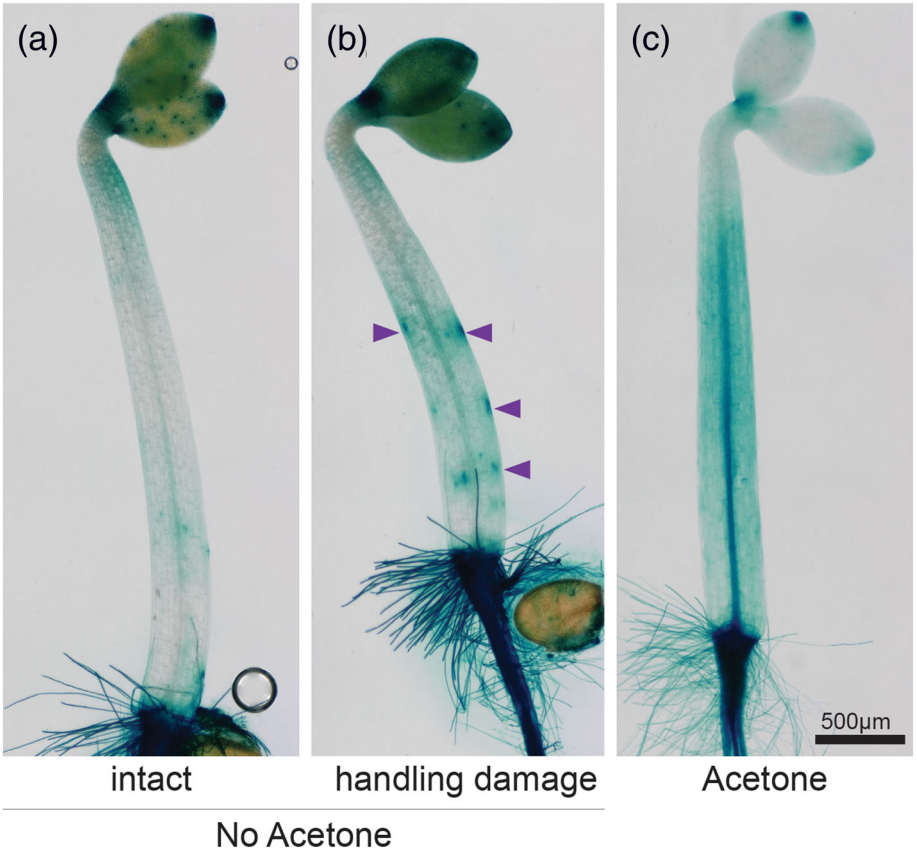

FIGURE 1 Acetone improves GUS staining in dark-grown hypocotyls. *p35S::GUS* 48HPG dark-grown seedlings treated stained without acetone fixation, undamaged (a), without acetone with accidental damage (b), and acetone fixed, undamaged (c). Scale bar = 500 um. Purple triangles indicate accidental damage sites. GUS, β-glucuronidase gene

completely remove this barrier but using 100% acetone can damage cellular integrity (Myung et al., 2013). Given that GUS staining was intensified at areas of accidental damage (Figure 1b), we decided to treat seedlings with acetone, minimize accidental damage, and introduce deliberate physical damage to improve penetration of the GUS staining solution in recalcitrant areas.

To start, we developed an easy, inexpensive way to minimize accidental damage to dark-grown hypocotyls. We created small mesh-bottomed baskets by melting the top 1.5 cm of 1,000-µl pipette tips onto pieces of 1-µm nylon mesh (see Methods S1; Figure S6). Once placed in these baskets, hypocotyls can be transferred between solutions without accidentally damaging the tissue with forceps. After acetone fixation, the samples were more prone to bending and our baskets also minimized such disturbance during transfer between solutions. We note that the mesh size may be varied easily during creation based on the target sample size; 1-µm mesh worked well for dark-grown *Arabidopsis* hypocotyls from germination to 48HPG.

