Chapter 19

Tissue-Printing Methods for Localization of RNA and Proteins that Control Seed Dormancy and Germination

Wioletta E. Pluskota, Kent J. Bradford, and Hiro Nonogaki

Abstract

A number of genes and proteins are expressed in a tissue- or cell layer-specific manner. Spatial patterns of gene expression are critical to understanding gene function. Tissue printing provides a simple and rapid method to analyze localization of mRNA and protein at the tissue and cellular levels. This is especially convenient for gene expression analysis in hard tissues, such as seeds that are often difficult to section. Seed RNA or protein can be transferred onto a suitable membrane by printing the cut surface of a bisected seed. This method has been used successfully to determine mRNA and protein localization in seed research. The resolution of printed seed images and RNA and protein signals in tissue printing is sufficient to identify embryo- or endosperm-specific expression of various genes and proteins. In some cases, these studies have contributed to elucidating the spatial characteristics of hydrolytic enzymes putatively involved in the completion of germination and/or early postgerminative growth. By the same principle, tissue-printing methods could also be valuable for elucidating the spatial characteristics of genes/proteins that control the inception, maintenance, and termination of seed dormancy.

Key words: Tissue printing, Seed, mRNA, Protein, Localization, Gene expression, Dormancy, Germination

1. Introduction

Many genes involved in seed dormancy and germination have been identified (1–3). To understand the physiological roles of those genes and gene products and their regulatory mechanisms, it is necessary to characterize the timing and localization of gene expression in seeds. However, preparation of high-quality seed tissue sections for in situ hybridization is difficult and time consuming. Dissection of separate seed tissues, such as the embryo and endosperm, to examine tissue-specific gene expression by RNA gel blotting or RT-PCR is also laborious. Tissue printing (4) is a

Allison R. Kermode (ed.), *Seed Dormancy: Methods and Protocols*, Methods in Molecular Biology, vol. 773,
DOI 10.1007/978-1-61779-231-1_19,

convenient alternative method to in situ hybridization or analysis of RNA samples extracted from various tissues.

Tissue-specific expression of gene family members appears to be a common feature of seed biology; seed tissue printing provides an excellent method to visualize and quantify localization of gene expression (5–7).

In this chapter, the value and application of seed RNA tissue printing are demonstrated by using the following examples (Subheading 3.5): (1) differentiation of the spatial expression patterns of *LeMAN1* and *LeMAN2* encoding endo-β-mannanases in tomato seed that share ~70% identity at the amino acid level (5) and (2) side-by-side comparison of a specific mRNA and the corresponding gene product (the protein) using the example of LeVA-P1, a binding domain of the vacuolar ATPase of tomato.

2. Materials

2.1. Seed Printing

1. Membranes: Positively charged nylon membrane (e.g., Amersham Hybond N$^+$, GE Healthcare, Piscataway, NJ) for RNA printing. Nitrocellulose membrane for protein printing (Optitran BA-S 85, Schleicher and Schuell, Keene, NH).
2. Double-edged razor blades.
3. Paper towels.
4. Powder-free gloves.
5. Parafilm.

2.2. RNA Detection (Northern)

2.2.1. Hybridization

1. Prehybridization buffer: 50% (v/v) deionized formamide, 2% (w/v) blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN), 0.2% (w/v) SDS, 5× SSC. Store at −20°C. Prehybridization buffer should be equilibrated to the temperature used for hybridization before use.
2. Digoxigenin (DIG)-labeled antisense RNA probes.
3. Plastic bag (0.11 mm thick; KAPAK, Minneapolis, MN).
4. Plastic bag sealer (e.g., TEW Electric Heating Equipment Co., Ltd., Taipei, Taiwan).

2.2.2. Washing (After Hybridization)

1. Washing buffer 1: 2× SSC, 0.1% (w/v) SDS (prepare from 20× SSC and 10% SDS).
2. Washing buffer 2: 0.2× SSC, 0.1% (w/v) SDS (prepare from 20× SSC and 10% SDS).

