Cycling of Gametes *in Vitro*: Proof of Concept (Development of cell cycling protocol preceding experimentation towards *in vitro* gametogenesis induction)

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ABSTRACT

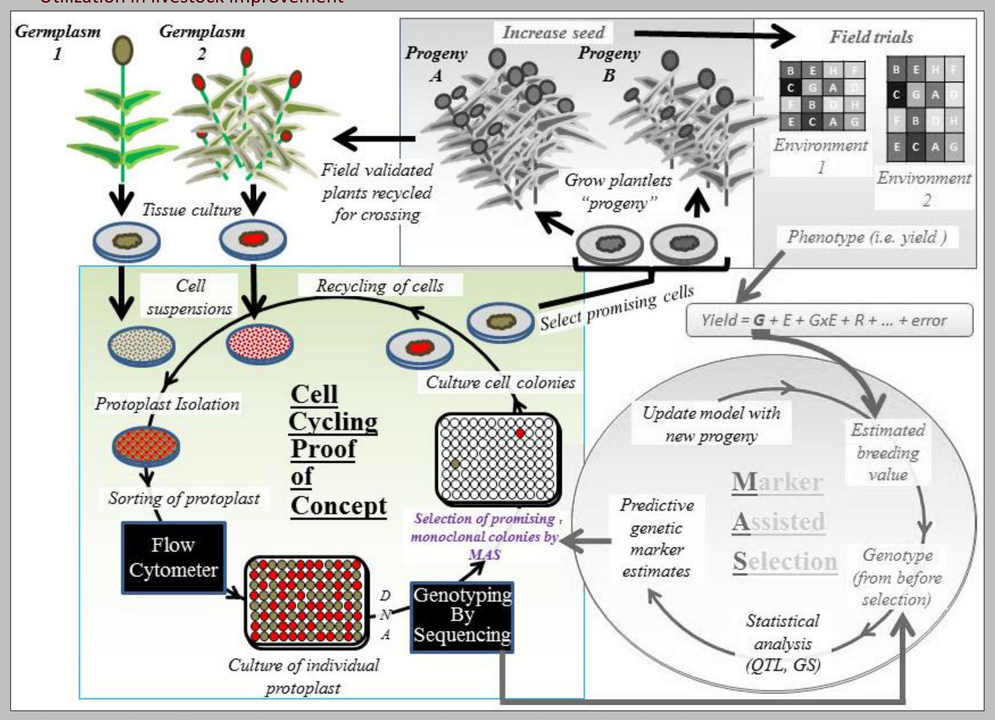
An ever growing human population accompanied with continuous environmental shifts demands the development of improved cultivars vital in providing the necessary food and fiber. Currently, cultivar progression requires years of development prior to performance trails; reducing such time requirements, for instance through the use of off season nurseries and grafting, has been important to the production of improved cultivars. Cycling of plant cells *in vitro* will lead the way in production of improved cultivars through substantial reductions in generation times required for new cultivar development. Carrot (*Daucus carota)* and tobacco (*Nicotiana tabacum)* species were selected for their extensive tissue culture history and high tolerance toward *in vitro* manipulation. Isolation of parental protoplasts from cell suspensions will be followed by sorting of individual cells through fluorescence activated cell sorting (FACs); expectantly allowing for production of totipotent single cell derived colonies. Supposed colonies can be designated, with the assistance of marker assisted selection (MAS), for further *in vitro* cycling or regeneration and advancement to performance trials. Demonstrating that single cells can be isolated, divide to form cell colonies, genotyped and regenerated is central to proclaiming the proposed hypothesis of Cycling of Gametes *in Vitro (CoGiV)* as a conceivable feat. This will warrant further testing towards development of procedures intended for *in vitro* gametogenesis induction.