To introduce deliberate damage in seedlings, we took two approaches: (1) de-foliation to allow solution access to the hook and apical regions through an apical cut surface, and (2) introducing points of damage along the hypocotyl length to allow periodic solution access. Seedlings constitutively expressing GUS (*p35S::GUS*) were harvested at 24HPG and 48HPG, subjected to deliberate damage and compared with nondeliberately damaged controls (Figure 2). Importantly, none of the deliberate damage methods used resulted in false-positive staining in control plants at either time point (Figure S4). De-foliation resulted in intense local staining at the apical cut surface, but this increase was very localized and only spread 149.5 ± 30 µm down the hypocotyl (Figures 2c,d and S2). We also tried segmenting hypocotyls into 3–4 pieces and saw a marked improvement in staining intensity (Figure S3), but because segmenting removes all spatial context and individual segments can easily escape through the nylon mesh of the transfer baskets, we did not pursue this as an ideal method. It is plausible that a complete vertical cut along the hypocotyl length may allow for full solution penetration of the hypocotyl, but this is technically challenging due to the small size of the seedlings.

To introduce deliberate points of damage along the hypocotyl length, we performed roughly equidistant stabbing of the hypocotyl using a 5-µm needle. At both 24HPG and 48HPG, needle stabs improved GUS staining along the hypocotyl including resulting in positive staining in areas that did not stain well when left intact (apical segment, hook, and cotyledons) (Figures 2e,f and S2). Interestingly, staining in recalcitrant areas was localized around the stab with little diffusion, similar to the result from de-foliation; this suggests that the issue of solution penetration is not solely due to cuticle resistance, but that tissue structure may also limit solution movement. This can also be seen in 0HPG seedlings, which stained uniformly without damage, but still exhibit increased staining intensity at stab sites (Figure S5). The consistent staining in basal and middle region vasculature also supports this hypothesis as vascular tissue may provide a more "open" route for solution movement.

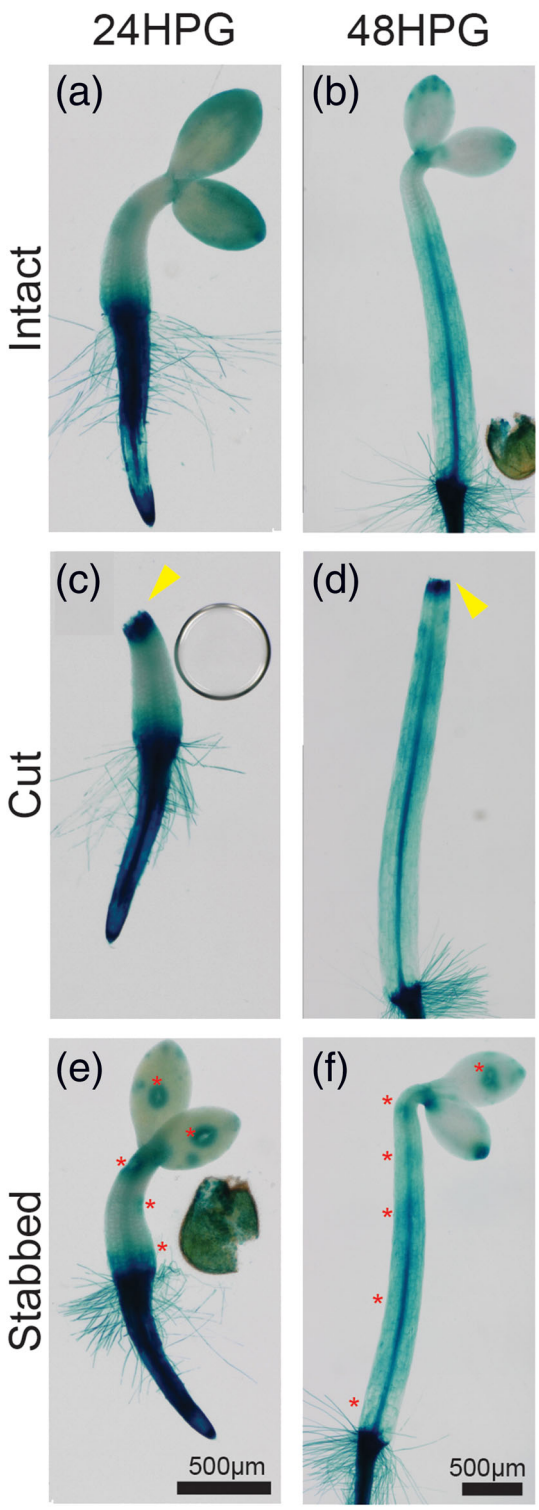

FIGURE 2 Deliberate physical damage of hypocotyls improves GUS staining in localized areas. *p35S::GUS* dark-grown seedlings stained after no physical damage (a & b), removal of cotyledons (c & d), and equidistant needle stabs (e & f) at 24HPG and 48HPG. Scale bars = 500 um. Red asterisks indicate needle stab sites. Yellow triangles indicate cut sites. GUS, β-glucuronidase gene