2.2.3. DIG Antibody Reaction

1. Maleic acid buffer (10×): 1 M maleic acid, 1.5 M NaCl, 2 M NaOH. Heat is generated due to the exothermic reaction.

Cool down to room temperature before adjusting the pH to 7.5 with NaOH. Store at 4°C.

2. DIG antibody reaction buffer (buffer A): 1× maleic acid buffer containing 0.3% (v/v) Tween 20. Stir gently until Tween 20 is uniformly mixed. Store at 4°C.
3. DIG blocking buffer: Buffer A containing 5% (w/v) nonfat dry milk (stir at room temperature for 30 min).
4. Anti-DIG-alkaline phosphatase (AP) Fab fragments (0.75 U/μL; Roche Molecular Biochemicals). Store at 4°C.

2.2.4. Washing (After DIG Antibody Reaction)

Buffer A: 1× maleic acid buffer containing 0.3% (v/v) Tween 20.

2.2.5. Signal Detection (Alkaline Phosphatase Reaction)

Option A: Colorimetric detection

1. Equilibration buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
2. Tris–HCl buffer 1 M, pH 8.8.
3. $MgCl_2$ solution: 1 M $MgCl_2$.
4. Nitroblue tetrazolium chloride (NBT): 1 mg/mL in 0.2 M Tris–HCl, pH 8.8. Preparing fresh solution is recommended.
5. 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP): 10 mg/mL dimethylformamide (DMF). Preparing fresh solution is recommended.
6. Colorimetric enzyme reaction mixture: 0.18 M Tris–HCl, pH 8.8, 2 mM $MgCl_2$, 0.025 mg/mL BCIP, and 0.1 mg/mL NBT (prepare from 1 M Tris–HCl, pH 8.8, 1 M $MgCl_2$, NBT, and BCIP stock solutions).
7. Plastic bag (0.06 mm thick; KAPAKA, Minneapolis, MN).

Option B: Chemiluminescent detection

1. Equilibration buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
2. Chemiluminescent substrates (e.g., CDP-star Ready for Use, Tropix Inc, Bedford, MA or LumiPhos, Lumigen, Southfield, MI).

2.3. Protein Detection (Western)

1. Tris-buffered saline (TBS): 25 mM Tris, 0.15 M NaCl, adjust pH with HCl to 7.4.
2. TBS-Tween 20 (TBST): TBS containing 0.05% (v/v) Tween 20.
3. TBST blocking buffer: TBST containing 1% (w/v) bovine serum albumin (BSA).
4. Plastic bag (0.11 mm thick; KAPAK, Minneapolis, MN).
5. Primary antibodies (e.g., rabbit IgG specific to genes of interest).
6. TBST.
7. Secondary antibodies (e.g., anti-rabbit goat IgG conjugated with alkaline phosphatase).

3. Methods

In this section, methods for RNA detection using nonradioactive RNA probes and for protein detection using primary and secondary antibodies are described. The first step of seed printing is common for both RNA and protein detection, except for the nature of the membranes. Likewise, the final steps of both RNA and protein detection are similar in that the detection makes use of an enzyme (alkaline phosphatase) reaction for visualization of signals.

3.1. Seed Printing

1. Place an imbibed seed (see Note 1) on wet filter paper on a stage (e.g., a lid of plastic Petri dish can be used as a stage).
2. Cut a seed into halves by holding the seed vertically with forceps and using one side of a double-edged razor blade. (A double-edged razor blade can be wrapped with Kimwipes and cracked into halves with caution.) The thin blade of the razor gives a smoothly cut surface. Longitudinally bisected half seeds as well as transversally bisected seeds can be used for tissue printing. This procedure is best performed under a dissection microscope. To obtain a high-quality cut surface, each edge of the razor blade should not be used more than several times.
3. Place the half seed with its cut surface face down on a membrane. (A positively charged nylon membrane is used for RNA; nitrocellulose membrane is used for protein, see Subheading 2.)
4. Cover the membrane and half seed with parafilm and press hard with a thumb for ~15 s (see Note 2). Placing multiple layers of soft paper towels under the membrane is important to obtain a deep print of the seed (Fig. 1a). If the membrane is kept on a hard stage, the printed image will not be deep enough to visualize seed morphology. It is recommended that the resolution of printed images is examined under a dissection microscope before proceeding with hybridization.
5. Release pressure from the thumb gently and remove half seed with forceps. Avoid touching the membrane with the forceps to prevent extraneous marks on the prints and make sure that seed parts are not left on the membrane.
6. For tissue-printing RNA detection (Northern), cross-link RNA to the membrane using a UV cross-linker (see Note 3) and go to Subheading 3.2.

