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Commentary

An Improved Clearing Method for GUS Assay in *Arabidopsis* Endosperm and Seeds

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Abstract. Precise cellular localization of the GUS stain is notoriously difficult in *Arabidopsis* seeds. Here we report an improved protocol for the clearing of seeds after GUS staining. Incubation in ethanol–acetic acid (EtAc) and Hoyer's medium allows reliable cellular localization of the GUS, even in seeds from late developmental stages. This method also leads to the staining of nucleoli in the endosperm and embryo, facilitating nuclear counts in endosperm development.

Key words: *Arabidopsis*, chloral hydrate, clearing, endosperm, GUS, Hoyer's medium, lactophenol, seed.

Introduction

Recent advances in enhancer (Boisnard-Lorig et al., 2001; Sørensen et al., 2001; Vielle-Calzada et al., 2000) and promoter trapping technology (Topping et al., 1994) have facilitated gene discovery in developing endosperm of *Arabidopsis thaliana*. Clearing of GUS-stained seeds has been described previously, but clearing of fragile endosperm tissues and later-stage seeds has remained a problem (Luo et al., 2000; Topping et al., 1994; Vielle-Calzada et al., 2000). Improvements of clearing procedures have focused on optimizing screening to enable quick and easy GUS detection in the embryo. In the endosperm, factors masking GUS expression include the presence of chloroplasts after cotyledon initiation and the gradual darkening of the seeds as they mature. In particular, low levels of GUS staining in the chalazal endosperm in older seeds have been difficult to detect. To overcome these problems, we have optimized a traditional clearing procedure with Hoyer's medium. This protocol allows improved detection of GUS-stained seeds from all developmental stages. We illustrate the use of the improved method by selected examples from a promoter trap screening experiment recently initiated in our laboratory (Stangeland et al., 2001). It was motivated by inadequacies of existing methods to discriminate between GUS in different regions of the endosperm, including the micropylar endosperm (MEN), peripheral endosperm (PEN), and chalazal endosperm (CZE) (Brown et al., 1999).

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Methods

Plant material and growth conditions

Transgenic T-DNA lines were generated in the C24 ecotype of *Arabidopsis thaliana* by root transformation as explained in Mandal et al. (1995). Progeny seeds were selected on commercially available MS medium (Duchefa) containing 50 mg per liter of kanamycin. Plants were transferred to soil and grown at 23±3°C and 70% humidity, under 40-W cool white fluorescent lights (16 h light, 8 h dark).

GUS assay and image processing

GUS assay was performed (Jefferson et al., 1987) on dissected nonfixed *Arabidopsis* siliques in different developmental stages. Shortly after harvesting, siliques were slit twice longitudinally and incubated in the GUS assay buffer (10 mM phosphate buffer [pH 7], 0.5 % Triton X-100, 1 mg/mL X-Gluc A, 2 mM potassium ferricyanide) for 1 day at 37°C. Siliques were incubated in ethanol–acetic acid and cleared in Hoyer's medium (Liu and Meinke, 1998) or lactophenol (Beeckman and Engler, 1994). Dissected ovules were observed under the Zeiss Axioplan microscope equipped with Nomarski optics. Photographs were taken with a Zeiss Microscope camera MC 100 using a T64 Kodak film.

Results and Discussion

Traditional clearing of *Arabidopsis* seeds in Hoyer's medium (Liu and Meinke, 1998; Motzfeldt-Laane and Lie, 1992) takes a long time and often fails, with or without GUS staining. We used Hoyer's medium, which contains 100 g of chloral hydrate dissolved in 30 mL of water (Liu and Meinke, 1998). Hoyer's "light" contains 60 mL of water rather than 30 mL. Some Hoyer's media can be used for both clearing and mounting (Motzfeldt-Laane and Lie, 1992). When Hoyer's medium is used as a mounting medium, it contains more glycerol, gum arabicum, and glucose. In our experiments, endosperm appeared milky, embryos were green, and integuments were brown, making it difficult to recognize organ shapes. Seeds at and after the heart stage of embryo development were not cleared properly, even after several days (Figure 1A).