2.3 | Staining of endogenously promoted GUS lines is improved by deliberate, periodic, physical disruption

To test our deliberate-damage GUS staining methods and check for GUS staining accuracy, we employed both the defoliation and needle-stab methods on two GUS-GFP lines under the control of endogenous promoters, *pSAUR19* (Procko et al., 2016) and *pYUCCA8* (*pYUC8*; Robert et al., 2013). The reporter protein fusion between GUS and GFP allowed us to compare GUS staining patterns with those observed by confocal microscopy-based visualization of GFP.

GUS staining using *pSAUR19* has been shown to exhibit a hypocotyl-specific staining pattern in 5-day-old light-grown seedlings (Spartz et al., 2012); 4-day-old dark-grown *gSAUR19-GFP* seedlings also indicated hypocotyl-specific expression (Wang et al., 2020). In our hands, intact *pSAUR::GUS:GFP* 48HPG seedlings exhibited hypocotyl-specific GFP and GUS staining (Figure 3a,b), though there was some distinction between GFP and GUS patterning (Figure 3b blue arrowheads); there was a lack of GUS staining in the underside of the apical hook and a general spottiness to the staining along the hypocotyl that was not seen with GFP. De-foliation did intensify the staining at the top of the hypocotyl (Figure 3c), but there was still a lack of consistent staining in the base of the hypocotyl. Interestingly, we did not observe the same sharp cut-off of GUS staining signal improvement at the cut site that we saw with the *p35S* promoter (Figure 2c,d). This may be due to *pSAUR19* being epidermis specific (Procko et al., 2016); thus, buffer penetration into the internal cell layers does not intensify the staining. In contrast, periodic needle stabbing increased the intensity of the staining along the hypocotyl and helped resolve the differences between GFP and GUS seen with the other two methods, making the staining both more intense and more accurate (Figure 3d).

In contrast to *pSAUR19*, *pYUC8::GUS* has been shown to have a cotyledon and root tip-specific staining pattern with a marked absence of hypocotyl staining (Hentrich et al., 2013; Rawat et al., 2009). In intact *pYUC8:GUS-GFP* 48HPG dark-grown seedlings, we observed a GFP signal in the cotyledons only, consistent with the literature (Figure 3e). A matching pattern was observed with GUS staining (Figure 3f). Neither defoliation (Figure 3g) nor needle stabs (Figure 3h) changed this pattern. This result further supported our conclusion that deliberate damage did not induce GUS staining in areas that should not have stained nor did the process of stabbing transfer significant amounts of active GUS protein between stabbed regions.

Finally, we tested *pXTH18::GUS* (Vissenberg et al., *2005)*. Previously, *pXTH18* was shown to be active in the elongation and differentiation zones of the *Arabidopsis* root (Osato et al., 2006), leaf vasculature, and throughout 6-day-old dark-grown seedlings (Becnel et al., 2006). Intact *pXTH18::GUS* 48HPG seedlings exhibited staining in the leaf vasculature and collet, but not in the hypocotyl counter to

