

Single-Cell Complementation of Barley *mlo* Mutants Using a PDS-1000/He Hepta™ System

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Abstract

Transient expression via particle bombardment is widely used as a means of gene transfer to bacteria, yeast, animals, and plants. In this study we describe the use of the Hepta adaptor for the PDS-1000/He biolistic system for transformation and transient expression of *Mlo* in single epidermal cells of detached barley leaves. Barley *Mlo* is known to dampen plant defense and its expression in single epidermal cells of *mlo* resistant mutants restores susceptibility to attack from the powdery mildew fungal pathogen, *Blumeria graminis*. Consistently high transformation efficiencies were obtained upon delivery of a plasmid carrying both Green Fluorescent Protein (GFP) and *Mlo*. After bombardment and fungal spore inoculation, we found leaf sectors with numerous green fluorescent epidermal cells supporting growth of the pathogen. Likewise, we observed a high transformation efficiency of *Arabidopsis* leaf cells upon delivery of a GUS (β -glucuronidase) reporter gene construct. Application of the Hepta adaptor reduces the number of biolistic transfers necessary to obtain sufficient numbers of transformed plant cells for quantitative scoring of single-cell traits.

Introduction

Mutation induced recessive alleles (*mlo*) of the barley *Mlo* gene confer broad spectrum resistance against *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew. Conversely, the presence of wild-type *Mlo* leads to susceptibility upon attack from this obligate biotrophic fungal pathogen. *Mlo* encodes the founder of a novel family of plant-specific integral membrane proteins (Büschges et al. 1997). The 7-transmembrane-domain protein resides in the plasma membrane and is presumed to act as a negative regulator of a basal defense mechanism (Devoto et al. 1999).

We have previously shown that transient single-cell expression of wild-type *Mlo* in *mlo* resistant leaves restores susceptibility to *Blumeria graminis* (Shirasu et al. 1999). This was based on cobombardment experiments involving 2 plasmids harboring either *Mlo* or GFP (Figure 1).

Here we describe a modification of the transformation procedure by using the PDS-1000/He Hepta adaptor. This device fits into the shocking chamber of the PDS-1000/He unit and splits the helium shock wave over 7 outlets, permitting a more even dispersal of DNA-coated particles and a greater target area.

Methods

Plant and Fungal Material

Leaves of *mlo* resistant barley (*Hordeum vulgare* cv. BC Ingrid *mlo*-5) and *Arabidopsis thaliana* (ecotype Ms-0) were used for this study. Barley plants were grown in a controlled environment at 18°C (16 hr light/8 hr darkness), whereas *Arabidopsis* plants were grown in short-day (8 hr light/16 hr darkness) conditions. First leaves of 8-day-old barley plants or rosette leaves of approximately 5-week-old *Arabidopsis* plants were used for the experiments. Barley leaves (apical 5–7 cm segments) were harvested and kept on 1% agar containing 10% sucrose 4 hr prior to bombardment.

Blumeria graminis f. sp. *hordei* K1 was propagated on *H. vulgare* cv. Golden Promise as previously described (Shirasu et al. 1999).

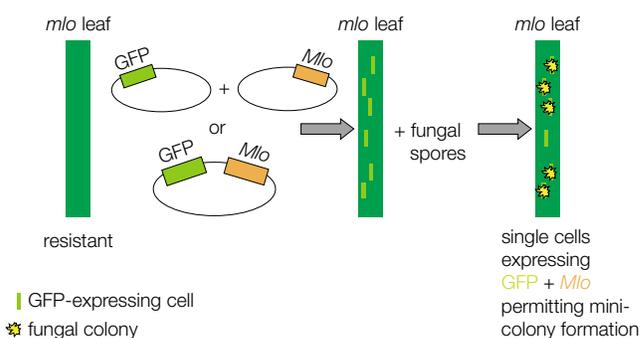


Fig. 1. Scheme of the transient single-cell expression system. Detached resistant (*mlo*) barley leaves were bombarded with either 2 separate plasmids carrying GFP and *Mlo* coding sequences (Shirasu et al. 1999) or a single plasmid carrying both genes (pUGLUM, this study). After particle bombardment, leaves were inoculated with powdery mildew spores. Single leaf epidermal cells expressing both genes are fluorescent green and support growth of fungal colonies.