6′. For tissue-printing protein detection (Western), follow Subheading 3.3.

3.2. RNA Detection (Northern)

To analyze localization of gene expression, mRNA transferred to the membrane is hybridized with DIG-labeled antisense RNA probes, which are synthesized from cDNA by in vitro transcription

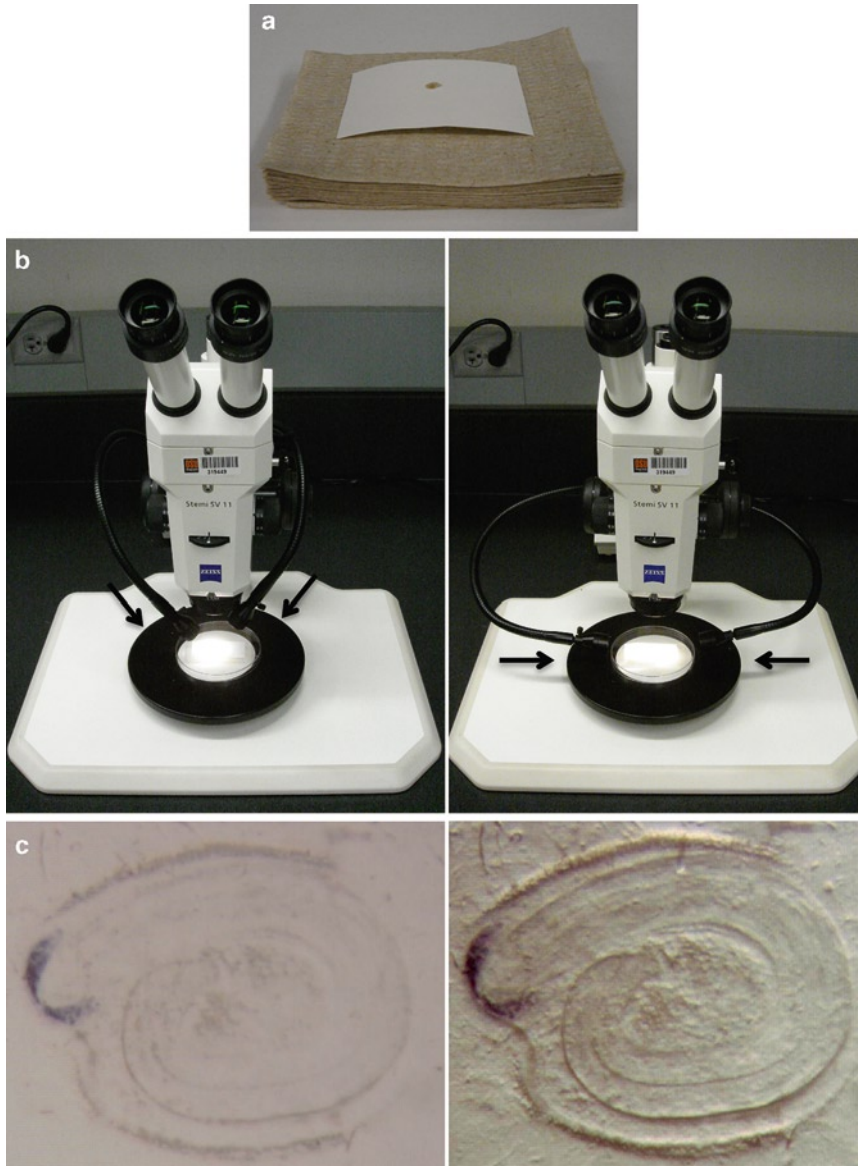

Fig. 1. Seed printing and image capturing. (**a**) A membrane placed on paper towels for tissue printing. (**b**) Examples of vertical (*left*) and horizontal (*right*) lighting to the tissue print membrane under a dissection microscope. (**c**) Tissue print images captured under vertical (*left*) and horizontal (*right*) lighting shown in (**b**). Note that the image captured under horizontal lighting has higher resolution of seed morphology (*right*) than the image under vertical lighting (*left*). Image: *LeMAN2* endo-β-mannanase mRNA expression in tomato seed.