Improved clearing was achieved by combining clearing in Hoyer's medium with an additional incubation in a mixture of ethanol and acetic acid (EtAc). Although this step is included in some procedures (Berleth and Jurgens, 1993; Boisnard-Lorig et al., 2001), neither the optimization nor its use in combination with the GUS assay has been reported. The use of this preincubation step in EtAc for one to several hours resulted in improved clearing in Hoyer's medium. When the ovules (up to and including the heart stage of embryo development) were precleared in EtAc, Hoyer's medium produced good clearing of embryos but variable clearing of the endosperm. Occasionally, we observed pearllike structures in the endosperm (Figure 1B), and the endosperm sometimes was detached from the integument (Figure 1E). The chalazal endosperm cyst and the nodules sometimes detached from the chalazal proliferating tissue and the integument (Figure 1F). Partitioning of the chalazal endosperm cyst was also observed (Figure 1F). We

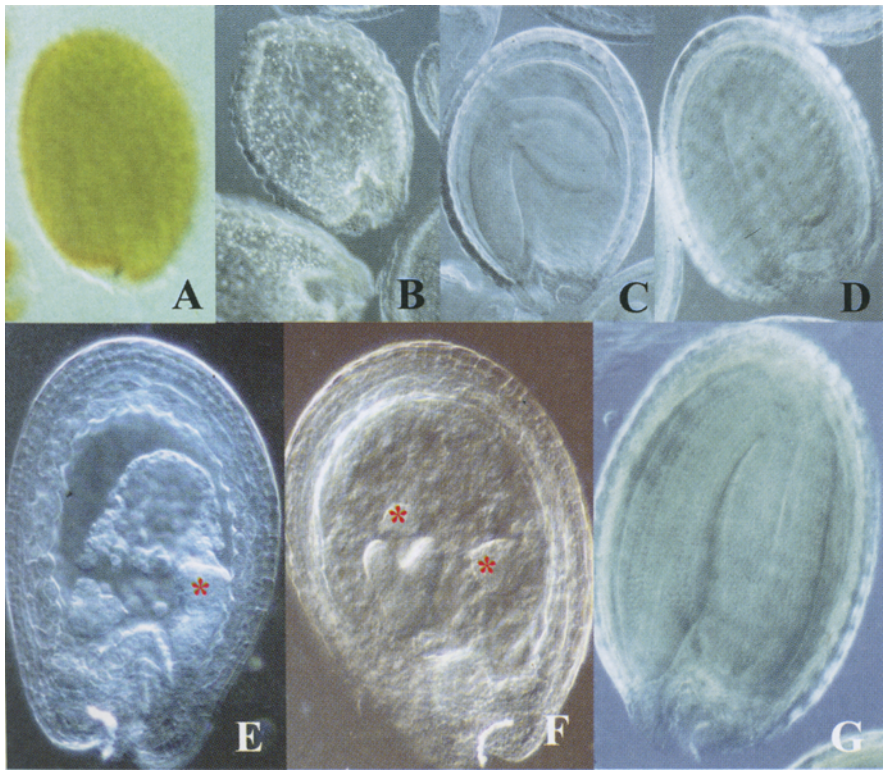

Figure 1. Clearing of wild-type *Arabidopsis* ovules with Hoyer's medium. (A) Clearing of cotyledon stage seed could not be obtained by traditional clearing methods. (B) Hoyer's reagent can damage fragile endosperm tissue and cause the appearance of "pearls." C, D, G: Seeds incubated in EtAc 1:1 for (C) 4 h, (D) 6 h, and (G) 8 h before clearing in Hoyer's overnight. Ages of the seeds were (C) walking stick and (D, G) cotyledon stage. (E) Detached endosperm. (F) Chalazal endosperm (red asterisk), detached from the chalazal proliferating tissue.

interpret the damage of the free nuclear endosperm to be caused by the strength of the Hoyer's medium. Therefore, we used Hoyer's light when clearing ovules up to and including the heart stage of embryo development. Even though better results were obtained after preclearing was included, the results were variable. We concluded that the duration of incubation in EtAc and the ratio of ethanol to acetic acid both needed further optimization.

