Chapter 25

Analyzing Somatic DNA Repair in Arabidopsis Meiotic Mutants

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Abstract

Meiotic and somatic recombination share a common set of factors. Thus, the analysis of somatic DNA repair in meiotic mutant lines should be of special interest. Growth defects of mutant plants induced by specific genotoxins can thereby hint to DNA repair functions of the affected proteins. Here, we describe two kinds of approaches to characterize deficiencies in DNA repair in mutant lines of *Arabidopsis thaliana*, after genotoxin treatment.

Key words *Arabidopsis thaliana*, Cisplatin, DNA repair, Genotoxin, Methyl methanesulfonate, Mitomycin C, Root meristem

1 Introduction

Homologous recombination is essential for the correct course of meiosis. As meiotic recombination is initiated by the introduction of double strand breaks and shares a multitude of mechanisms with somatic double strand break repair, many factors involved in meiosis also possess an important role in somatic DNA repair [1, 2]. Therefore, it is highly recommended to test whether Arabidopsis mutants affected in meiosis also have somatic DNA repair defects. A prominent example is the proteins of the RTR (RECQ4A/TOP3α/RMI1/2) complex [3]. The complex is involved in the dissolution of recombination intermediates like double Holliday junctions, thus playing an important role in DNA repair via homologous recombination. RTR-mutant lines exhibit characteristic hypersensitivities against a number of genotoxins [4–6]. Furthermore, *top3α* and *rmi1* mutants are characterized by a unique meiotic phenotype, with extended chromosome fragmentation leading to an arrest after meiosis I [5, 7]. Similarly, the nuclease MUS81 is involved in resolving class II crossovers in meiosis, whilst also being important for somatic DNA cross-link repair [8]. Another example is the cohesion cofactor PDS5

Mónica Pradillo and Stefan Heckmann (eds.), *Plant Meiosis: Methods and Protocols*, Methods in Molecular Biology, vol. 2061,
https://doi.org/10.1007/978-1-4939-9818-0_25, © Springer Science+Business Media, LLC, part of Springer Nature 2020

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(characterized as meiotic factor SPO76 in a number of organisms), shown to be involved not only in meiosis in Arabidopsis but also exhibiting an involvement in DNA double-strand break repair [9]. This demonstrates the close link between meiotic recombination and DNA repair. For the analysis of DNA repair, mutant analysis testing the hypersensitivity of plants against certain genotoxins is a well-established and reliable method. By the selection of specific genotoxins, different kinds of DNA damage can be induced. Commonly used genotoxins for the induction of genotoxic stress are the methylating agent methyl methanesulfonate (MMS) and the two DNA cross-linking agents cisplatin and mitomycin C. DNA cross-links pose a dangerous threat for genome stability by blocking essential processes like replication and transcription. While cisplatin induces mostly intrastrand cross-links that affect only one DNA strand and leave the complementary strand as a repair template, mitomycin C mainly causes interstrand cross-links that are highly toxic by connecting both DNA strands [10, 11]. Mutants defective in the repair of specific DNA damages, exhibit a slower growth in the presence of the causal genotoxin, thus resulting in a reduced fresh weight compared to wild-type plants treated with the same substance [4, 12]. This allows for the conclusion to be made whether or not the affected protein is involved in the repair of this kind of DNA damage. As the repair of specific DNA damages require specialized pathways, depending on the involvement of the affected factor, mutant lines can vary in their response to different genotoxins. A second approach for the characterization of genotoxin sensitivity is based on the analysis of cell death in the root meristem. Meristematic cells are characterized by a high proliferation rate, but at the same time, an accumulation of mutations has to be prevented. As plant stem cells form precursors of all tissues during the whole lifetime of a plant, DNA damage protection is especially important here. Thus, cells of the root and apical meristem are highly sensitive against genotoxic stress. This results in a selective appearance of programmed cell death in meristematic tissue, even at a low level of DNA damage [13]. Due to its transparency and ease of accessibility for microscopy, the root meristem is an ideal system to analyze the effect of DNA damage. Because of their high proliferative rate, root cells are particularly suitable indicators of replication-associated DNA damage [14]. Propidium iodide (PI) is a fluorescent dye that intercalates into nucleic acids, but is only able to penetrate the perforated cell membrane of dead cells, thus enabling the differentiation between living and dead cells. The application of PI staining enables the analysis of cell death in root cells for the quantification of replication-associated DNA damage in mutant lines. Depending on the strength and the nature of the defect to process aberrant DNA replication intermediates, enhanced cell death is detectable under normal plant growth conditions [15, 16]. An additional

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genotoxin-treatment might be advisable in cases where no clear defect is visible under standard conditions [17, 18].

In the following, we describe the analysis of Arabidopsis mutant lines in response to genotoxic stress. Thereby, two different kinds of protocols are described. The sensitivity assay relies on the reduction in total fresh weight in response to the treatment with genotoxic agents. The second method is based on the analysis of cell death in the root meristem, using microscopic analysis of PI stained roots that can be genotoxin treated.