with T7 or T3 RNA polymerase. DIG UTPs are incorporated into RNA molecules during synthesis. DIG molecules on the RNA probes are detected by anti-DIG antibody conjugated with alkaline phosphatase. The final detection of signals can be done using colorimetric or chemiluminescent substrates for alkaline phosphatase (see Note 4).

3.2.1. Hybridization

1. Incubate the membrane in prehybridization buffer equilibrated to an optimal temperature for 15 min. Temperature for hybridization depends on the probe used. For a specific probe of 1–2 kb length, 60°C in the presence of formamide works for hybridization and is stringent enough to avoid background. This step can be performed in a closed container.
2. Place the membrane between sealable plastic sheets (or bag). Add the appropriate volume of prehybridization buffer (0.1 mL/cm^2 membrane). Add the RNA probe (100 ng/mL) and incubate for 16 h (see Note 5).

3.2.2. Washing After Hybridization

1. Rinse the membrane briefly with washing buffer 1 and then wash with fresh washing buffer 1 equilibrated at 60°C once for 20 min.
2. Wash the membrane with washing buffer 2 at 60°C twice for 20 min (see Note 6).

3.2.3. DIG Antibody Reaction

1. Rinse the membrane with buffer A at room temperature.
2. Block the membrane with DIG blocking buffer at room temperature for 30 min using a shaker.
3. Add anti-DIG antibody (1:15,000) to the DIG blocking buffer and incubate at 25°C for 1 h.

3.2.4. Washing (After DIG Antibody Reaction)

1. Rinse the membrane briefly with buffer A.
2. Wash the membrane with buffer A at room temperature three times for 20 min using a shaker.

3.2.5. Signal Detection

1. Incubate the membrane in equilibration buffer at room temperature for 5 min to adjust pH environment (pH 9.5) for alkaline phosphatase.

Option A: Colorimetric detection

2. Place membrane in the colorimetric enzyme reaction mixture for 2 h to overnight with agitation (see Note 7).
3. Wash the membrane with water to remove the substrates and dry it at room temperature. The dried membranes can be kept at room temperature without losing signals or the original resolution of printed images.

Option B: Chemiluminescence detection

2′. Place the membrane with RNA side facing up on a plastic sheet.

3′. Apply chemiluminescent substrates for alkaline phosphatase (~10 μL/cm of membrane) and incubate at room temperature for 5 min.

4′. Cover the membrane with a plastic sheet (see Note 8).

5′. Expose to X-ray film for at least 15 min (CDPstar) or 30 min (LumiPhos).

3.3. Protein Detection (Western)

For protein detection, a combination of primary antibodies raised against a protein of interest and commercial secondary antibodies can be applied. The following is an example of protein detection using alkaline phosphatase-conjugated secondary antibodies.

3.3.1. Primary Antibody Reaction

1. Incubate membranes in TBST blocking buffer at room temperature for 30 min (see Note 9).
2. Add primary antibodies (e.g., 1:1,000) and incubate the membrane at 25°C for 1 h.

3.3.2. Washing (After Primary Antibody)

1. Rinse the membrane briefly with TBST.
2. Wash the membrane with TBST at room temperature three times for 10 min using a shaker.

3.3.3. Secondary Antibody Reaction

1. Add secondary antibody to TBST blocking buffer (e.g., 1:5,000) and incubate the membrane at 25°C for 1 h.

3.3.4. Washing (After Secondary Antibody)

1. Rinse the membrane briefly with TBST.
2. Wash the membrane with TBST at room temperature three times for 10 min using a shaker.

3.3.5. Signal Detection

See Subheading 3.2.5.