Plasmids

Plasmid pUGLUM (Zhou et al. 2001) carrying GFP and *Mlo* coding sequences under the control of the strong constitutive maize ubiquitin 1 promoter was used for the barley experiments. For transformation of *A. thaliana*, plant binary vector pPam-GUS (T Rademacher and R Panstruga, unpublished) was used. In this plasmid the β -glucuronidase reporter gene is driven by a double constitutive cauliflower mosaic virus 35S promoter.

Preparation of DNA-Coated Gold Microcarriers

The preparation of DNA-coated gold microcarriers (1 μ M particle size) was carried out according to the manufacturer's instructions (Bio-Rad PDS-1000/He manual). Briefly, gold particles were soaked in 70% ethanol (v/v), washed thoroughly with sterile water, and resuspended in 50% glycerol at a concentration of 60 mg/ml. To coat the particles with plasmid DNA, 50 μ l (3 mg) of microcarriers were mixed with 5 μ l DNA (1 μ g/ μ l), 50 μ l 2.5 M CaCl_2 and 20 μ l 0.1 M spermidine (free base). After 2 washing steps with ethanol (first 140 μ l 70%, second 140 μ l 100%), the coated particles were finally resuspended in 48 μ l 100% ethanol.

Conditions for Particle Bombardment and Fungal Inoculation

Each of the 7 macrocarriers of the Hepta adaptor was loaded with 6 μ l of the coated microcarriers corresponding to 0.62 μ g plasmid DNA. Hence, a total of approximately 4.3 μ g plasmid DNA was delivered per shot. The target shelf carrying the petri dish with detached leaf segments was placed 6 cm from the Hepta adaptor. Usually 8–10 leaf sections (barley) or 10–20 rosette leaves (*A. thaliana*) were placed side by side in a single 9 cm petri dish for the biolistic transfer. After evacuating the shocking chamber to 27" Hg, specimens were bombarded with a helium pressure of 900 or 1,100 psi. Immediately after bombardment, the vacuum was released and leaf segments were kept on the plates 24 hr for recovery. The leaves were transferred to fresh plates (1% agar, 0.002 g/L benzimidazole) and inoculated with a high density of conidiospores of *B. graminis* f. sp. *hordei* K1. Petri dishes carrying the detached leaf sections were incubated in a growth cabinet under conditions described above.

Staining for β -glucuronidase (GUS) Activity

Arabidopsis rosette leaves were stained for GUS activity 3–4 days after bombardment by vacuum infiltration (1 hr) in 100 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0, 0.1% Triton X-100, 2 mM $\text{K}_3\text{Fe}(\text{CN})_6$ containing 0.5 mg/ml 5-bromo-4-chloro-3-indoxyl- β -D-glucuronic acid and incubated overnight at 37°C. The stained leaves were cleared thereafter in ethanol (96%) for several hours.