2 Materials

2.1 Reagents

Prepare all solutions with ultrapure water. Prepare and store all reagents at room temperature (unless indicated otherwise).

1. Germination medium (GM) for cultivation of *Arabidopsis thaliana*: 4.9 g/l Murashige and Skoog medium including vitamins and MS buffer, 10 g/l sucrose, adjust pH to 5.7 with KOH and sterilize by autoclaving. Add 7.6 g/l (10 g/l for vertical cultivation) Plant Agar before sterilization for solid media (*see* **Note 1**). Pour solid media, cooled to 50 °C, after sterilization into usual round petri dishes for horizontal cultivation or rectangular petri dishes for vertical cultivation.
2. 5 μg/ml propidium iodide (PI) solution: Dissolve in water and store in a lightproof container at 4 °C.
3. 1 mM *Cis*-diamminedichloridoplatinum(II) (cisplatin): Dissolve in water and store in aliquots at −20 °C.
4. Methyl methanesulfonate (MMS): Prepare aliquots at room temperature.
5. 1 mg/ml mitomycin C (MMC): Dissolve in water immediately before use.

3 Methods

Surface-sterilize your Arabidopsis seeds with your favorite method and incubate the seeds over night at 4 °C in water for stratification. All steps until weighing/slide preparation should be performed under sterile conditions in a laminar flow hood to prevent microbial contamination. Pay special attention to the handling and disposal of genotoxins and all material contaminated with genotoxins. Prevent aerosol formation especially during the disposal of liquid genotoxin waste by using a pump.

3.1 Sensitivity Assay

1. Sow 100 seeds per line on solid GM, evenly spaced. Incubate the plates horizontally in a plant growth chamber for 7 days (*see* **Notes 2** and **3**).

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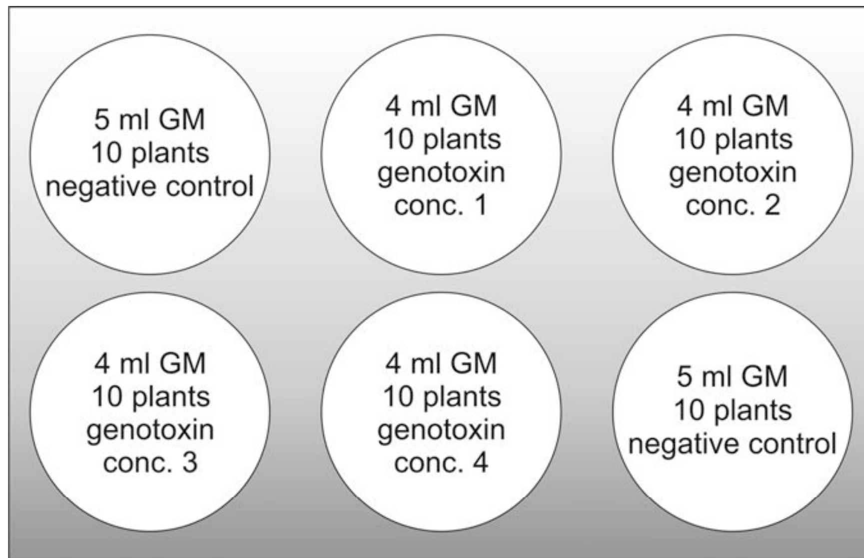

Fig. 1 Six-well plate setup for sensitivity assays. The two outermost wells are used for negative controls; the other wells can be used for treatment with four different genotoxin concentrations. The wells for negative controls are filled with 5 ml liquid GM medium; the wells for genotoxin treatments are filled with 4 ml GM, and the appropriate genotoxin can be added diluted in 1 ml GM. Every well is equipped with 10 plants

2. Use an individual six-well plate for each line and fill two wells with 5 ml liquid GM for negative controls; fill the other wells with 4 ml GM (*see* **Note 4**). Using forceps, transfer ten seedlings into each well (Fig. 1, *see* **Note 5**). Incubate the six-well plates in a plant growth chamber overnight.
3. Dilute your genotoxin stock in liquid GM medium. Add 1 ml of appropriately diluted genotoxin to the wells with 4 ml GM for a total of 5 ml (*see* **Note 6**). Thus, four different genotoxin concentrations can be tested per six-well plate. Incubate the six-well plates in a plant growth chamber for 13 days.
4. Transfer the plantlets onto tissue paper, place another tissue on top and press with the lid of the six-well plate to remove excess liquid. Determine the fresh weight of the plants in each well with a fine scale balance (*see* **Note 7**).
5. For data analysis, form the mean weight from both negative controls and calculate the fresh weight of the treated samples in relation to the control. This enables the elimination of the influence of basic growth differences from different lines.

3.2 Analysis of Cell Death in the Root Meristem

1. Sow three lines of 13 seeds on rectangular petri dishes with solid GM. Seal the plates with Parafilm and incubate them vertically in a plant growth chamber for 4 days.

