

## Measuring Homologous Recombination Frequency in *Arabidopsis* Seedlings

Marisa Rosa\* and Ortrun Mittelsten Scheid

Gregor Mendel Institute of Molecular Plant Biology, Vienna, Austria

\*For correspondence: [massrosa@berkeley.edu](mailto:massrosa@berkeley.edu)

**[Abstract]** Somatic homologous recombination (SHR) is a major pathway of DNA double-strand break (DSB) repair, in which intact homologous regions are used as a template for the removal of lesions. Its frequency in plants is generally low, as most DSB are removed by non-homologous mechanisms in higher eukaryotes. Nevertheless, SHR frequency has been shown to increase in response to various chemical and physical agents that cause DNA damage and/or alter genome stability (reviewed in March-Díaz and Reyes, 2009). We monitor the frequency of SHR in transgenic *Arabidopsis* seedlings containing recombination substrates with two truncated but overlapping parts of the  $\beta$ -glucuronidase (GUS) reporter gene (Orel *et al.*, 2003; Schuermann *et al.*, 2005). Upon an SHR event, a functional version of the transgene can be restored (Figure 1A). A histochemical assay applicable to whole plantlets allows the visualization of cells in which the reporter is restored, as the encoded enzyme converts a colorless substrate into a blue compound. This type of reporter has been extensively used to identify gene products required for regulating SHR levels in plants. We analyze plants stimulated for SHR by treatments with DNA damaging agents (bleocin, mitomycin C and UV-C) and compare them to non-treated plants.

### Materials and Reagents

1. *Arabidopsis thaliana* seeds of reporter lines IU.GUS-8 and DGU.US-1 (Figure 1) (Orel *et al.*, 2003) [introgressed into various genetic backgrounds and genotyped for homozygosity of reporters and mutations (Rosa *et al.*, 2013). As an example, we use wild type Columbia-0, and mutants' *arp6-3* and *swc6-1*. Both mutants lack subunits of the *Arabidopsis* homolog of the SWR1 complex and, additional to being sensitive to DNA damaging agents (Rosa *et al.*, 2013), have pleiotropic developmental defects (for more information see Reference 5)]
2. Sodium hypochlorite (Sigma-Aldrich, catalog number: 425044)
3. Tween-80 (Sigma-Aldrich, catalog number: 4780)
4. Sterile H<sub>2</sub>O
5. Solid growth medium
6. Liquid plant growth medium (same as solid growth medium but without agar)
7. Hydroxyurea

8. 70% ethanol
9. Seed sterilization solution (see Recipes)
10. Bleocin (commercial name for bleomycin) (EMD Millipore, catalog number: 203408) (see Recipes)
11. Mitomycin C (Duchefa Biochemie BV, catalog number: M0133) (see Recipes)
12. GUS staining solution (see Recipes)
13. 1 M Sodium Phosphate Buffer (see Recipes)

### **Equipment**

1. Plastic petri dishes for plant culture (see Notes) (round 200 x 15 mm, with 20 ml of solid growth medium)
2. Sterile hood, preferably a biological safety cabinet to avoid exposure to the genotoxins
3. Bench top block shaker (*e.g.* Eppendorf Thermomixer®)
4. UV crosslinker (254-nm UV light bulbs, 15 watts each) (Stratagene, model: Stratalinker 2400)
5. Forceps
6. Box or aluminum foil
7. Parafilm
8. 1.5 ml Eppendorf tubes
9. 50 and 15 ml Falcon tubes
10. Vacuum applicator (*e.g.* desiccator)
11. Plant growth facilities with 16-h-light/8-h-dark cycles, at 21 °C
12. Incubator at 37 °C
13. Stereomicroscope

### **Procedure**

1. Wash the seeds in 1.5 ml Eppendorf tubes with 1 ml of the sterilization solution, shake in a benchtop block shaker at 300 rpm for 6 min at room temperature. Allow seeds to set in the bottom of the Eppendorf and remove supernatant. Wash with 1 ml of sterile water, 5 times for 5 min in the shaker. After the final wash, remove supernatant and dry overnight in the closed sterile hood, leaving the tubes open.

*Note: Sterilize approximately 200 seeds per genotype and treatment. All subsequent steps involving seed or plantlet manipulation should be carried in the sterile hood.*

2. Plate seeds on 200 x 15 mm petri dishes with growth medium ( $\approx$  75 to 100 per plate and 2 plates per genotype and treatment), using flame-sterilized and cooled forceps. Stratify

in the dark (in a box or wrapped in aluminum foil) at 4 °C for 2 to 4 days and transfer to the growth chambers with illumination.

*Note: Any other sterile plating techniques are suitable.*

### 3. Treatments

- a. Bleocin and mitomycin C: Grow plants until day 7. Add 10 ml of drug-free liquid media, or media with 0.1 µg/ml of bleocin or 10 µg/ml of mitomycin C. Seal with parafilm and return to normal growth conditions until analysis. Shaking is not necessary.

