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Using CRISPR/Cas9 to produce haploid inducers of carrot through targeted mutations of centromeric histone H3 (CENH3)

Frank Dunemann[1*], Katharina Unkel[1] and Thorben Sprink[2]

[1] Julius Kühn-Institute (JKI), Institute for Breeding Research on Horticultural Crops, Quedlinburg, Germany
[2] Julius Kühn-Institute (JKI), Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany
* Email: frank.dunemann@julius-kuehn.de

Julius Kühn-Institut
Federal Research Centre for Cultivated Plants

Introduction

The production of genetically homogeneous parental lines for F_1 hybrid breeding through several subsequent steps of inbreeding is long lasting and inefficient. Manipulating the centromere-specific histone H3 (CENH3) has been proposed as universal novel method for the production of haploid and doubled haploid crop plants [1].

Actually two different approaches are used for CENH3-based uniparental genome elimination *in vivo* by crosses with 'haploid inducers' (**Fig. 1**):

- **One-step**: genetic modifications of the endogenous (native) CENH3 gene by creating point mutations through TILLING or genome editing techniques including the CRISPR/Cas9 system
- **Two-step**: lethal CENH3 knockout mutants are rescued by transformation with modified CENH3 transgenes or native CENH3 genes isolated from related species.

Methods

Binary vector construction

- CRISPR/Cas9 construct (One-step and Two-step): gRNA for target site 'C4' within exon 5 of the *DcCENH3* coding sequence; pDE-Cas9 vector [2].
- *Panax ginseng* CENH3 construct (Two-step): *PgCENH3* foreseen to complement carrot CENH3 knockout mutants; binary expression vector p6i-d35S [3].

The plasmids were introduced each into *Agrobacterium rhizogenes* strain 15834.

Transformation of carrot with *A. rhizogenes* and plant regeneration

- Bacterial inoculums were either used as single inoculum (CRISPR target 'C4', One-Step) or mixed 1:1 for co-transformation experiments (Target 'C4' and *P. ginseng* CENH3, Two-Step).
- The regenerated hairy root lines were cultivated on appropriate selection media and used as starting material for plant regeneration via somatic embryogenesis (**Fig. 2**).

Molecular and cytogenetic analyses

- Test for integration of the CRISPR/Cas9 expression cassette (pDE-Cas9-C4 plasmid) with PCR primer (SS42/43, Bar, Cas9)
- Test for integration of *P. ginseng* CENH3 gene with specific primer
- High resolution melting (HRM) analysis with a Real-Time thermocycler
- Sequencing: PCR fragments (directly and cloned) Sanger sequenced
- Immunofluorescence analyses: based on polyclonal antibodies developed for specific peptides corresponding to the N-terminus of *DcCENH3* and *PgCENH3.*

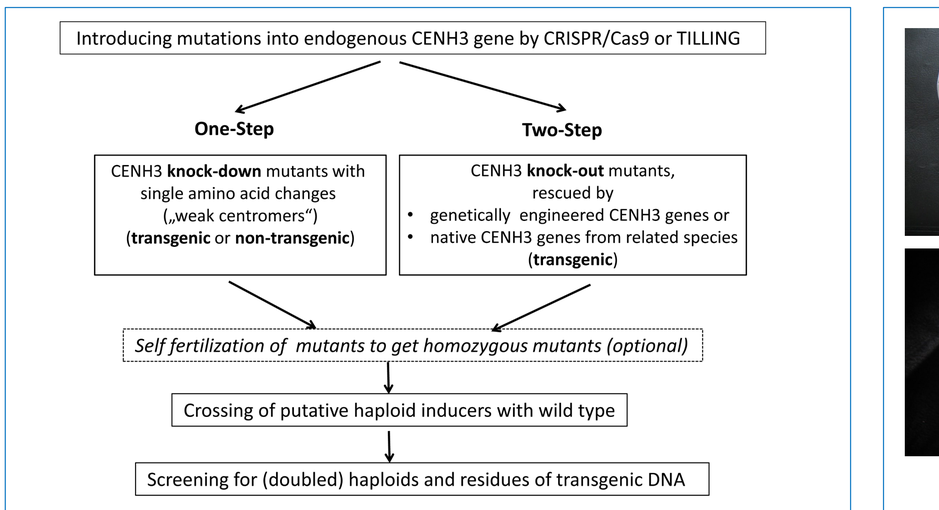

Fig. 1
Schematic presentation of the two different strategies to create 'haploid inducer' genotypes through manipulation of CENH3

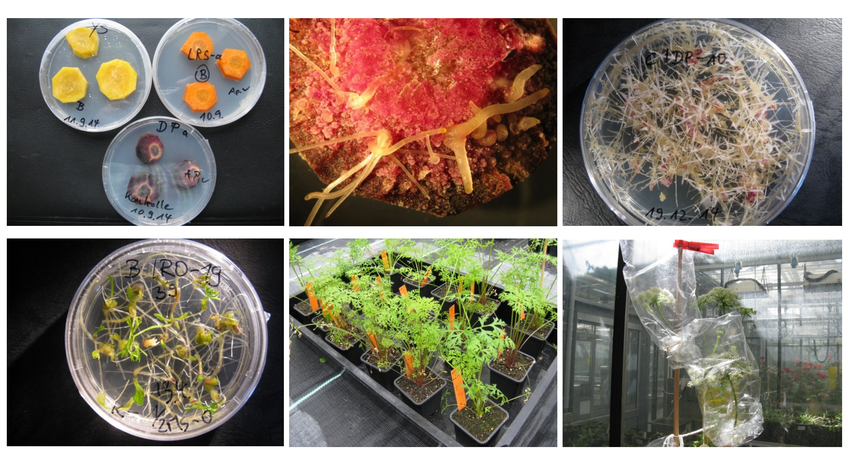

Fig. 2
Regeneration of hairy roots and transformed carrot plants via somatic embryogenesis