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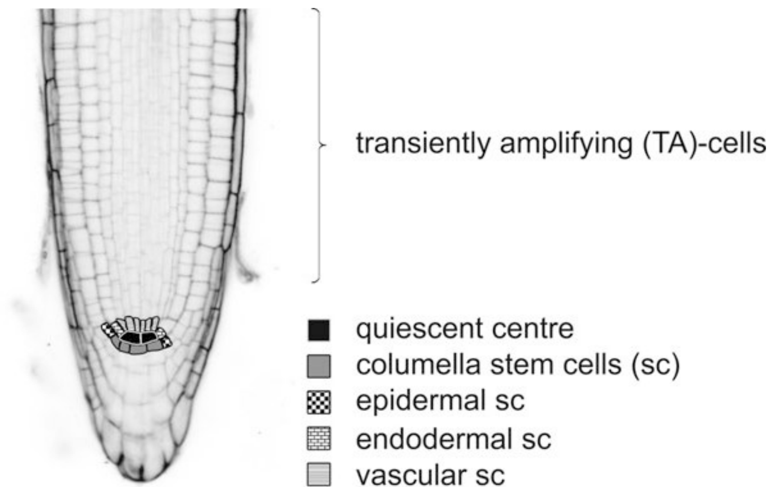

Fig. 2 Different cell types in the root meristem. The root meristem contains the stem cell niche, consisting of the quiescent center surrounded by four different stem cell types. These stem cells give rise to the different organs of the root. At the apex of the stem cell niche, the transiently amplifying cells are located, that are characterized by a high division activity

2. Transfer ten seedlings into a well of a six-well plate filled with 5 ml liquid GM for untreated approaches or 4 ml liquid GM for genotoxin treated approaches (*see* **Note 8**).
3. Dilute your genotoxin stock in liquid GM medium. Add 1 ml of appropriately diluted genotoxin to the wells containing 4 ml liquid GM (*see* **Note 9**). Incubate the six-well plates in a plant growth chamber for at least 24 h.
4. The next day, carefully wash the seedlings by dipping them shortly into water. Then remove the leaves with forceps and transfer the root onto a microscope slide with 180 μl PI solution. Place 7–10 roots on one slide and add a coverslip (24 × 40 mm, *see* **Note 10**).
5. Analyze the roots with a confocal laser scanning microscope using an appropriate filter set and count the number of dead cells per root in the anterior 200 μm (*see* **Notes 11–13**). By taking the special arrangement of the cells around the quiescent center into account, different cell types can be distinguished (Fig. 2).

4 Notes

1. Make sure to use media with at least 10 g/l agar for vertical cultivation, as medium with less agar tends to sag in petri dishes.

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2. For plant growth in a growth chamber, we use a day–night rhythm of 16 h illumination at 22 °C and 8 h darkness at 20 °C. The number of days for all incubation steps is optimized for these growth conditions. You can alter the incubation time according to your needs, but make sure to stay consistent with time periods once decided upon.
3. It is possible to elongate incubation time to 2 weeks, for example for segregating lines that enable phenotypic identification of homozygous plants. Then transfer only five plants per well in the next step.
4. We always use two wells, as far away from each other as possible, for negative controls, as the six well plates are contamination prone and the experiment relies on at least one suitable negative control for each line.
5. Be careful not to tear the plant roots off during transfer. If this is a recurring problem in your experiment, consider using less agar for your GM plates.
6. As a starting point for final genotoxin concentrations we use 40–100 ppm MMS, 5–20 μg/ml MMC and 5–20 μM cisplatin. Try different ranges of concentrations for your lines to find a suitable sensitivity window. Of course the assays described here are not limited to these genotoxins only, there is a wide selection of different genotoxins available. If your genotoxin is not soluble in water, make sure to add the maximal amount of solvent contained in your treated wells also to the negative control.
7. This assay also allows the determination of plant dry weight. For this purpose, write down the empty weight of each weighing dish before weighing plant fresh weight. After fresh weight determination, dry the plants in the individual weighing dishes at 60 °C at least overnight. Then weigh the weighing dishes including the dry plants and subtract the empty weight of the weighing dish.
8. Be extremely careful with plant roots as the lightest touch can damage root cells, which can affect results.
9. As a starting point for final genotoxin concentration, we use 40 μM cisplatin, 3 μg/ml MMC or 75 ppm MMS. Try different concentrations for your lines to find a suitable sensitivity window (*see* **Note 14**).
10. Prepare the slides immediately before analysis and do not press on the coverslip as roots are highly sensitive.
11. Dead cells are apparent by a red fluorescence. As PI also penetrates the intercellular space, this allows for the visualization of the different cells in the root.
12. Make sure to check all focal planes for dead cells.

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13. For an alternative evaluation method, you can count the number of roots exhibiting at least one dead cell per cell type.
14. In the case that a DNA repair defect can be revealed by the described protocols, it might be advisable to also test the efficiency of intrachromosomal homologous recombination (HR) in somatic cells. Several powerful transgenic assay systems based on the restoration of the ß-glucuronidase marker by HR are available for Arabidopsis, and a detailed protocol of their application has already been published in this series [19, 20].

Acknowledgments

This work was supported by the European Research Council (Advanced Grant "COMREC" and "CRISBREED").

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