RATIONAL

- Development of new cultivars currently requires 5-40 years (Murray et al., 2013), reduction in generation times is key to rapid development of crops with performance improvements tailored to current needs.
- Bypassing biological limits (sexual maturity period, photoperiod sensitivity, ect.) of cultivar improvement will drastically increase frequency of performance advancement
- Time and resources necessary to complete field level plant breeding limits the amount of material breeders can realistically manage

BENEFITS OF GAMETE CYCLING

- Artificial induction of gametogenesis sidesteps biological reproductive requirements
- Cultivar development can be conducted entirely in a lab setting, with field experiments beginning at the performance trial phase
- Facilitates trait introgression of exotic genes into superior cultivars
- Provides prospect of producing novel genetic variability through fusion of currently incompatible gametes
- Utilization in livestock improvement



Schematic of CoGiV proof of concept (Murray et al., 2013) indicated by colored region

RESEARCH APPROACH

Cell Suspensions

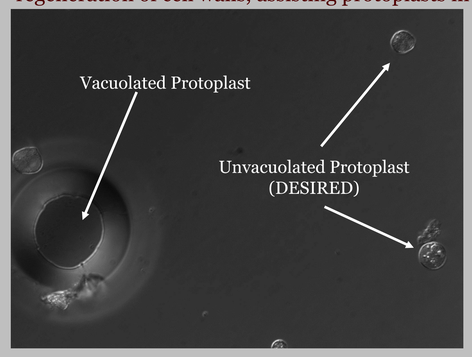
- Carrot calli: Etiolated seedling hypocotyl segments placed on MS media with 0.2 ppm 2,4-D
- Tobacco calli: Square leaf segments placed upon MS media with 1.0 ppm 2,4-D and 0.1 ppm kinetin
- Friable callus placed into liquid media and agitated (29° C , 130 rpm) within an Innova 42 orbital shaker

Tobacco callus

Carrot callus

Protoplast Isolation

- Carrot:
  1.5 % (m/v) Macroenzyme R-10, 1.0% (m/v) Cellulase R-10 (Grzebelus et al., 2012 and Lee et al., 1989)
- Tobacco:
  0.3 % (m/v) Macroenzyme R-10, 1.0% (m/v) Cellulase (Kirchhoff et al., 2012)
- Isolated protoplast stored at 26° C dark conditions within 35mm petri dish for 72 hours to allow for partial regeneration of cell walls, assisting protoplasts in surviving cell sorting

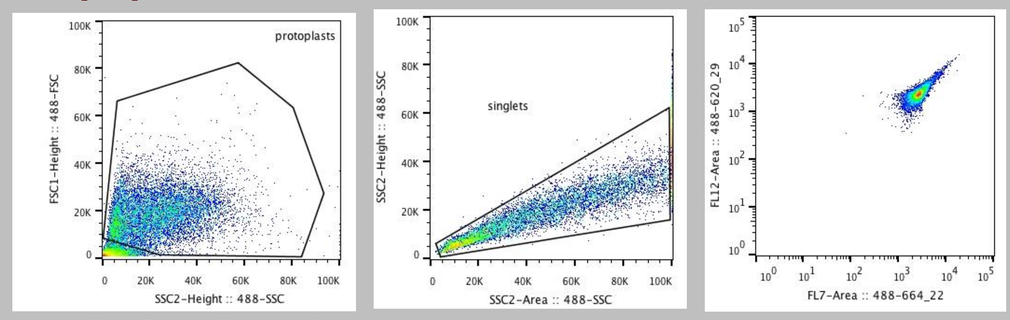


Enzymatic isolated carrot protoplast

Beckman Coulter MoFlo Astrios Cell Sorter

Cell Sorting

- Beckman Coulter MoFlo Astrios Cell Sorter
- Detected red auto fluorescence emitted by endogenous chlorophyll
- Gates places on:
  - Side Scatter(SSC) vs Forward Scatter (FSC)
  - Side Scatter(SSC) vs Side Scatter(SSC)
- Isolate protoplasts selected from Fluorescence (488-620) vs Fluorescence (288-664)



Scatter plot and gate calibrations of B7262B carrot protoplast for FACs

FUTURE WORK

- Confirmation of singular protoplast within individual wells of 96 well microplate using Olympus IX71 inverted microscope
- Induce cell division and monoclonal cell colony growth using conditioned media and nurse cell cultures
- Genotype monoclonal cell colonies
- Regenerate monoclonal colonies into differentiating plant tissues

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