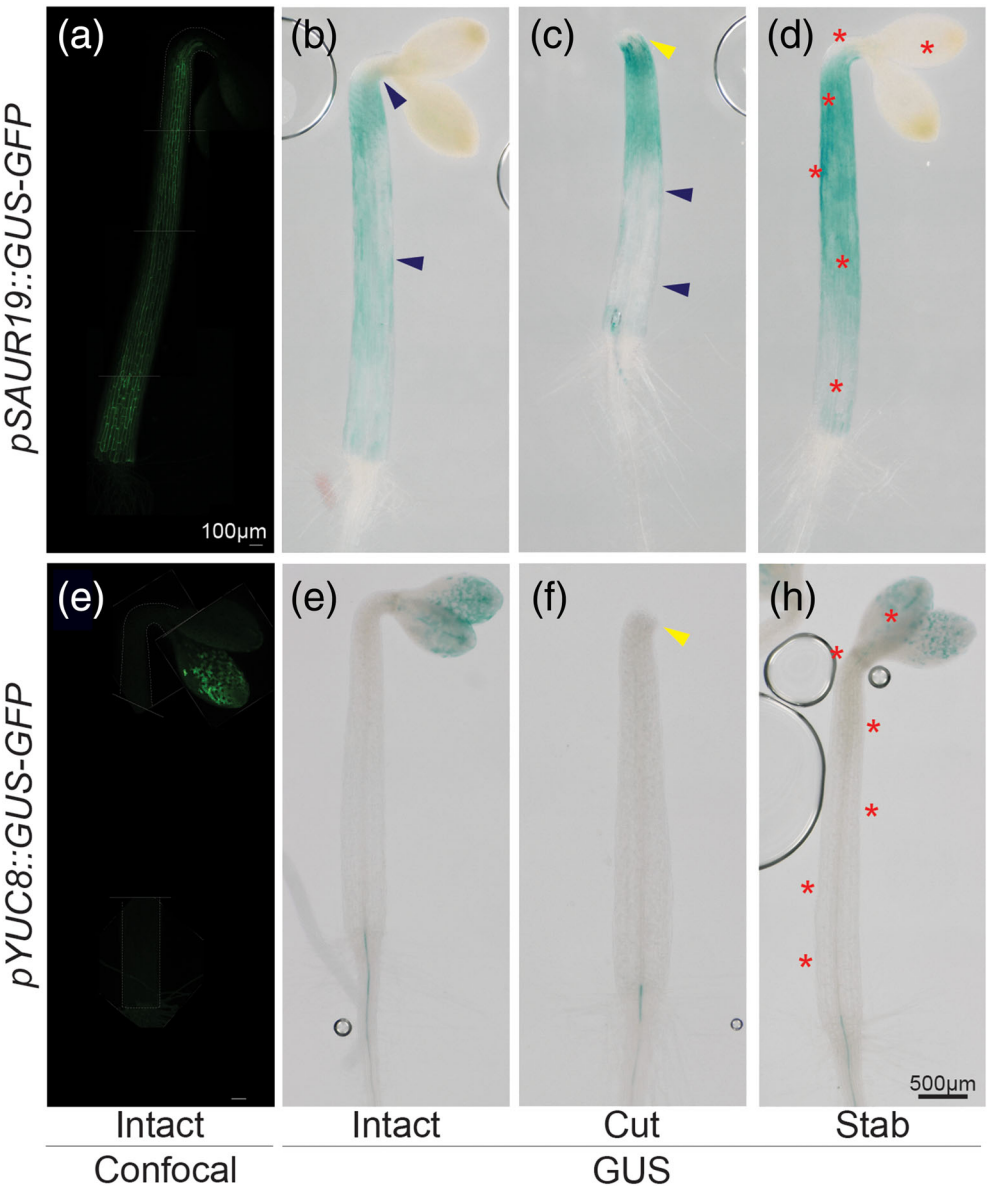

FIGURE 3 Deliberate damage of hypocotyls provides more accurate GUS staining. 48HPG dark-grown seedlings of two GUS-GFP lines were imaged for GFP fluorescence and GUS stained to compare the effect of deliberate damage on staining patterns. (a–d) *pSAUR19::GUS-GFP*. (e–h) *pYUC8::GUS-GFP*. Intact seedlings were imaged with confocal (a, e) or GUS stained (b, f). Cotyledons removed (c, g) or needle stabbed (d, h). Red asterisks indicate needle stab sites. Yellow triangles indicate cut sites. Blue triangles indicate areas where GFP and GUS pattering do not match. Dotted line outlines hypocotyl; vertical white lines indicate image breaks in stitched images. White scale bar = 100 um. Black scale bar = 500 um. GFP, green fluorescent protein; GUS, β-glucuronidase gene

published results (Figure 4a). De-foliation dramatically revealed GUS staining in the hypocotyl with a basipetal pattern diminishing towards the hypocotyl base (Figure 4b). When hypocotyls were stabbed at periodic intervals along the seedling, the basipetal pattern was recapitulated (Figure 4c) allowing us to conclude that it was likely a true reflection of promoter activity as opposed to an artifact of the staining method.

3 | DISCUSSION

Taken together, our results support the use of acetone fixation and deliberate physical damage to improve GUS staining accuracy in dark-grown *Arabidopsis* hypocotyls. Although both de-foliation and equidistant needle stabbing improved hypocotyl GUS staining, we recommend needle stabbing as it ensures that buffer penetration of the hypocotyl is as uniform as possible, and it retains the cotyledons for staining that may be relevant for the experimenter. We also strongly recommend the inclusion of both a negative control and a positive control, such as *p35S::GUS*, when performing all GUS staining experiments to allow for assessment of both false positives and false negatives due to endogenous GUS activity and suboptimal buffer penetration, respectively.