Optimization of the ratio and the incubation time

We tested different ratios of ethanol to acetic acid (9:1, 3:1, 1:1, 1:3, 1:9) in the preclearing solution after the GUS assay by incubating seeds in EtAc for 30 min to 4 h and clearing in Hoyer's light overnight. Results were scored the next day. Incubation in solutions of 3:1, 1:3, and 1:9 improved clearing of young seeds after 1 h. Young ovules were sensitive to prolonged exposure to acetic acid. The best clearing of the endosperm and the easiest localization of the GUS stain were observed in solutions of 1:1 and 1:3 after several hours.

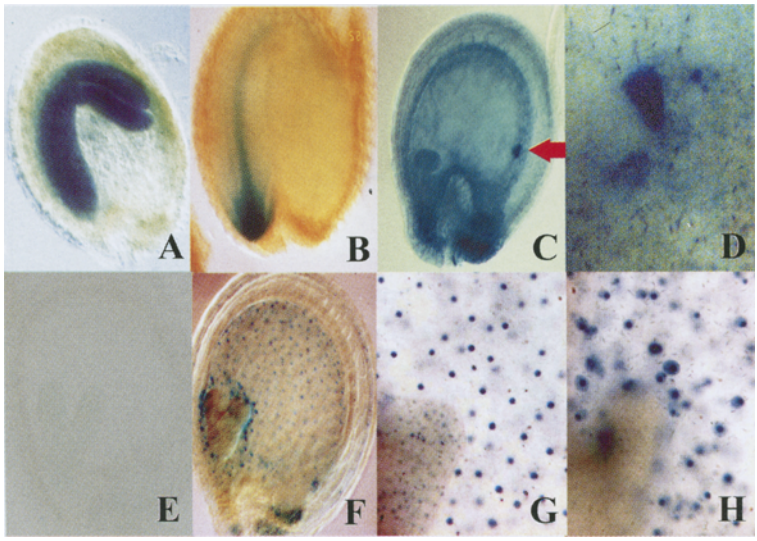

Figure 2. Clearing of GUS-stained seeds. A-E: Seeds were incubated for 4-8 h in EtAc 1:1 and cleared with Hoyer's medium overnight. A-B: Clearing of seeds after the heart stage of embryo development with Hoyer's medium. (A) walking-stick embryo of marker line A showing GUS activity in the embryo. (B) In marker line B, GUS activity was detected in the hypocotyl. Good clearing of the seed allowed precise localization of the GUS. (C) Marker line C; GUS was detected in the whole seed but was very strong in the endosperm nodule (red arrow). (D) Close-up of the GUS-stained endosperm nodule from C. (E) GUS staining of the wild type ovule at the heart stage of embryo development. F-H: Prolonged incubation in Hoyer's medium can cause staining of endosperm nucleoli. (F) Nucleolar staining in the marker line F after prolonged clearing. (G) Close-up of the endosperm from F; GUS activity was detected in endosperm nucleoli. (H) Marker line H; real GUS activity was detected in endosperm nucleoli, nucleoplasm, and cytoplasm.

A series of similar clearing experiments without GUS assay showed that non-stained older seeds were as difficult to clear as GUS-stained seeds. Extreme combinations of ethanol–acetic acid (9:1 and 1:9), pure acetic acid, and pure ethanol resulted in poor clearing. Incubation in EtAc 1:1 for several hours greatly improved clearing and enabled successful clearing of almost mature seeds (Figure 1C-D, 1G). The optimal ratio of ethanol to acetic acid for older seeds was 1:1.

Incubation in 1:1 EtAc for 15 min improved clearing of young seeds up to and including the heart stage of embryo development. Optimal incubation time was 1-4 h for these young ovules. Clearing enabled precise detection of the GUS, as shown for embryos of marker lines A-C (Figure 2A-C). Clearing of older seeds, which usually appear brownish, was best after 4-8 h in EtAc, before clearing with Hoyer's medium (Figures 1C-D, 1G, 2A-B). Incubation times were proportional to the age of the seeds (Figure 1C-D, 1G). This was the case for both GUS-stained and non-stained seeds. Good clearing and GUS detection at later developmental stages are illustrated in Figure 2A-B. We detected GUS activity in cotyledons (Figure 2A, line A) and hypocotyls (Figure 2B, line B) of late-stage

embryos. In the transgenic line C, we detected intense GUS staining of the endosperm nodule (Figure 2C-D).

