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Targeted Mutagenesis of CCoAOMT1 in Petunia using CRISPR/Cas9 Technology.

Abstract:

The aim of this research is to introduce a mutation in the CCoAOMT1 gene from Petunia x hybrida by using the CRISPR/Cas9 mechanism and to determine whether the CRISPR/Cas9 technique (Figure 1) is functional in petunia.

The CCoAOMT1 gene suppresses the production of anthocyanin that provides purple flower colour in petunia [Shaipulah *et al.* Plant Physiol. 2016]. By mutating the CCoAOMT1 gen, the production of anthocyanins is no longer suppressed, allowing young plants to become purple.

For CRISPR/Cas9 an sgRNA and a Cas9 enzyme are required. Therefore, an sgRNA sequence encoding for the CCoAOMT1 gene has been cloned in a vector. By performing 3 PCR'S, digestion and ligation, the desired plasmid will be constructed. These plasmids are transformed in protoplasts together with the plasmids encoding for GFP and Cas9. The transformed protoplasts will be regenerated.

The CRISPR/Cas9 complex will introduce a ds-break in the CCoAOMT1 gene. This break will lead to a mutation ensuring the CCoAOMT1 gen is no longer functional.
We cloned three sgRNA sequences and verified their sequence. We increased the protoplasts transformation efficiency to 70%. The transformed protoplasts are regenerating into microcalli that will develop into plants in the coming months.

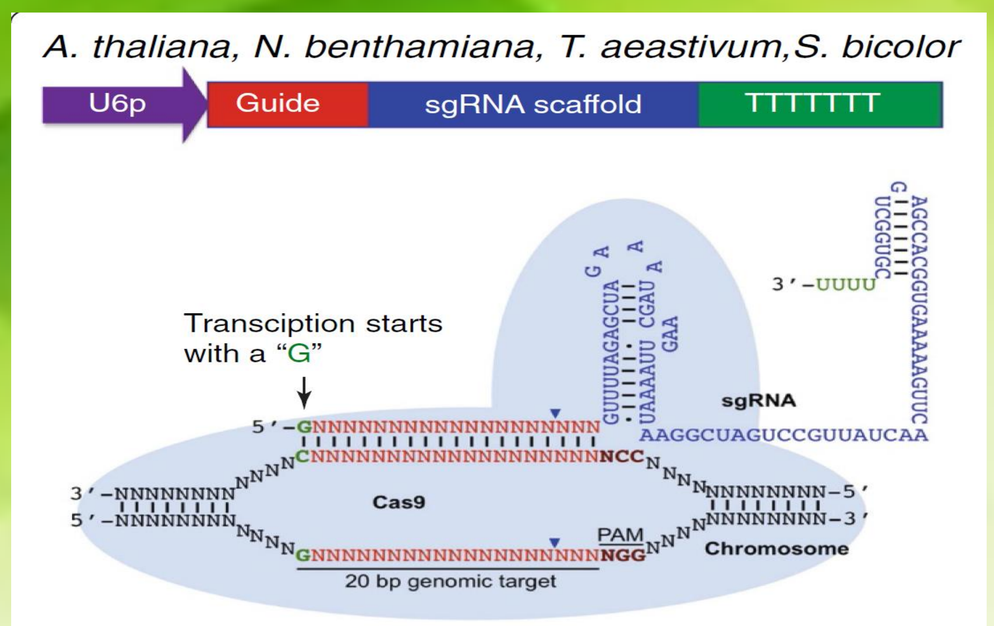


Figure 1: CRISPR/Cas9 technique
The blue shape represents the CAS9 protein, a nuclease, which ensures the Double-Strand Break (DSB). The Cas9 is controlled by the sgRNA, consisting of a fusion between a crRNA (containing the recognition sequence of the gene that had be mutated (here in red)) and the tracrRNA responsible for the interaction with Cas9 (here in blue). The Cas9 protein cuts 3nt for the PAM sequence in the genome. The cell will restore the DSB by NHEJ that is prone to mutations, thereby switching the gene off.
[Belhaj *et al*. Plant Methods, 2013]

Methods:

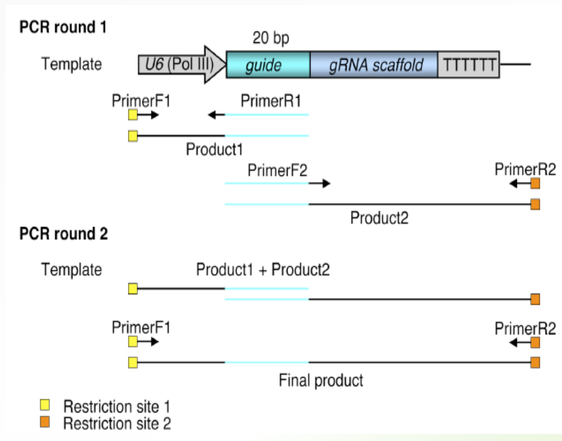
Design
- Gene sequence analysis
- sgRNA design for CCoAOMT1 mutagenesis

