Protocol

Phenotypic Analysis of *Arabidopsis* Mutants: Trypan Blue Stain for Fungi, Oomycetes, and Dead Plant Cells

Saskia van Wees

This protocol was adapted from "How to Analyze a Mutant Phenotypically," Chapter 4, in Arabidopsis*: A Laboratory Manual* (eds. Weigel and Glazebrook). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2002.

INTRODUCTION

Trypan blue stains vasculature, dead plant cells, and fungal and oomycete hyphae. It is useful for assessing the extent of colonization of tissue, and for detecting microlesions present in certain lesion-mimic mutants. Trypan blue staining requires chloral hydrate for destaining, which is inconvenient, because it is a controlled substance. The chloral hydrate can be replaced with 1:2 lactophenol:ethanol, but the background staining will be higher than it is when chloral hydrate is used.

RELATED INFORMATION

For information about obtaining a mutant in *Arabidopsis*, see **Setting Up *Arabidopsis* Crosses** (Weigel and Glazebrook 2006), **Genetic Analysis of *Arabidopsis* Mutants** (Weigel and Glazebrook 2008), and the Discussion. See Keogh et al. (1980) for a description of trypan blue staining in fungal tissue. For a description of lesion-mimic mutants, see Dangl et al. (1996).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

<**!**>Chloral hydrate solution (25 g of chloral hydrate in 10 mL of H_2O)
Ethanol
Glycerol (70% w/v)
<**R**>Lactophenol
Tissue sample for staining
<**!**>Trypan blue

Equipment

Microscope (compound)
Plate (12-well microtiter)
Water bath (boiling)

METHOD

1. Prepare the staining solution as follows:
   i. Add trypan blue to lactophenol to a concentration of 2.5 mg/mL.
   ii. Add 2 volumes of ethanol to the trypan blue-lactophenol solution.
2. Place the tissue to be stained in the wells of a 12-well microtiter plate. Cover the tissue with the staining solution from Step 1.ii, and heat in a boiling water bath for 1 min.
3. Allow the plate to sit at room temperature for 1-24 h. (Begin with 1 h; if samples are not stained well enough, increase the time of incubation.)
4. Remove the staining solution, and cover the tissue in chloral hydrate solution. Incubate for 2-6 h at room temperature.

   The staining solution may be saved and reused. This practice helps to minimize the amount of phenol waste generated.
5. Replace the chloral hydrate solution, and incubate overnight at room temperature.
6. Remove the chloral hydrate solution, and cover the tissue in 70% glycerol.
7. Examine the tissue using a compound microscope.

   For examples of trypan blue staining of dead plant cells, Peronospora parasitica *in leaves, and* Fusarium oxysporum *in leaves, see Rate et al. (1999), Petersen et al. (2000), and Epple et al. (1997), respectively.*

DISCUSSION

Two methods of obtaining a mutant are by directed screening for plants with a certain phenotype and by knocking out a gene of interest. The phenotypes of knock-out mutants are difficult to predict, and it is also quite common for mutants, first isolated because of a specific phenotype, to have other, pleiotropic defects, i.e., multiple defects in addition to that of primary interest. Thus, it is often necessary to characterize a variety of phenotypic parameters. This is particularly important as the field of molecular genetics expands. If a number of groups, each looking for a different phenotype, independently identify different mutations in the same gene, an awareness of additional defects will often show that the new mutation is allelic to existing mutants.

Before embarking on an extensive phenotypic characterization, it is important to confirm that the various phenotypes of an individual are genetically linked (see **Setting Up *Arabidopsis* Crosses** [Weigel and Glazebrook 2006] and **Genetic Analysis of *Arabidopsis* Mutants** [Weigel and Glazebrook 2008]). Strictly speaking, all of the phenotypes should be mapped, but it is normally sufficient to backcross a newly isolated mutant about five times to remove defects due to unlinked mutations. Phenotype mapping becomes more important, however, when dealing with a mutant that has been isolated by site-selected mutagenesis (T-DNA or transposon knock-out). In this case, it must be shown that any phenotype cosegregates with the induced mutation. Definitive proof that a phenotype is indeed due to the disrupted gene can only come from complementing the mutant with a wild-type copy of the disrupted gene or by constructing *trans*-heterozygotes carrying two independently isolated mutations.

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