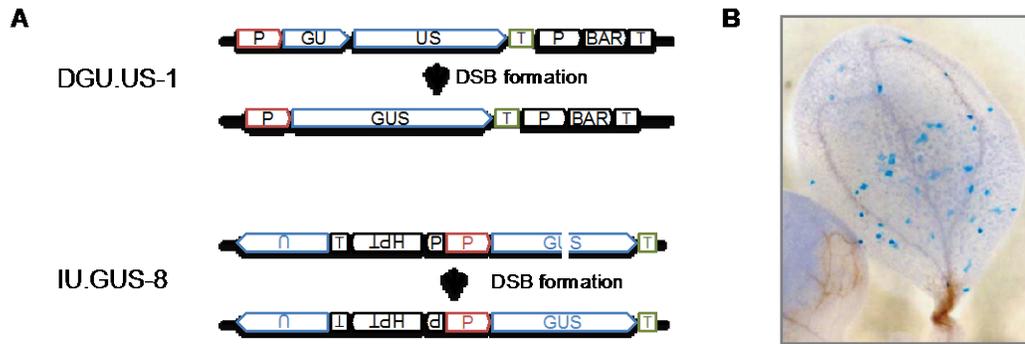
*Note: Grow until day 7 = 7 days after transfer to the growth chambers.*

- b. Hydroxyurea: Plate seeds on solid growth plates, without or with 1 mM of the chemical, and grow at standard conditions until analysis.
- c. UV-C: Grow seedlings until day 4 of growth and treat with 8 kJ/m<sup>2</sup> UV-C in a Stratalinker. Return to standard conditions until analysis.

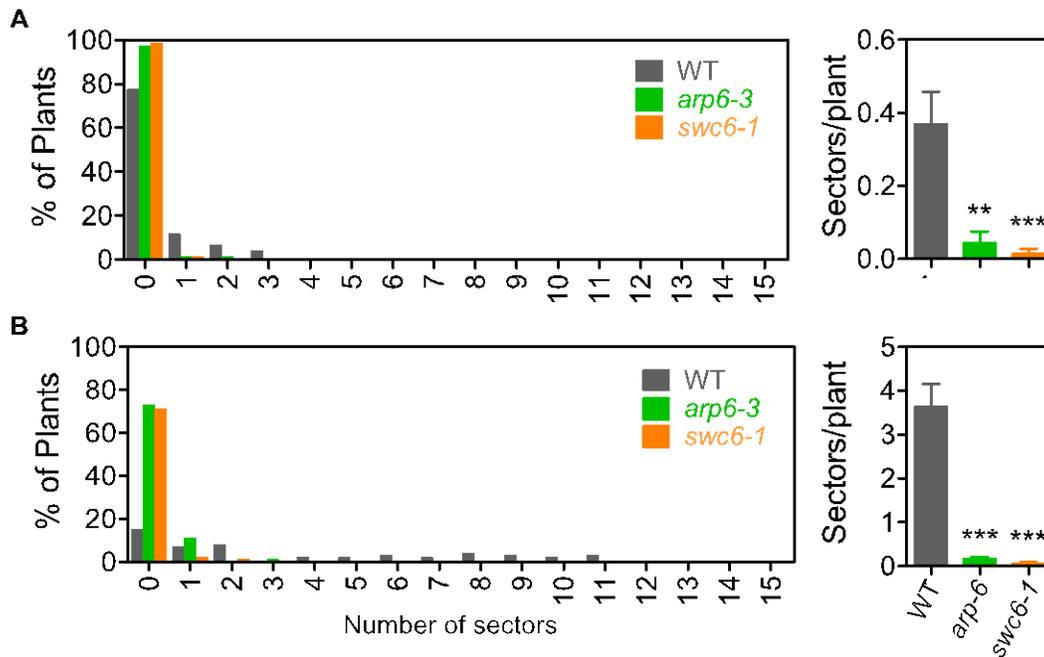
*Note: Before the treatment sterilize the interior of the Stratalinker with 70% Ethanol and run it for 3 min with the maximum dose of UV-C. Then place plates inside and remove the lid, keeping it inside the Stratalinker. Quickly close the door, to avoid contamination, and run the desired dosage of UV-C. When the treatment is finished close the plates still inside the Stratalinker and return them to normal growth conditions.*

### 4. GUS histochemical assay

- a. With forceps, carefully collect the 12-day-old plants of each plate in 15 ml Falcon tubes and submerge them in 10 ml of GUS staining solution. Be careful not to break the roots, cotyledons or leaves. Vacuum infiltrate for 15 min and incubate overnight at 37 °C.
- b. De-stain the plants in 70% ethanol overnight at 37 °C. Continue to change the ethanol twice a day until most plants tissue is white.
- c. Under a stereomicroscope score the number of GUS positive blue sectors indicating recombination events per whole plant (Figure 1B). To analyze the data, generate histograms with the frequency of seedlings with different numbers of blue sectors, and the total frequency in each line and treatment (Figure 2).