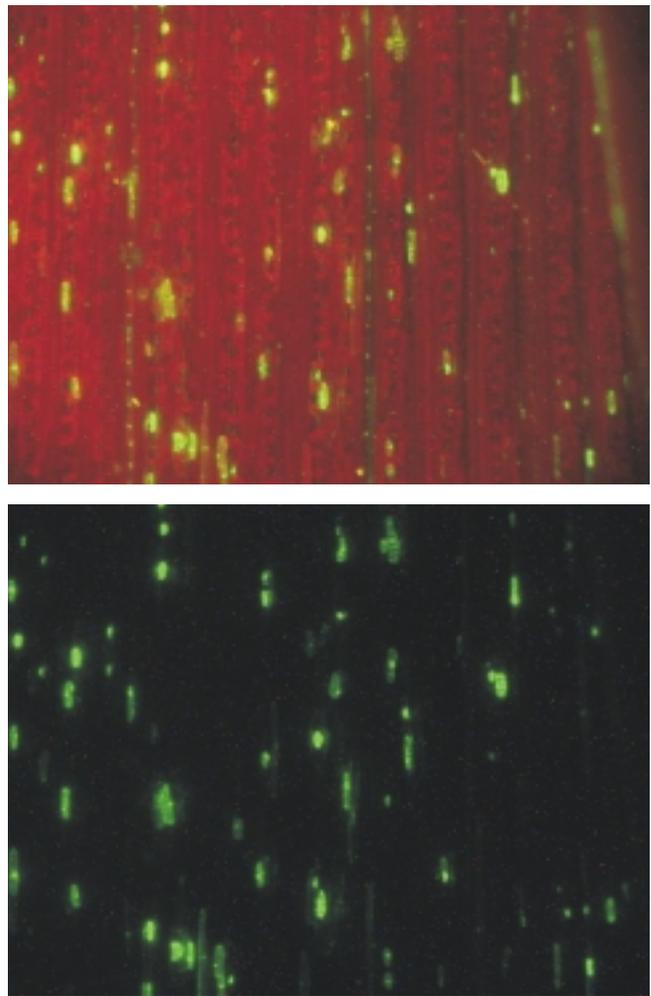


Fig. 2. Sector of a barley leaf expressing GFP in single epidermal cells. GFP expression in different epidermal cell types 18 hr after bombardment with pUGLUM was visualized by analysis with a Leica fluorescence stereo microscope using (A) Leica filter GFP1 (425/60 nm excitation filter, 480 nm barrier filter) or (B) Leica filter GFP3 (470/40 nm excitation filter, 525/50 nm barrier filter). The red color in (A) results from chlorophyll autofluorescence of leaf mesophyll cells.

Results and Discussion

Detached leaves of *mlo* resistant barley cultivar Ingrid containing the null mutant allele *mlo-5* were bombarded with plasmid DNA of pUGLUM as described in Methods (Figure 1). The plasmid harbors the *Mlo* wild type and the GFP reporter genes whose expression is driven by a ubiquitin promoter. Approximately 24 hr after the biolistic transfer, we observed leaf sectors with numerous green fluorescent epidermal cells and occasionally single fluorescent cells outside of the clusters (Figure 2). Optimal transformation efficiencies were obtained with He pressures of 900 psi and 1,100 psi. Higher He pressures (1,350 psi) resulted in disruption of foliar tissue, whereas lower pressures (450 or 650 psi) led to a reduction in the number of detectable green fluorescent cells. GFP expression was observed in different cell types of the leaf epidermis, e.g., guard cells and interstomatal short and long cells (Figure 2).

