DEVELOPMENT OF A PROTOPLAST SYSTEM FOR NON-TRANSGENIC, TARGETED GENOME EDITING IN *S. TUBEROSUM*

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INTRODUCTION

Genome editing technologies based on the CRISPR-Cas9 programmable nuclease are emerging as invaluable tools for research and plant breeding. Crops modified using this technology have recently been deemed non-regulated by U.S. regulatory agencies for process and product. Transfecting protoplasts with preassembled Cas9 ribonucleoprotein complexes and regenerating plants from these edited protoplasts offers a potentially non-transgenic, DNA-free genome editing strategy for clonally propagated crops.

We will establish a protoplast regeneration platform in *Solanum tuberosum.* Here we report on protoplast isolation and plant regeneration from two potato clones: *S. tuberosum* Group Tuberosum *cv.* Desiree and *S. tuberosum* Group Phureja *cv.* DM1-3, as well as our gene target design strategy and progress. We anticipate that edited lines produced from our pipeline will provide useful material for potato functional genomics.

OBJECTIVES

- To identify and implement an efficient protoplast regeneration system and compare response in two potato backgrounds; Desiree and DM1-3.
- Design a strategy for identifying gene candidates to implement DNA-free genome editing, via preassembled Cas9 ribonucleoproteins (RNPs).

PROTOPLAST REGENERATION METHODS

Donor material	Species	Group	Chromosome number	Ploidy
Desiree	*S. tuberosum*	Tuberosum	2n = 4x = 48	Tetraploid
DM1-3	*S. tuberosum*	Phureja	2n = 2x = 24	Doubled monoploid

Table 1: Genotype background information

- Potato plants propagated *in vitro* on Murashige and Skoog medium supplemented with Gamborg vitamins and 2% sucrose. Plants grown at 24°C, 16hr day length.
- Roughly 1g of healthy leaf tissue was subject to enzyme digestion and protoplast isolation by flotation.
- Protoplast density measured via hemocytometer and re-suspended at 0.5 x 10^6 protoplasts/mL.
- Protoplasts embedded in alginate gel and incubated in the dark until callus production.
- Callus transferred to light conditions for shoot regeneration and rooting.

PROTOPLAST ISOLATION EFFICIENCY

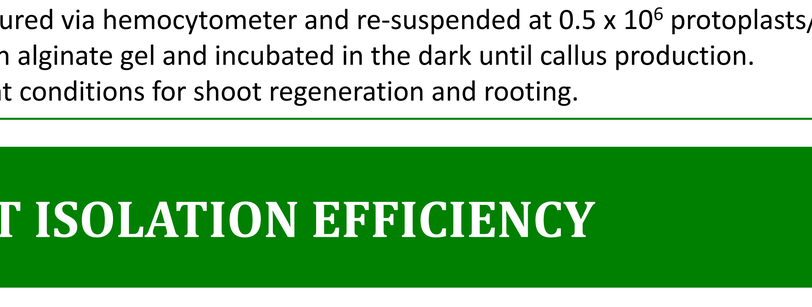
Donor material	Avg. number protoplasts per isolation
Desiree	7,756,666
DM1-3	8,300,000

Table 2: Donor material protoplast isolation efficiency

- Successful protoplast isolation in both backgrounds provides platform for RNP editing and ability to rapidly test new guide RNAs, possible without plant regeneration.

MULTICELLULAR DEVELOPMENT

Figure 1: Multicellular development 19 days post isolation a) DM1-3 development b) Desiree development



- Both Desiree and DM1-3 protoplasts develop to multicellular stages, however Desiree displays a faster, more robust growth than DM1-3 at equal time points post isolation (Figure 1).
- DM1-3 development arrests in early multicellular stages (Figure 1a), while Desiree development continues (Table 3, Figure 2).

References Heyer, Arnd G., et al. "Function of phytochrome A in potato plants as revealed through the study of transgenic plants." Plant Physiology 109 (1995): 53-61.

CALLUS PRODUCTION AND PLANT REGENERATION

- Unlike DM1-3, Desiree protoplasts continue to develop through multicellular stages to produce callus, shoots and fully regenerated, rooted plants (Table 3, Figure 2).

