

Special Research Report # 313: Use of CRISPR to Develop Powdery Mildew Resistance in Gerbera

Category: Plant Breeding and Genetic Engineering

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BACKGROUND

Powdery mildew (PM) is a common fungal disease of gerbera that affects its marketability. While some gerbera cultivars exhibit partial resistance to the causal agent *Podosphaera xanthii*, the disease is usually controlled through the use of fungicide¹. Progress has been made in breeding gerbera for PM-resistance¹, although this approach is lengthy and not applicable to current cultivars. The introduction of disease-resistance genes through genetic engineering has been examined², but the regulatory costs of commercializing transgenic gerbera can make this strategy less attractive. Gene editing technology³ provides the means to obtain PM-resistance efficiently and potentially without regulatory costs.

Our first goal was to develop gene editing technology for gerbera using the CRISPR/Cas system. This system uses sequence guide RNAs (sgRNAs) to direct the Cas nuclease to a specific gene target, where it cuts the DNA. Misrepair of the DNA break causes mutations that inactivate the gene. To investigate gene editing in gerbera, *in vitro* regeneration and gene transfer protocols were needed. Once these procedures were established, we could test CRISPR/Cas in gerbera by targeting a model gene, *phytoene desaturase* (*PDS*). The inactivation of *PDS* results in an albino phenotype that is readily observable.

Our second goal was to use CRISPR/Cas to inactivate the *MLO* genes that makes gerbera susceptible to PM. *MLO* genes are present in plants as a small gene family and specific members facilitate PM infection. PM-resistance has been developed through *MLO* inactivation in diverse crops such as tomato, wheat, and grape. To date, the application of this strategy for developing PM-resistance in ornamental species is limited to recent work with petunia⁴.

MATERIALS & METHODS

Gerbera tissue culture: Sterile shoot cultures of gerbera cultivars were established following the disinfestation of capitula of greenhouse-grown plants ('Flori Line Maxi Yellow', Garvinea 'Sweet Love') or seeds (Revolution 'Bicolor Red Lemon', Majorette 'Pink Halo'). Shoots were induced from explants exposed to Murishige and Skoog (MS) medium containing 0.1 mg/L indole butyric acid (IBA), 1.0 mg/L benzylaminopurine (BAP), and 0.5 mg/L glutamine. Leaves from shoot cultures were cultured on MS with 0.1 mg/L IBA and 1.0 mg/L meta-topolin for *de novo* shoot development. Rooting was induced on shoots dipped in a 1 mg/ml IBA solution for 10

seconds and cultured on MS medium with 0.1 mg/L IBA, 1.0 mg/L BAP, and 250 mg/L activated charcoal.

Gerbera transformation: Leaf explants from shoot cultures were incubated with LBA4404 (OD₆₀₀ 0.6) on MS medium with 0.5 mg/L glutamine, 0.1 mg/L IBA, 2.0 mg/L BAP, and 250 µM acetosyringone. After 72 hours, the explants were washed and plated on MS medium with MS with 0.1mg/L IBA, 2.0 mg/L meta-topolin, 0.5 mg/L glutamine, 400 mg/L timentin, and 7.5 mg/L hygromycin. Antibiotic selection was conducted in the dark at 23°C for 3 months. Following callus induction, the hygromycin was reduced to 3.75 mg/L, and buds that developed were isolated for shoot development.

Gene identification and CRISPR construct development: A partial gerbera *PDS* sequence (JQ894780.1) was aligned with Asteraceae *PDS* sequences to inform PCR primer design. PCR products from cDNA and genomic DNA of *G. jamesonii* 'Flori Line Maxi Yellow' were sequenced. Six Cas12 guide sequences were identified, synthesized, and incorporated into CRISPR/Cas12 constructs by Golden Gate cloning. Gerbera *MLO* genes were identified from NCBI SRA reads (PRJNA647707) of the leaf transcriptome. Full-length transcripts were assembled and BLAST analysis with sunflower *MLO* sequences detected corresponding gerbera orthologs. Four Cas9 targets were identified in exons 3 and 4 of *G. hybrida* 'Flori Line Maxi Yellow' *MLO* genes. Sequence guides were synthesized and incorporated into two CRISPR/Cas9 constructs using Golden Gate cloning.

PDS mutation analysis: Genomic DNA was isolated from leaves of plants exhibiting a *PDS* deficient phenotype. PCR was conducted to amplify the CRISPR/Cas target regions and the resulting amplicons were sequenced. Sequence data from *PDS* mutants and wild-type plants were compared to characterize the induced mutations.

RESULTS

Gerbera tissue culture and transformation: Shoot cultures of four gerbera cultivars were established after disinfestation procedures were determined (Fig. 1). Multifactorial experiments were conducted to optimize organogenic regeneration. Factors that were examined included explant type, basal media, plant growth regulators, agar concentration, and light quality and quantity. The optimized protocol is shown in the Materials and Methods section.



Figure 1. Sterile shoot cultures established for (A) 'Flori Line Maxi Yellow', (B) Revolution 'Bicolor Red Lemon', (C) Garvinea "Sweet Love", and (D) Majorette 'Pink Halo'. (E) Plant regenerated from leaf explant of Majorette 'Pink Halo'

Leaf explants were used for gene transfer experiments, with the visual markers GFP and GUS used to confirm transformation (Fig. 2). Variables examined included the gerbera cultivar, *Agrobacterium tumefaciens* strain and co-culture duration, timentin level, and antibiotic type and level for plant selection. The optimized transformation protocol for the gerbera cultivars that we used is described in the Materials and Methods section.

