Development of Salinity Tolerant Petunia Through CRISPR/Cas9 GeneEditing

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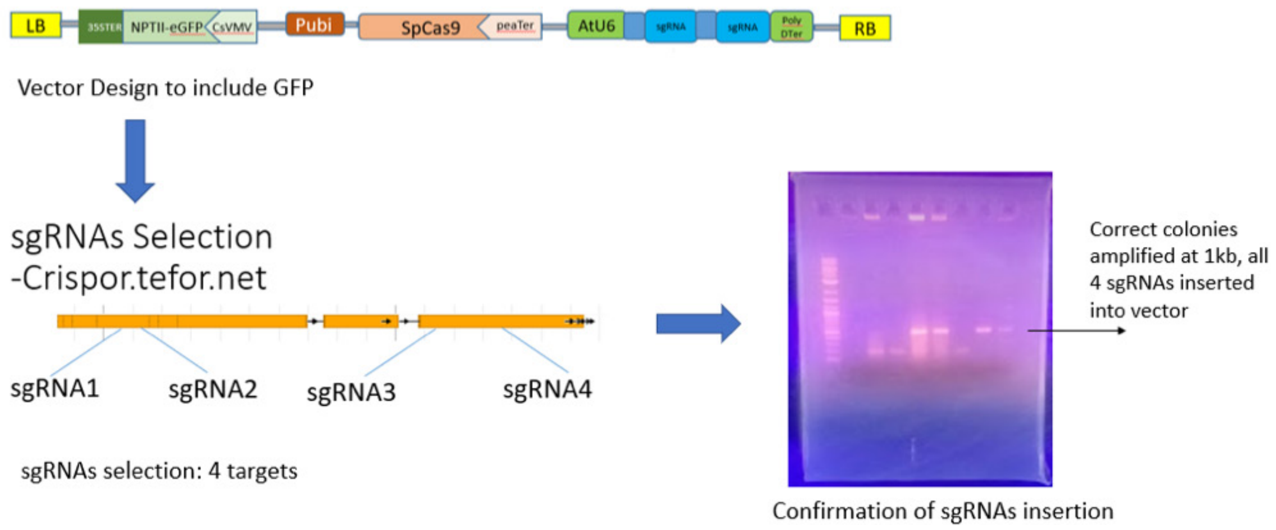
ABSTRACT

Florida's beaches are among the most popular vacation destinations for visitors. Likewise, more than three quarters of Florida's population lives in coastal counties. Due to extreme conditions such as salt spray, wind, poor soil, storms and salt-water intrusion; landscaping is difficult around coastal areas. Additionally, the use of recycle water for irrigation is becoming more common for nursery plant production as well as many neighborhoods. The high concentration of salt in the soil cause significant adverse effects in plant growth thus limiting plant production for landscaping. Petunia (*Petunia* × *hybrid*) is one of the most popular bedding plants nationwide owing to its colorful flowers. Current petunia cultivars cannot be utilized for bedding plants in Florida coastal regions; and the lack of salinity-tolerant germplasms greatly hinders the development of salinity-tolerant petunia through conventional hybridization method. Therefore, the purpose of the project is to create mutation in the *GIGANTEA* (*GI*) gene which has been reported to increase salt tolerance in other plant species. In this study, CRISPR/Cas9 was successfully used to knock out *GI* gene. With the unique design of our CRISPR/Cas9 vector to include NPT-GFP, newly regenerated T0 plants as well as T1 seedlings were easily screened by visualizing GFP to select high Cas9 endonuclease activity and increased gene editing efficiency. Sanger sequencing results showed two out of four lines sequenced are carrying heterozygous and homozygous mutations at the sgRNA loci of *GI* gene. Currently, the homozygous *pegi* mutants are subjected to further investigations on their sensitivity to NaCl treatments and alteration of their flowering in response to photoperiods. We expect that a transgene-free salt tolerant petunia mutant can be released once we have fully characterizing their phenotypic parameters under saline conditions, and we believe that this novel germplasm may generate a substantial impact on petunia breeding.