Although we have quantified GUS staining intensity using histochemical images, we advise extreme caution when using this technique; the absolute intensity of GUS staining is influenced by a wide variety of factors, and thus quantification of these images can be extremely variable and can easily lead to misinterpretation of results. We highly advise that histochemical staining be primarily used for promoter activity localization and not quantification. As an alternative, fluorometric techniques using the GUS substrate 4-methylumbelliferyl ß-D-glucuronide (4-MUG) are widely used to accurately and

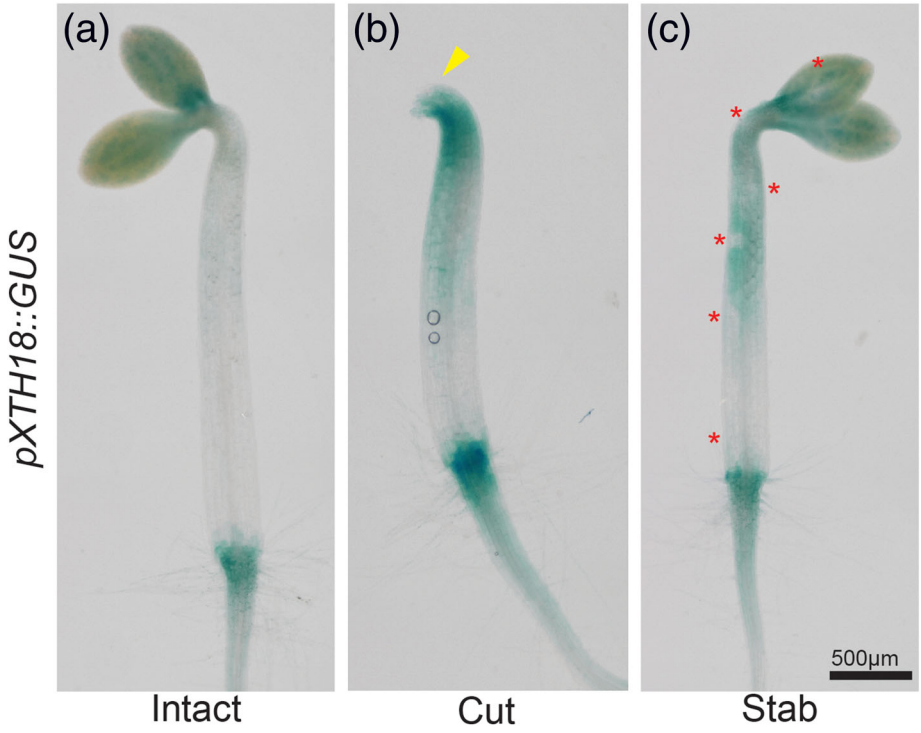

FIGURE 4 Deliberate damage of hypocotyls reveals obscured GUS staining in dark-grown hypocotyls. 48HPG dark-grown *pXTH18::GUS* seedlings stained after no physical damage (a), removal of cotyledons (b), and equidistant stabbing with a 5-um needle (c). Scale bar = 500 um. Red asterisks indicate needle stab sites. Yellow arrowheads indicate cut sites. GFP, green fluorescent protein; GUS, β-glucuronidase gene

quantitatively measure GUS activity and can be performed on extracts and in intact seedlings as desired (Blázquez, 2007; Halder & Kombrink, 2015).

We do not put forward this methodology as the be-all-and-end-all of GUS staining protocols and expect to continue improving it as we move forward in our research. We fully anticipate encountering unforeseen barriers to staining accuracy in other contexts but believe the inclusion of controls and alternative assessment methods (e.g., RNA-based gene expression data) will allow for cumulative accuracy in our methods. We hope that this work provides other researchers with a starting methodology for their work and perhaps even a map for staining improvement in other systems.