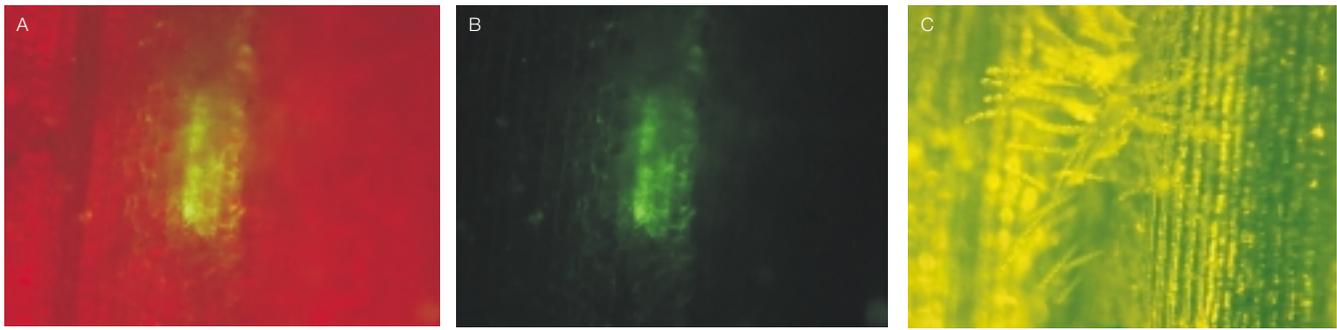


Fig. 3. GFP expression in and powdery mildew conidial formation on the surface of a transformed epidermal cell. GFP expression was visualized by analysis with a Zeiss Axiophot microscope using (A), filter GFP1 (450–490 nm excitation filter, 510 nm dichromatic mirror, and 520 nm barrier filter) or (B), filter GFP2 (470/20 nm excitation filter, 493 nm dichromatic mirror, and 505–530 nm barrier filter). C, growth of conidiophores on the epidermal surface was visualized by bright-field microscopy. The red color in (A) results from chlorophyll autofluorescence of leaf mesophyll cells.

Detached leaf sections were heavily inoculated with spores of *B. graminis* f. sp. *hordei* isolate K1 approximately 24 hr after bombardment. Microscopic fungal growth was visible 3–4 days after inoculation as the formation of aerial hyphae and conidiophores. In the vast majority of sections, formation of fungal colonies originated from single infected cells expressing GFP (Figure 3). Because plasmid pUGLUM contains both GFP and *Mlo*, our data suggest that transient expression of wild-type *Mlo* complements *mlo* resistance at the single cell level. This is consistent with results obtained after cobombardment of 2 plasmids containing GFP or *Mlo* (Shirasu et al. 1999).

A particle inflow gun with a single outlet and a much smaller target area was used in a previous study (Shirasu et al. 1999). Under those conditions, leaf sections of 3 cm length could be bombarded in a single shot. About 20 leaf segments and several bombardments were necessary to obtain 100–300 GFP expressing cells (Shirasu et al. 1999).

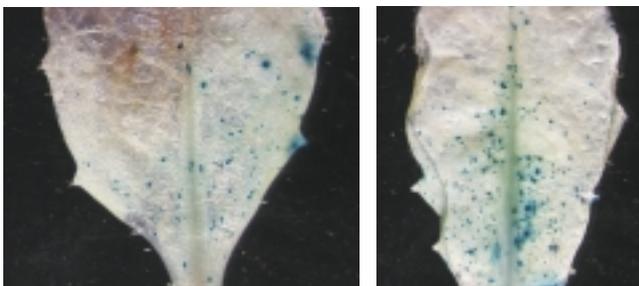


Fig. 4. GUS expression in *A. thaliana* leaves. Detached rosette leaves of approximately 5-week-old *A. thaliana* ecotype Ms-0 were bombarded with plasmid pPam-GUS using the same conditions described for transformation of barley leaves. The activity of the GUS reporter gene is visualized by the indigo stain as described in Methods.

Modification of the experimental setup described here generated a comparable number of cells expressing the GFP reporter gene after a single bombardment. Application of the Hepta adaptor resulted in consistently high numbers of transfected cells in several independent experiments. Generally, we found multiple clusters of transfected epidermal cells in bombarded leaf segments as shown in Figure 2. Thus, the Hepta adaptor is useful for experiments in which several independent constructs need to be tested or in which high numbers of transformed cells are desired.

Next we tested the efficiency of the Hepta system transformation procedure in the dicot plant *A. thaliana* by delivering a plasmid expressing GUS to detached leaf tissue. In this case we used plasmid pPam-GUS driving expression of the reporter gene from the doubled 35S promoter. Experimental conditions were identical as described above for the transformation of barley leaves. We obtained a high density of GUS-stained cells that were clustered in multiple leaf areas (Figure 4) 3–4 days following bombardment.

Our findings demonstrate the usefulness of the Hepta particle delivery system in obtaining high transformation efficiencies in leaves of monocot and dicot plant species.

References

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