3.4. Capturing the Images

While tissue-printing signals and seed morphology are relatively stable on the membranes, it is recommended to take photographs of tissue prints with signals under a dissection microscope shortly after the signals are detected. To visualize the seed prints, it is helpful to give light laterally from both sides of a membrane rather than exposing light from above (Fig. 1b). The best resolution can be obtained by keeping membranes wet (or semidry) to visualize both the dark signal of RNA or proteins and the deep prints of seed tissues very clearly (Fig. 1c). Digital images can be analyzed by appropriate software for quantification of signal intensities.

3.5. Application of the Techniques for Understanding Seed Biology

1. Differentiating the spatial expression patterns of related genes: Tissue-specific expression of gene family members is a common feature of seed biology (8–11). As an example, seed RNA tissue printing was used to differentiate the spatial expression patterns of *LeMAN1* and *LeMAN2* encoding endo-β-mannanases in tomato seed that share ~70% identity at the amino acid level ((5); Fig. 2). The germination-specific gene *LeMAN2* exhibits expression confined to the micropylar region of the endosperm

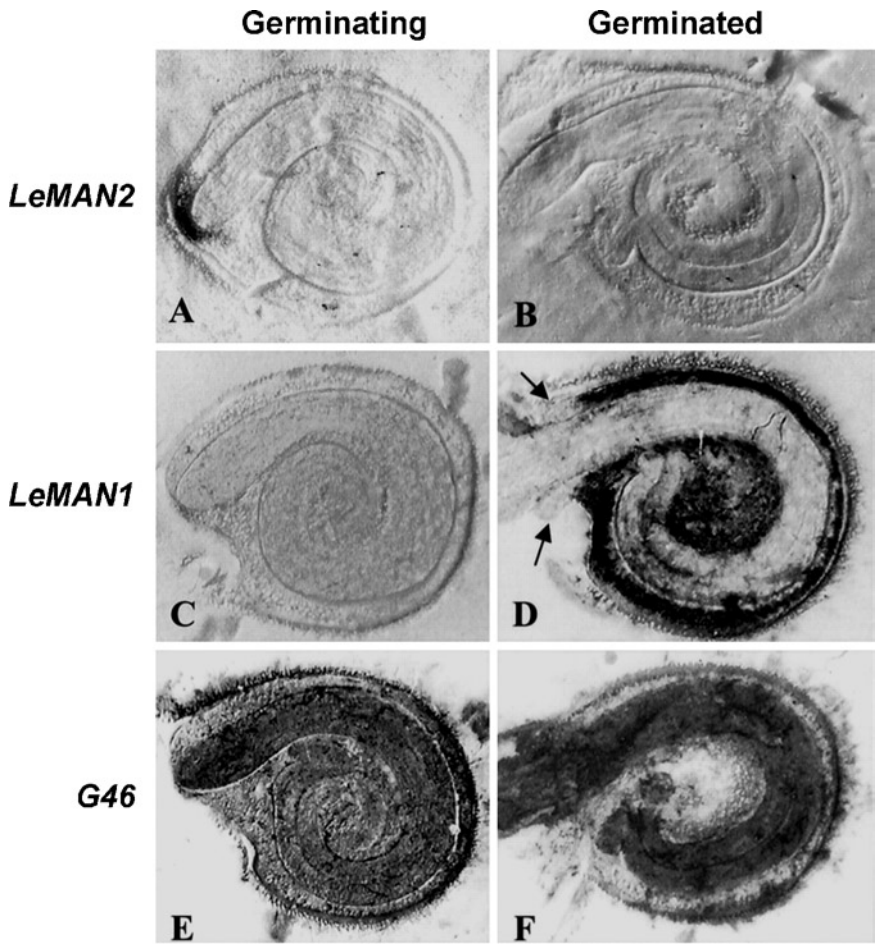

Fig. 2. Tissue printing and hybridization of germinating (24-h imbibed) and germinated tomato seeds. Prints were hybridized with antisense riboprobes for *LeMAN2* (**a**, **b**), *LeMAN1* (**c**, **d**), and *G46* encoding a constitutively expressed ribosomal protein used as a control (**e**, **f**). The *arrows* in (**d**) indicate the remaining endosperm cap tissue in the germinated seeds, which do not hybridize with the *LeMAN1* probe. From ref. 5; used with permission of the American Society of Plant Biologists.