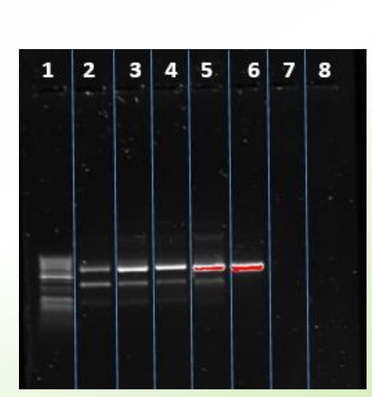
sgRNA vector cloning
- PCR fragments 1,2 and fusion PCR fragment 3 (sgRNA) are obtained
- Fragment 3 digestion en ligation into the plasmid backbone

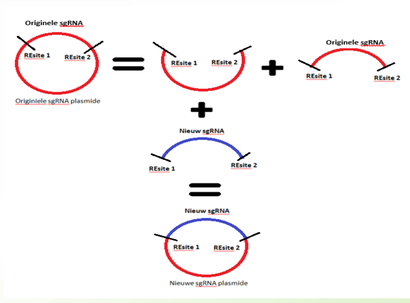
Transformation
- Plasmids transformation in *E.coli* bacteria and sequence verification
- Protoplasts isolation
- Protoplast transformation

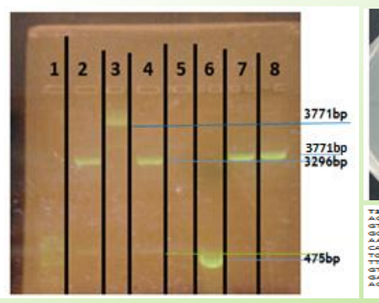
Regeneration
- Protoplast regeneration
- T7 Restriction analysis for mutant detection

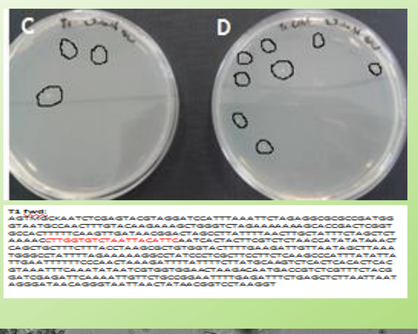
Results:

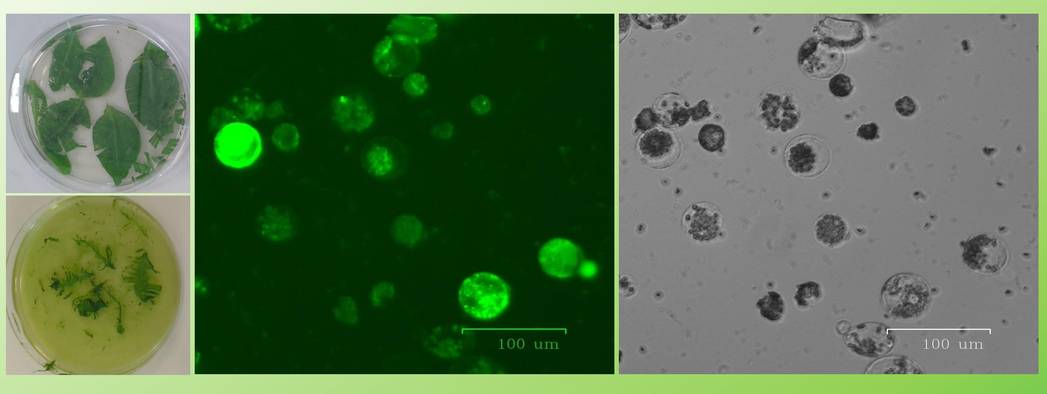


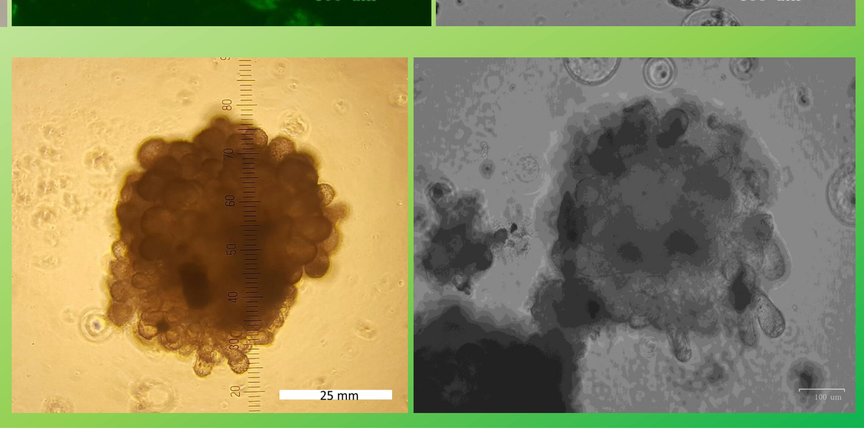












Conclusions:

- Functional primers are designed for three SG-targets.
- SG-targets are ligated into plasmid backbones.
- Plasmids are transformed and amplified in *E.coli* bacteria.
- Plasmids are sequence verified
- Protoplast transformation efficiency is increased to 40-70%.
- The first stages of protoplast regeneration to microcalli is successful.

Future recommendations:

- DNA isolation from protoplasts has to be optimized to perform restriction analysis.
- T7 restriction analysis to detect mutations in the CCoAOMT1 gene.
- Fine-tune protoplast regeneration to ensure microcalli will be grown to plants and to be able to observe the altered phenotype of the mutated plants.