Preventing seeds from browning

We noticed that seed browning was prevented when the clearing solution did not contain gum arabicum. However, cleared seeds had to be mounted in Hoyer's containing gum arabicum to prevent drying. Browning usually occurred in seeds that were left for more than 2 d on the slide. Therefore, if a micrograph of the seed is needed, photographs should be taken soon after the mounting. In addition, ovules mounted in Hoyer's medium shrunk or flattened, making endosperm studies impossible. To achieve better results, seeds should be cleared in Hoyer's medium without gum arabicum and mounted in Hoyer's containing gum arabicum. Clearing in mounting Hoyer's (Motzfeldt-Laane and Lie, 1992) did not work well for clearing the seeds (results not shown), but was used for mounting seeds that were cleared in Hoyer's medium and Hoyer's light.

Nucleolar staining

After incubating siliques in Hoyer's for 4 wk, most of the transgenic lines had a similar appearance (Figure 2F-G). The blue CIBr-indigo could be localized to the nucleoli of the endosperm, embryo, and integuments. We analyzed 6 marker lines that showed GUS activity in the endosperm and embryo. GUS activity was localized in both the nucleoplasm and the nucleolus in only one line (Figure 2H), probably indicating real nuclear localization. All other lines looked more or less the same after prolonged clearing, with strong GUS staining localized to nucleoli and the chalazal proliferating tissue (Figure 2F-G). Possible explanations of false-positive GUS staining in the chalazal proliferating tissue may include strong localized activity of the lytic enzymes present in the maternal tissues. In nucleoli, pH values different from those of the surrounding subcellular compartments might have caused the false nucleolar staining. This could have been the case in previously studied marker lines but was masked by staining in the cytoplasm because nucleolar staining became obvious only after the cytoplasm was cleared. It was reported that the endogenous b-glucuronidase is active in flowers and ovules of *Arabidopsis* below pH 5 (Martin et al., 1992). While performing thousands of GUS assays in our promoter trap screen, we occasionally observed weak GUS staining of the endosperm and embryo in wild-type seeds that could not be recorded by the microscope camera (Figure 2E). This weak staining of the endosperm and embryos was diffuse and could be distinguished from what was believed to reflect the true GUS activity.

We tested to see if the nucleolar staining could be observed in wild-type seeds that normally did not show GUS activity. We stained wild-type seeds for GUS activity and cleared them in EtAc and Hoyer's medium (Figure 3). After a few days in Hoyer's medium, seeds did not show GUS activity (Figure 2E). After 2 wk in Hoyer's medium, weak blue staining of young siliques was visible. After 4-6 wk in Hoyer's medium, nucleolar staining and staining of the chalazal proliferating tissue was observed (Figure 3). Some artifacts due to the prolonged incubation in the clearing agent, lactophenol, were also observed (Figure 3A-C). In