OBJECTIVES AND METHODS

Objective 1: To design sgRNAs targeting GIGANTEA (GI) gene and insert them into CRISPR/Cas9 vector for knockout mutation.

Method: By using NCBI BLAST and Crispor.tefor.net, four sgRNAs were selected to target GI gene and they were inserted into CRISPR/Cas9 Vector via Gateway method. Q5 PCR was used to confirm the correct size at 1kb, where all four sgRNAs were successfully inserted into the vector



Vector Design to include GFP

sgRNAs Selection
-Crispor.tefor.net

sgRNA1 sgRNA2 sgRNA3 sgRNA4

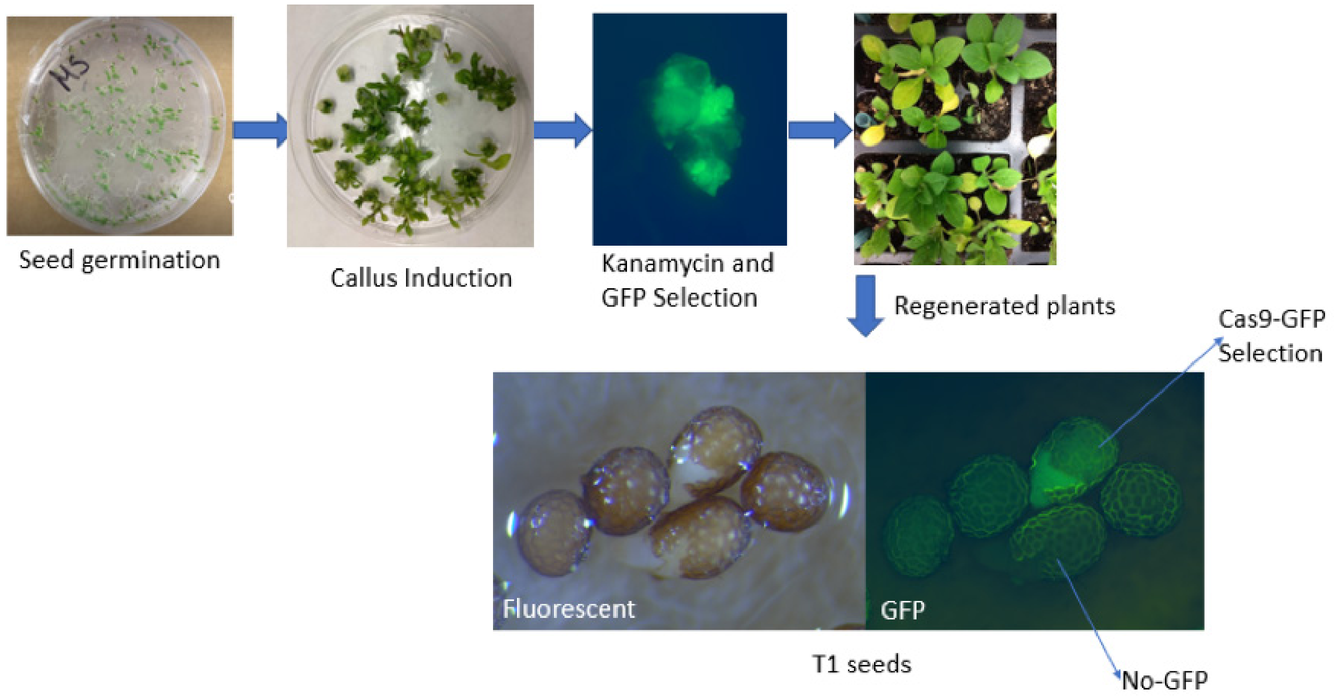
sgRNAs selection: 4 targets

Correct colonies amplified at 1kb, all 4 sgRNAs inserted into vector

Confirmation of sgRNAs insertion

Objective 2: To regenerate Petunia var. Mitchell gene edited plants through *Agrobacterium-mediated* transformation.