4 | METHODS

4.1 | Plant materials and growth

Arabidopsis thaliana seeds were sterilized using 70% ethanol for 5 min and plated onto 0% sucrose 1/2 MS with Gamborg's B5 vitamins (Duchefa Biochemie, Haarlem, The Netherlands) with .8% plant agar media (MilliporeSigma, St. Louis, MO, USA). Plates were wrapped in aluminum foil and placed at 4°C for 2 days to stratify. Plates were then unwrapped and transferred to a Percival Growth Chamber (Percival Scientific, Perry, IA, USA) set for long days (16-h light, 8-h dark) at 22°C for approximately 24 h. Newly germinated seeds (0HPG) were identified based on radicle penetrance of the endosperm and transferred to 1.5% sucrose 1/2 strength MS with Gamborg's B5 vitamins with plant agar media and oriented with the radicle pointing downward (for more details, see Bou Daher et al., 2019). Plates were once again wrapped in foil to simulate darkness and placed in a Percival Growth Chamber set for constant darkness and 20°C. Seedlings were harvested for staining and confocal imaging at 0HPG, 24HPG, and 48HPG.

Genotypes used were as follows: Columbia-0, *p35S::GUS* (J.J. Harada, UC Davis; gift), *pXTH18::GUS* (Vissenberg et al., 2005), *pYUC8::GFP-GUS* (CS69897; Robert et al., 2013), and *pSAUR19::GFP-GUS* (Procko et al., 2016).

4.2 | Small strainer baskets

The small strainer baskets were made by cutting off a 1.5-cm piece of the wide end of a 1000-μl pipette tip using a hot scalpel. The base of the 1.5-cm piece was then heated using a flame to melt a small amount of the plastic; the still-melted face was then pressed onto a piece of nylon mesh to form the basket and seal it. Once cooled, excess nylon mesh was trimmed away (Figure S6). For more complete instructions, see Method S1: GUS Staining of Dark-Grown Hypocotyls Step #1.

4.3 | GUS staining

The conditions for GUS staining were initially based on previous protocols used for staining dark-grown hypocotyls (Pelletier et al., 2010). Briefly, seedlings were harvested at the indicated time points and either placed in ice-cold 90% acetone for at least 2 h after having been equidistantly stabbed using a 5-um tungsten cat whisker needle (Signatone, Gilroy, CA, USA), having the cotyledons

removed using a 5-mm Micro Knife (Fine Science Tool, Foster City, CA, USA), or having no physical disruption. Seedlings not fixed using acetone were placed in ice-cold GUS Staining Buffer without X-GLUC (GSB-XGLUC; 14-mM NaH2PO4, 36-mM Na2HPO4, 10% Triton, 2-mM K4[Fe(CN)6], 2-M K3[Fe(CN)6], 10% Triton). Acetone-treated seedlings were placed under vacuum for 5 min at room temperature (RT) and allowed to sit at RT for 20–30 min after the vacuum was released. Acetone was removed from acetone-treated seedlings and replaced with GSB-XGLUC. Seedlings were then placed on ice and placed under vacuum for 5 min. GSB-XGLUC was replaced with GSB with X-GLUC (GSB + XGLUC; 14-mM NaH2PO4, 36-mM Na2HPO4, 10% Triton, 2-mM K4[Fe(CN)6], 2 M K3[Fe(CN)6], .2% Triton, 2-mM X-GLUC) and samples were again treated with vacuum on ice for 5 min. Seedlings were then transferred to a 37°C incubator (New Brunswick, San Diego, CA, USA) for 15 h (overnight) and subsampled at 30 min, 1 h, 2 h, and 3 h to determine the optimal incubation time per genotype. Finally, GSB + XGLUC was removed and replaced with 70% ethanol for storage. For detailed step-by-step protocol, see Data S1.

4.4 | Imaging

GUS stained seedlings were mounted on slides using water and imaged using a Keyence VHX-6000 (Keyence, Itasca, IL, USA). Confocal Images were obtained using a Zeiss 710 Confocal Microscope (Zeiss, Oberkochen, Germany).

4.5 | Signal quantification

For both GUS and RFP signal intensity quantification, hypocotyls were split into three regions: base, middle, and top (as indicated in Figures 2 and S1). GUS staining intensity was quantified from the light microscopy images using the method presented in Béziat et al. (2017). H2B-RFP signal was quantified using the ImageJ "Analyze Particle" tool (Schneider et al., 2012). Briefly, Z-stack images were compressed, background signal was subtracted, a Gaussian blur was applied, and brightness and contrast were adjusted. A binary mask was created from the RFP image and "Analyze Particles" used to create particle outlines that were overlaid on the original image. Particle mean gray value was measured for 20–40 nuclei per region. Graphs were made using plots of difference (Postma & Goedhart, 2019).

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CONFLICT OF INTEREST

The authors did not report any conflict of interest.

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