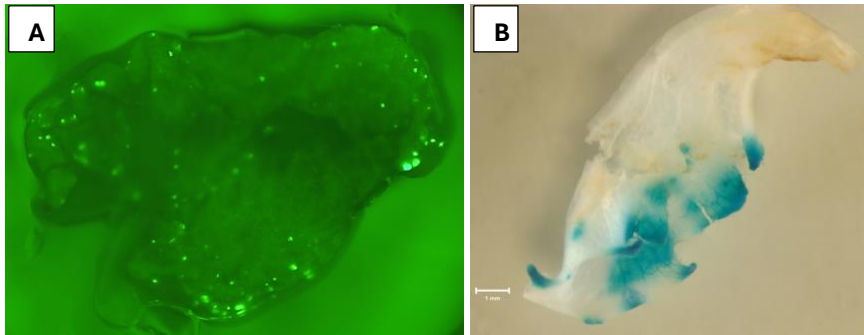


Figure 2. (A) Expression of green fluorescent protein in leaf of 'Flori Line Maxi Yellow' after transformation with CaMV:eGFP and NOS:HPH. (B) Expression of GUS (blue color) in leaf of Revolution 'Bicolor Red Lemon' transformed with CaMV35S:GUS and NOS:NPTII.

PDS identification and CRISPR/Cas12 construct development

Comparison of a gerbera *PDS* sequence (JQ894780.1) with other Asteraceae *PDS* sequences enabled PCR primers to be designed for the isolation of the gene from *G. jamesonii* 'Flori Line Maxi Yellow'. Cas12 targets were identified from the *PDS* gene sequence and guide sequences were synthesized. Two guide sequences were subcloned into each CRISPR/Cas12 construct (Fig. 3). The CRISPR/Cas12 constructs were introduced into *A. tumefaciens* and transferred into *G. jamesonii* 'Flori Line Maxi Yellow'.

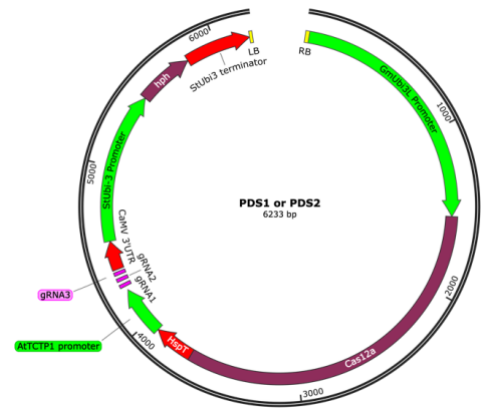
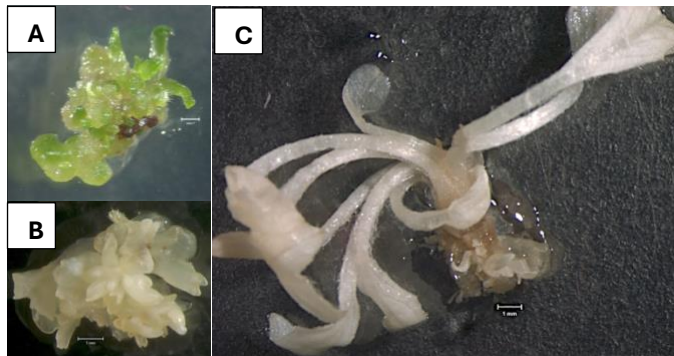


Figure 3. CRISPR/Cas12

construct for GpPDS

PDS mutation analysis



Hygromycin-resistant cultures were recovered that exhibited the albino phenotype typical of *PDS* mutations (Fig. 4). In some cases, the subculture of chimeric lines was required to obtain purely white cultures. Analysis of DNA from albino plants was used to confirm *PDS* mutations.

Figure 4. Phenotype of PDS mutations.

Shoot cultures of a control (A) and PDS-mutant (B). Regenerated plant with PDS mutation.

Gene editing could enable the development of non-transgenic cultivars with specific mutations for traits of interest, like powdery mildew resistance. There are still technical issues that need to be addressed. For most gerbera cultivars, the regeneration of transgenic plants is inefficient at best. The introduction of morphogenic regulators (e.g. *WOX*, *GRF-GIF*) during gene transfer has promoted the *in vitro* regeneration of recalcitrant crop species⁵. Whether these work in gerbera as well could be investigated. To obtain transgene-free, gene edited plants, an approach that is showing promise is the delivery of CRISPR/Cas components as RNA or ribonucleoprotein. The nanoparticle delivery of these macromolecules is currently being examined in model and crop plants⁶. Finally, the absence of genome sequence data for gerbera complicated the identification of the *PDS* and *MLO* genes. The recent publication of a sequenced gerbera genome⁷ will facilitate gene editing in this species in the future.

IMPACT OF RESEARCH TO THE INDUSTRY

To our knowledge, this report is the first example of gene editing in gerbera. Genome editing through CRISPR technology has tremendous potential for improving ornamental traits of plants³.

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