Method: Petunia seeds were surface sterilized and germinated on MS media. Cotyledons were excised and dipped in EHA105 harboring the CRISPR/Cas9 GI knockout vector for 5 mins and were co-cultivated for 3 days. Afterward, transformed explants were placed on callus/shoot induction media containing Kanamycin and Timetin antibiotics for 3 weeks and longer to regenerate new shoots. Two-inches or longer shoots were placed on Rooting media with the same antibiotics to select transgenic plants that rooted. Rooted plants were grown in the greenhouse and T1 seeds were collected. Germinated seeds were screen for GFP fluorescent signals, seeds showing GFP signals were considered as CRISPR/CAS9 carrying seeds for further analysis.



Seed germination

Callus Induction

Kanamycin and GFP Selection

Regenerated plants

Cas9-GFP Selection

Fluorescent

GFP

T1 seeds

No-GFP

Objective 3: To determine the LD50 of Petunia var. Mitchell WT

Method: WT petunia seeds were surface sterilized and germinated on MS media, five days after germination, seedlings were placed on new MS media with 0, 150, 250, or 350mM of NaCl. Pictures were taken after 2 weeks of treatment.

Objective 4: To determine gene edited plants in T1 seedlings.

Method: T1 seeds were germinated and DNA were extracted from cotyledon using the SDS method (Edward's Buffer). Q5 enzyme was used to amplify specific region covering the targeted sites and the PCR products were sent for Sanger sequencing through GENEWIZ. Sequencing data was analyzed using ice.synthego.com.

RESULTS

1. Since the petunia is a hybrid of *Petunia axillaris* and *Petunia inflata*, we have obtained the GI sequences from both genomes. Vector construction: we have successfully made a CRISPR/Cas9 vector containing four sgRNAs for targeting different copies of PeGIs in the petunia Mitchell.
2. Agrobacterium transformation: Transgenic plants were successfully regenerated and monitored using GFP and Kanamycin selection.
3. NaCl LD50 of Petunia WT: It was determined visually that at 250mM NaCl, petunia seedlings growth was stunted, and their leaves were noticeably narrowed due to stress. At 350mM NaCl, petunia seedlings were not able to grow, and their cotyledon was completely bleached **(Figure 1)**. Therefore, we concluded that 250mM NaCl would be the concentration to test gene-edited plants vs. WT.
4. Gene edited T1 plants: 2 out of 4 lines are gene edited based on Sanger DNA Sequencing which is 50% of gene editing efficiency. Homozygous lines will be grown in the greenhouse for seed collection and testing. Sequencing results from non gene-edited **(Figure 2)** vs. heterozygous gene edited **(Figure 3)** vs. homozygous gene edited **(Figure 4)** plants.