Donor material	Avg. # Callus produced/ isolation	Elongated shoots produced/ isolation	Rooted shoots/ isolation
Desiree	389	*83	*50
DM1-3	0	0	0

* Preliminary data. Data collection still in progress.

Table 3: callus production and shoot regeneration data

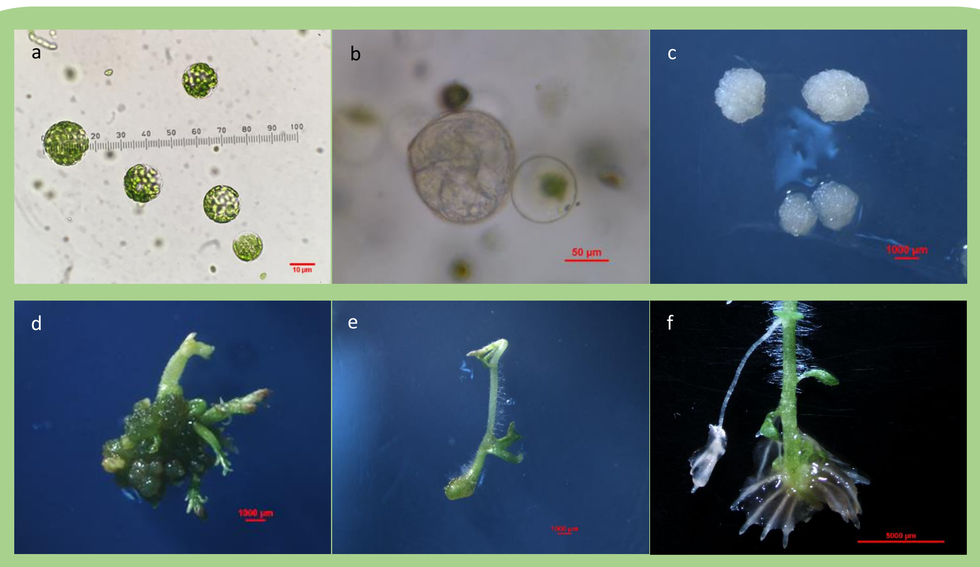


Figure 2: Desiree protoplast development (a) Protoplast isolation, day 0 (b) Multicellular development 12 days post isolation (c) Callus growth stage at transfer to shoot inducing medium (d) Shoot production from callus after 14 days on bud elongation medium (e) Elongated shoot after 14 days on shoot elongation medium (f) Rooted, regenerated shoot 4 months post isolation

- Active callus formation in Desiree occurs 6-9 weeks post isolation, but can continue as late as 16 weeks post isolation.
- Shoot primordia develop from callus 3.5-8.5 weeks post transfer to shoot inducing medium.
- Protoplasts develop into rooted, regenerated plants in as little as 4 months.
- Efficient protoplast creation and plant regeneration in Desiree makes it an ideal candidate to further explore genetic modification via introduction of Cas9 RNPs.

GENE TARGET DESIGN STRATEGY

- Criteria for choosing a gene target:
  - Visual phenotype of gene knockout early in development to screen for regenerated plants with successfully edited genomes.
  - Preferred single copy gene (though not essential, single copy in tetraploid genome provides higher probability of editing all alleles).
  - Minimal adverse effects on reproductive traits.

Candidate gene identification

- Candidate gene identified: Phytochrome A (Phy A)
  - Potato Phy A antisense lines are etiolated compared to WT controls when grown under low red/far-red light conditions (Heyer et al., 1995). We expect a similar phenotype in edited lines.
  - Single copy gene in potato genome.
- Potato orthologue identified using Arabidopsis sequence as a reference and BLAST search against potato transcripts.
- Transcripts aligned to DM1-3 reference genome exons on chromosome 10.

GUIDE RNA DESIGN AND FUTURE WORK

- 2 PAM sites identified in potato Phy A sequence.
- Verified no SNPs in our guide target region between the reference genome and Desiree.
  - Primers designed to PCR amplify each target region.
  - Cloned PHY A fragment from Desiree and sequenced 23 clones to verify no SNPs exist between Desiree genome and DM1-3 reference genome.

Future work

- Assemble and transform Cas9 and custom guide RNPs into Desiree protoplasts.
- Molecularly and phenotypically characterize regenerated, edited potato plantlets.