(the endosperm cap) before radicle emergence, whereas *LeMAN1* is expressed in the rest of the endosperm specifically after germination (Fig. 2). The signal from a constitutively expressed gene provides a good control for the efficiency of RNA transfer to the membrane and can be used for normalization of the signal intensity from individual seeds for quantification (Fig. 2e, f).

2. Side-by-side comparison of a specific mRNA and the corresponding gene product (protein): Another advantage of seed tissue printing is that multiple seed samples can be printed on a membrane and the signals from individual seeds for a specific mRNA or protein can be compared side by side across treatments or conditions (12). In addition, both mRNA and proteins can

be detected using tissue prints. For example, a print of one half of a bisected seed can be exposed to an RNA probe and the other half of the same seed (mirror image print) can be reacted with antibodies specific to the gene product. Figure 3a demonstrates an example of detection of mRNA and protein in individual seeds in the case of LeVA-P1, a binding domain of the vacuolar ATPase of tomato (A. Mella et al., unpublished results). In this case, two different reactions (RNA hybridization and antibody binding) were performed on prints of the opposing halves from individual seeds. The results obtained by tissue printing were then quantified based on the digital image intensities, which showed a good correlation between mRNA and protein accumulation in individual seeds (Fig. 3b). Alternatively, the same print can be stripped after hybridization

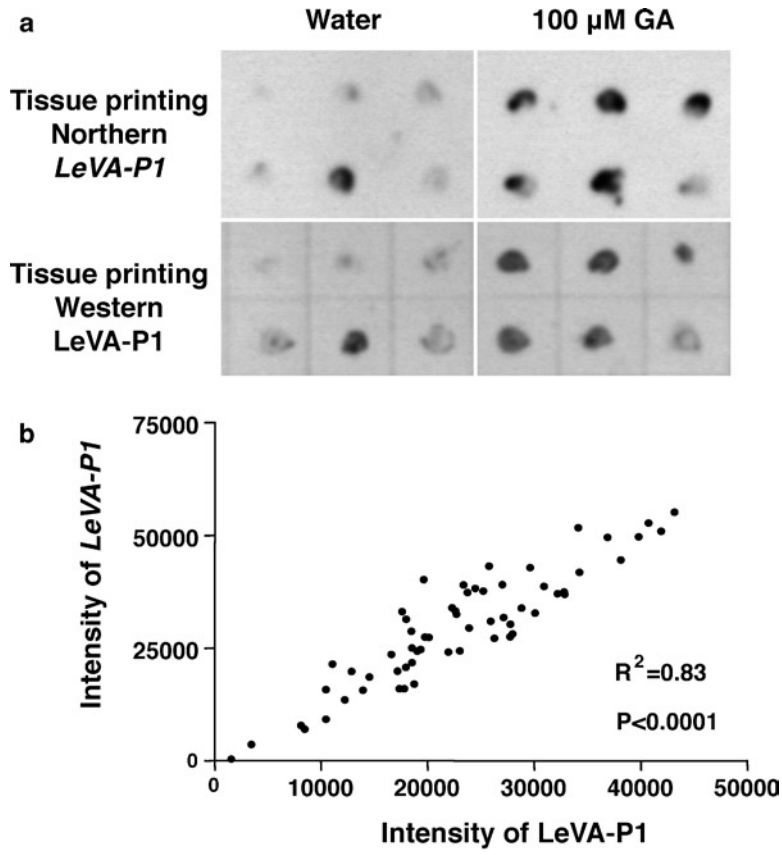

Fig. 3. Northern and Western tissue prints. The seeds were sliced in the middle and both halves printed in mirror membranes. One membrane was developed as Northern (*LeVA-P1*, bean vacuolar ATPase) and the other as Western (antibody against LeVA-P1). (**a**) Mirror images of seeds treated with water or 100 µM gibberellin (GA_{4+7}). (**b**) Regression of the digitized measurements of the mRNA and protein intensities of the mirror images of individual seeds (intensities in arbitrary units). Previously unpublished data of A. Mella et al.

with one probe (e.g., a probe to a gene of interest) and rehybridized with another probe (e.g., a probe to a constitutively expressed gene).