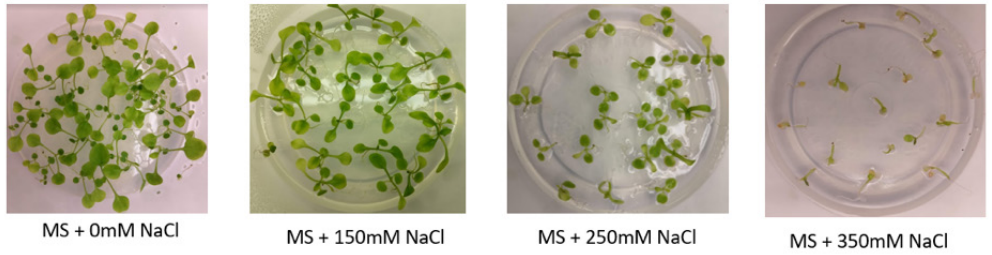
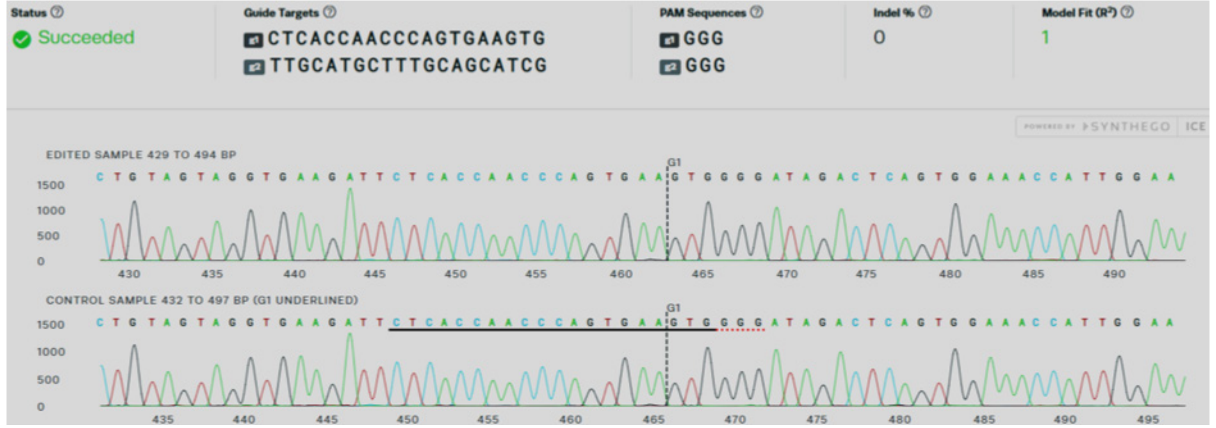

MS + 0mM NaCl
MS + 150mM NaCl
MS + 250mM NaCl
MS + 350mM NaCl

Figure 1. Effect of different concentrations on growth of petunia WT seedlings.



Status	Guide Targets	PAM Sequences	Indel %	Model Fit (R²)
Succeeded	1 CTCACCAACCCAGTGAAGTG 2 TTGCATGCTTTGCAGCATCG	GGG GGG	0	1

Figure 2. No sequencing differences between the control (bottom) and non-edited plants (top) in the chromatogram. The PAM and sgRNA were underlined in black and red dots.

Status	Guide Targets	PAM Sequences	Indel %	Model Fit (R²)	Knockout-Score
Succeeded	1 CTCACCAACCCAGTGAAGTG 2 TTGCATGCTTTGCAGCATCG	GGG GGG	50	0.96	50

Figure 3. A 4 bp deletion occurred to only one strand of DNA in gene edited plant (top), resulting in double chromatogram peaks around vertical black dashed line.

Status	Guide Targets	PAM Sequences	Indel %	Model Fit (R²)	Knockout-Score
Succeeded	1 CTCACCAACCCAGTGAAGTG 2 TTGCATGCTTTGCAGCATCG	GGG GGG	99	0.99	99

Figure 4. A base pair deletion occurred to both strand in the gene edited plant (top), resulting in clean shifted chromatogram peaks around vertical black dashed line, which suggested this gene edited mutation is homozygous.

CONCLUSIONS

We have accomplished our research goal of creating gene-edited petunia plants that have the knockout mutation in *GIGANTEA* (*GI*) gene. Due to the long processes of regeneration in tissue culture (3 months) and flowering/seed collection in the greenhouse (6 months), we were not able to fully examine the sensitivity of these gene edited seedlings to salt stresses at this moment; we will continue to endeavor our effort to characterize the phenotypic changes of these petunia mutants including salt tolerance and circadian rhythm alternation. Ultimately, we hope to select petunia mutants with improved salt tolerance to serve as a new germplasm for petunia breeding.

PUBLICATION

Nguyen LD and Heqiang Huo, Circadian Alteration and Salinity Tolerance Caused by Gene-Editing of *GIGANTEA* Gene in Petunia, ***in preparation***