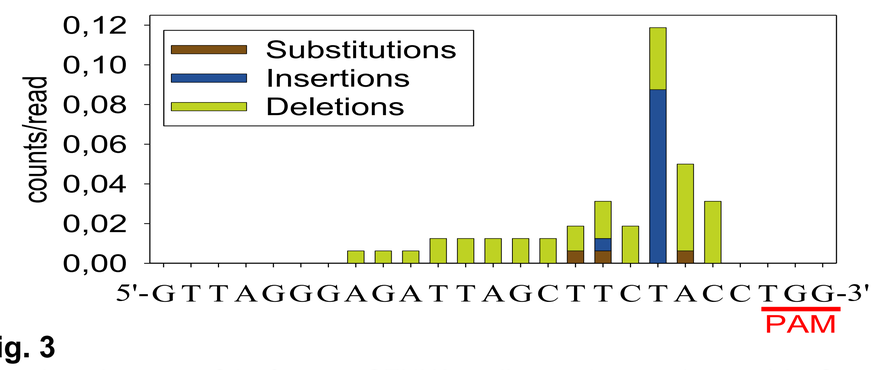

Fig. 3
Mutations in target C4 of carrot CENH3 coding region as detected in One-step experiments. Presented are the relative numbers of mutated samples as a fraction of total read numbers shown for the different sequence positions.

References

[1] Ravi M, Chan SWL (2010). Nature *464*, 615-618

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Results

Regeneration and analysis of hairy roots

Dependent of the experiment and the carrot cultivar used for transformation the number of hairy root lines varied considerably, but generally the regeneration capacity was high.

- PCR analyses confirmed the presence of the CRISPR/Cas9 construct in all hairy root lines that grew well at the end of the selection procedure.
- The *P. ginseng* CENH3 gene was present in almost all hairy roots resulting from the Two-Step experiments indicating that co-transformations have been induced quite efficiently in carrots.
- Transgenic lines carrying the CRISPR/Cas9 construct showed mutations such as insertions, deletions and substitutions within the C4 target (**Fig. 3**).
- Mutations within the *DcCENH3* gene appeared to be associated with a reduced CENH3 accumulation in the centromeres of some hairy root lines (**Fig. 4**).

Plant regeneration

The number of hairy root lines used for plant regeneration and the resulting number of transgenic carrot plants transferred successfully into a greenhouse is shown in **Table 1**.

Table 1. Number of hairy root lines leading to plant regeneration and number of regenerated T0 plants transferred into the greenhouse (*n.u.*, not used)

Method	Deep Purple	Yellowstone	Rotin	Blanche
One-step				
No. of hairy root lines	5	3	*n.u.*	1
No. of regenerated plants	20	39	*n.u.*	4
Two-step				
No. of hairy root lines	1	5	3	*n.u.*
No. of regenerated plants	0	35	8	*n.u.*

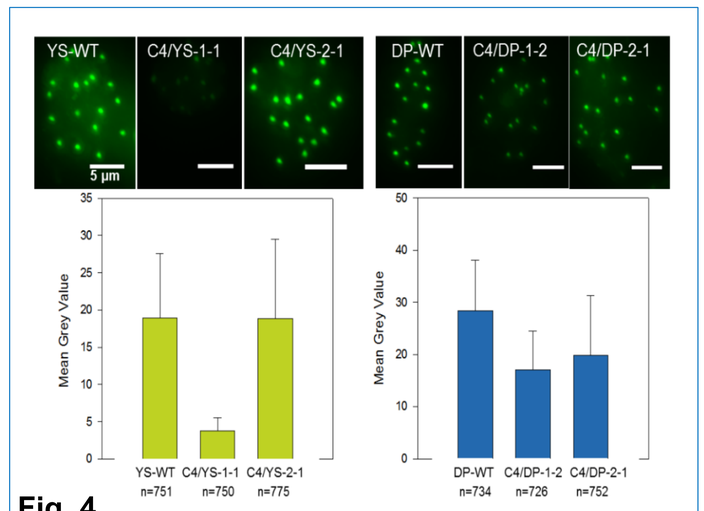

Fig. 4
CENH3 immunostaining of carrot nuclei of four hairy root lines generated in the One-step experiments. Centromere signal strength was determined by the software ImageJ-win32 (ImageJ.net).

Analysis of regenerated plants and crossing experiments

All regenerated T0 plants were analyzed by PCR analysis to verify their expected transgenic genotype. For instance, among the 35 plants regenerated within the Two-step program from hairy roots with YS origin (**Table 1**), 32 plants were shown to be transformed with both the CRISPR C4 construct and the *PgCENH3* gene.

T0 plants were crossed with several carrot cultivars and breeding lines in both directions to produce the next generations (T1 and T2). Within the Two-step approach about 1.000 plants have been produced from crosses with T0 or T1 parents, respectively, carrying both the CRISPR/Cas9 construct C4 and the ginseng *PgCENH3* gene.

Currently we are using flow cytometry, CENH3 immunostaining and root tip-based chromosome counting to identify haploid genotypes. To reveal putative doubled haploids, we are testing different types of molecular markers.

Conclusion

CRISPR/Cas9-based genome engineering was successfully used in cultivated carrots to induce mutations within the CENH3 gene. Cytogenetic analyses showed differences in the accumulation of CENH3 in mutant hairy root lines and regenerated plants. Actually putative 'haploid inducer' genotypes are tested, but similar as in other crop species, an efficient CENH3-based haploidization method has not yet been achieved in carrots.