4. Notes

1. Seeds must be imbibed sufficiently to transfer RNA or proteins efficiently to a membrane. Therefore, very early stages of imbibition (several hours) may not be suitable for tissue-printing analyses.
2. When seed printing is attempted for a long time (>15 s), the prints tend to result in fuzzy images probably due to small movement during imprinting.
3. To avoid the degradation of mRNA, do not keep membranes with prints for a long time before hybridization. It is better to use freshly prepared membranes for hybridization.
4. Detection sensitivity is high when chemiluminescent substrates are used and signals are detected on an X-ray film. However, seed morphology is not visualized in an X-ray film.
5. Add a probe directly to the prehybridization buffer without touching membranes or the inner sides of plastic sheets.
6. High stringency (high temperatures and presence of formamide) of washing gives highly specific signals with very low background in tissue prints.
7. Duration of the final enzyme reaction for signal detection varies depending on the amount of transcripts expressed or proteins synthesized and the concentration of the specific probes used for hybridization.
8. When CDPstar is used as a chemiluminescent substrate, gently squeeze out excessive substrate solution. This reduces background in an X-ray film.
9. There are some occasions, where 1.5% (v/v) Tween 20 works better than 1% BSA in the TBST blocking buffer for protein detection (Western) (J. Buitink, personal communication).

Acknowledgment

This work was supported by National Science Foundation grant IBN-0237562 (to H. Nonogaki).

References

1. Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y., and Yamaguchi, S. (2003) Gibberellin biosynthesis and response during Arabidopsis seed germination. *Plant Cell* **15**, 1591–604.
2. Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y., and Yamaguchi, S. (2004) Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds. *Plant Cell* **16**, 367–78.
3. Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y., and Nambara, E. (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant J* **41**, 697–709.
4. Varner, J. E., and Ye, Z. (1994) Tissue printing. *FASEB J* **8**, 378–84.
5. Nonogaki, H., Gee, O. H., and Bradford, K. J. (2000) A Germination-specific endo-β-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. *Plant Physiol* **123**, 1235–46.
6. Chen, F., and Bradford, K. J. (2000) Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiol* **124**, 1265–74.
7. Wu, C. T., Leubner-Metzger, G., Meins, F. Jr., and Bradford, K. J. (2001) Class I β-1,3-glucanase and chitinase are expressed in the micropylar endosperm of tomato seeds prior to radicle emergence. *Plant Physiol* **126**, 1299–313.
8. Yamaguchi, S., Kamiya, Y., and Sun, T.-p. (2001) Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during *Arabidopsis* seed germination. *Plant J* **28**, 443–53.
9. Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M., Nambara, E., and Marion-Poll, A. (2006) Functional analysis of Arabidopsis *NCED6* and *NCED9* genes indicates that ABA synthesised in the endosperm is involved in the induction of seed dormancy. *Plant J* **45**, 309–19.
10. Sawada, Y., Aoki, M., Nakaminami, K., Mitsuhashi, W., Tatematsu, K., Kushiro, T., Koshiba, T., Kamiya, Y., Inoue, Y., Nambara, E., and Toyomasus, T. (2008) Phytochrome- and gibberellin-mediated regulation of abscisic acid metabolism during germination of photoblastic lettuce seeds. *Plant Physiol* **146**, 1386–96.
11. Sawada, Y., Katsumata, T., Kitamura, J., Kawaide, H., Nakajima, M., Asami, T., Nakaminami, K., Kurahashi, T., Mitsuhashi, W., Inoue, Y., and Toyomasu, T. (2008) Germination of photoblastic lettuce seeds is regulated via the control of endogenous physiologically active gibberellin content, rather than of gibberellin responsiveness. *J Exp Bot* **59**, 3383–93.
12. Wu, C.-T., and Bradford, K. J. (2003) Class I chitinase and β-1,3-glucanase are differentially regulated by wounding, methyl jasmonate, ethylene, and gibberellin in tomato seeds and leaves. *Plant Physiol* **133**, 263–73.