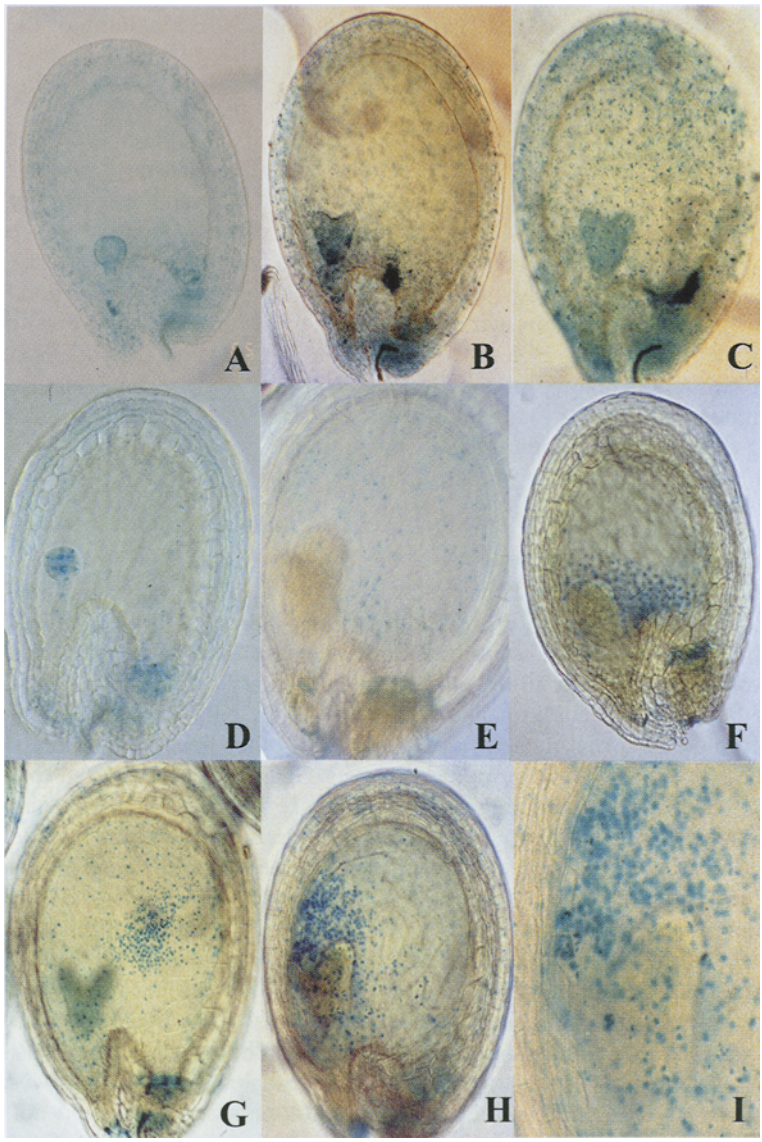

Figure 3. Staining artifacts in wild-type seeds caused by prolonged clearing. A-C: Prolonged clearing in lacto phenol results in blue staining of the embryo at (A) the globular stage and (B, C) "spotty" patterns in integuments at the heart stage. D-I: Prolonged clearing in Hoyer's medium/Hoyer's light. (D) Up to the heart stage, the blue stain is localized predominantly in the embryo and chalazal proliferating tissue. (E-I) In ovules at the heart stage, blue stain can be detected in endosperm and integuments. (F-H) Precipitation of endosperm nuclei after prolonged clearing. (I) Close-up of endosperm nuclei precipitated around heart stage embryo from H.

this case, spots were localized mostly to integuments and embryos (Figure 3A-C). In Hoyer's medium, wild type ovules at the globular stage of embryo development showed nucleolar staining in the embryo, endosperm, and proliferating

chalazal tissue (Figure 3D). False nucleolar GUS (Figure 3E-I) was weaker than in the marker lines (Figure 2F-G). In wild-type endosperm, nucleolar staining (Figure 3E-I) usually was not observed in the nucleoplasm of the endosperm nuclei. In a portion of wild-type seeds, the endosperm nuclei tended to precipitate (Figure 3F-I), probably as a result of gravity from being in Hoyer's medium for many weeks.

Conclusions

The clearing of GUS-stained seeds improves significantly when incubation in ethanol–acetic acid precedes clearing in traditional Hoyer's medium. This method is especially useful for precise GUS localization in older *Arabidopsis* seeds. Incubations of up to 4 h in EtAc for younger seeds and up to 8 h for older seeds are necessary to improve clearing. The optimal ratio of the 2 chemicals is 1:1. Prolonged clearing of GUS-stained seeds led to dye precipitation in the nucleoli. A chemical reaction that occurs in the nucleoli probably is the reason for this. Special attention should be taken when observing ovules after long incubations in GUS and clearing agents. Incubations in Hoyer's for up to 1 wk are most suitable for good and reliable GUS detection. In case of weak signals, embedding in resins might be a better choice. Several weeks of incubation results in nucleolar staining, which may be useful in studies of endosperm development and mutant screenings. Blue staining in the endosperm nucleoli can also serve as a useful marker to indicate the position of endosperm nuclear cytoplasmatic domains (NCD) (Brown et al., 1999).

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