**Figure 1. Principle of the assay.** (A) Recombination substrates in reporter lines for SHR DGU.US-1 and IU.GUS-8 (adapted from Reference 2). These lines were designed to distinguish different somatic recombination pathways (single-strand annealing and synthesis-dependent strand annealing) upon DSB repair after expression of the endonuclease I-SceI (Orel *et al.*, 2003; for more information about different SHR pathways see Reference 1). However, they are used here to measure SHR without I-SceI but after different exogenous DNA damage treatments. The DGU.US-1 substrate contains two fragments of the GUS ORF with homologous parts (GU, US) in direct orientation, harboring an overlap of 557 bp. In IU.GUS-8, two fragments of the GUS ORF (“GU”, “US”; blue) in direct orientation are separated, rendering the gene not functional. A fragment of 1087 bp partially homologous to “GU” and “US”, and containing “U” is located upstream in inverted orientation. SHR events between the repeats can restore a functional gene (GUS) in different ways: in line DGU.GUS-1, the sequence in common between the two repeats is deleted, while HR between indirect repeats in IU.GUS-8 results in the restoration of the GUS gene. P: constitutive 35S promoter of cauliflower mosaic virus; T: nopaline synthase terminator; HPT: hygromycin resistance gene, BAR: phosphinothricin acetyltransferase resistance gene, GUS:  $\beta$ -glucuronidase gene. (B) Histochemical GUS staining allows to visualize cells in which the GUS gene was restored by SHR (from Orel *et al.*, 2003).



**Figure 2. Examples of SHR frequency measurements.** Distribution of seedlings with different numbers of blue sectors and total frequency in line IU.GUS-8. A Mock and B 1 µg/ml Bleocin treatment. Sectors/plant refers to the average number of blue sectors, corresponding to recombination events, in the analyzed seedling population. Error bars correspond to the SE. SHR frequencies in each mutant population were significantly different to their wild-type (WT) counterpart, with a P value < 0.001. Asterisks indicate the significance between treated and mock populations according to P values from unpaired *t* tests: \*\*\*P < 0.001 and \*\*0.001 < P < 0.01.

**Notes**

1. For all treatments, always keep the plates belonging to one experiment (mock + treatment) growing side-by-side. For example, take plates for mock and UV-C treatment out of the growth chambers at the same time.
2. In this experiment we used Germination Media (GM) described in Reference 6 (detailed protocol available at <http://www.gmi.oeaw.ac.at/research-groups/ortrun-mittelsten-scheid/research-groups/ortrun-mittelsten-scheid/gm-medium-protocol>). We have not tested the use of regular MS plates but have no reason to believe the media will be a determining factor as long as the plates used in each experiment come from the same batch of media.

## Recipes

1. Seed sterilization solution
  - Consisting of 5% sodium hypochlorite and 0.05% Tween-80
  - Mix 500  $\mu$ l of sodium hypochlorite and 5  $\mu$ l of Tween-80 in 10 ml of sterile H<sub>2</sub>O
2. Bleocin (stock 10 mg/ml)
  - Dissolve 100 mg of Bleocin in 10 ml of H<sub>2</sub>O
  - Make 0.2 ml aliquots and keep at -20 °C until use
  - Avoid thawing/freezing cycles
  - Dilute to the desired concentration in liquid media, using disposable 50 ml falcon tubes
3. Mitomycin C (stock 0.5 mg/ml)
  - Dissolve 2 mg of Mitomycin C in 4 ml of H<sub>2</sub>O immediately before use
  - Dilute to the desired concentration in liquid media, using disposable 50 ml falcon tubes
4. GUS staining solution
  - Consisting of 1 mM sodium phosphate buffer (pH 7); 10 mM EDTA, 0.1% Triton-X, 100 mg/ml chloramphenicol; 2 mM potassium ferrocyanide; 2 mM potassium ferricyanide; 0.5 mg/ml X-glucuronide
  - Mix:
    - 10 ml of 1M Sodium Phosphate Buffer Solution (pH 7) (see below)
    - 4 ml of 0.25 M EDTA solution pH 8
    - 0.5 ml 20% (w/v) Triton X-100
    - 0.2 ml of 50 mg/ml Chloramphenicol
    - 1 ml of 0.2 M Potassium Ferricyanide
    - 1 ml of 0.2 M Potassium Ferrocyanide
    - 2.5 ml 20 mg/ml X-Gluc (kept in the dark at -20 °C)
  - Bring up to 100 ml with MilliQ water, filter-sterilize (0.2 micron filter) and keep at 4 °C wrapped in aluminum foil. If stored under these conditions, this solution will be stable for up to one month.
5. 1 M Sodium Phosphate Buffer (final pH 7)
  - Make 200 ml of 1 M Sodium Dihydrogen Phosphate (this is acid, about pH 4)
  - Put in a beaker and gradually add to this 1 M Di-Sodium Hydrogen Phosphate (pH about 9), until the mix reaches a pH of 7 at 20 °C
  - Autoclave

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