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Application of CRISPR/Cas9 to develop a CENH3-based haploidy induction system in carrot (*Daucus carota*)

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Introduction

The production of homozygous parental lines for F_1 hybrid breeding is costly and time consuming - especially in an open-pollinated crop plant like carrot (*Daucus carota*). Manipulating/Editing the centromere-specific histone H3 (CENH3) has been proposed as universal tool to produce haploid and doubled haploid crop plants through uniparental genome elimination [1, 2].

At JKI Quedlinburg we are investigating two different approaches for CENH3-based haploidy induction - described as '1-Step' and '2-Step' strategies [2].

- **1-Step**: genetic modifications of the endogenous (native) *DcCENH3* gene by creating non-lethal mutations (with a compromised function)
- **2-Step**: lethal CENH3 knock-out mutants are complemented by a CENH3 gene from the related plant species (in our case from *Panax ginseng*)

On this poster, we focus on the **1-Step** approach including attempts to develop a non-transgenic (DNA-free) method based on carrot protoplasts.

Methods

Binary vector construction

- CRISPR/Cas9 construct: gRNAs for target sites C3 and C4 of the *DcCENH3* coding sequence were cloned into the pDE-Cas9 vector [3]. The plasmids were introduced into *Agrobacterium rhizogenes* strain 15834.

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

- Bacterial inoculums were used for transformation of carrot root discs.
- The regenerated hairy root lines were cultivated on selection media and used as starting material for plant regeneration via somatic embryogenesis (**Fig. 1**).

Protoplast transformation

- Cotyledons from carrot plantlets were placed on 2,4-D containing media for about 6 months for callus culture (**Fig. 2**).
- Isolation and regeneration of protoplasts after the protocol of Grzebelus [4].
- PEG-guided transformation of protoplasts with RNPs (SpCas9::GFP + gRNA-C4)

Molecular analyses

- Test for integration of the CRISPR/Cas9 expression cassette (pDE-Cas9::C3/C4) with PCR primer (SS42/43, Bar, Cas9)
- High resolution melting (HRM) analysis using a Bio-Rad CFX96 system
- Sequencing:
  (1) PCR fragments were cloned and Sanger sequenced.
  (2) Illumina Next Generation Amplicon sequencing (A-EZ method, Genewiz, Leipzig) using a pooling technique

Cytogenetic analyses

- Immunofluorescence analyses: based on a polyclonal antibody developed for a specific peptide corresponding to the N-terminus of *DcCENH3*

Results

Regeneration and analysis of hairy roots

The experiment design and the transformed carrot cultivar both impacted the number of hairy root lines heavily. However, the overall regeneration capacity was high.

- PCR analyses confirmed the presence of the CRISPR/Cas9 construct in all hairy root lines that grew well on selective media.
- Transgenic lines carrying the CRISPR/Cas9 constructs C3 and C4 showed insertions, deletions and substitutions within the C3 and C4 targets.
- Mutations within the *DcCENH3* gene appeared to be associated with a reduced CENH3 accumulation in the centromeres of some hairy root lines (**Fig. 3**).

Plant regeneration from hairy roots

The number of T0 plants regenerated from hairy roots and transferred successfully into a greenhouse is shown in **Table 1**.

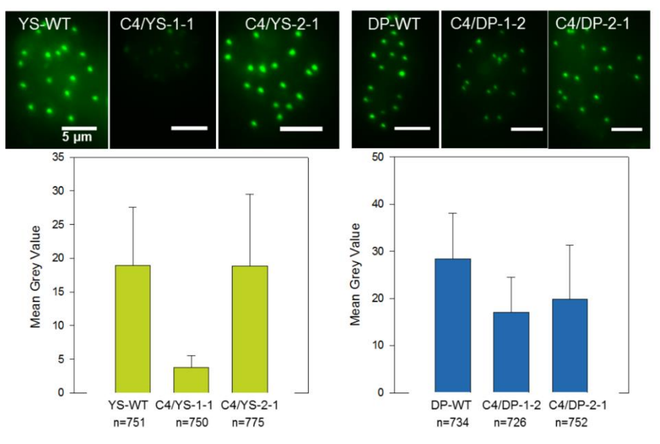

Fig. 3 CENH3 immunostaining of carrot nuclei of four hairy root lines with mutations within the C4 target of CENH3. Centromere signal strength was determined by the software ImageJ-win32 (ImageJ.net).

Table 1
Number of T0 plants regenerated from different hairy root lines originating from carrot cultivars ‚Blanche' and ‚Yellowstone'

Target	Cultivar	Hairy Root line	No. of To-plants
C3	Blanche	C3/BL-2	12
		C3/BL-3	12
		C3/BL-5	8
		C3/BL-6	1
C4	Yellowstone	C4/YS-2-1	6
		C4/YS-2-2	11
		C4/YS-2-3	21
	Blanche	C4/BL-5	3

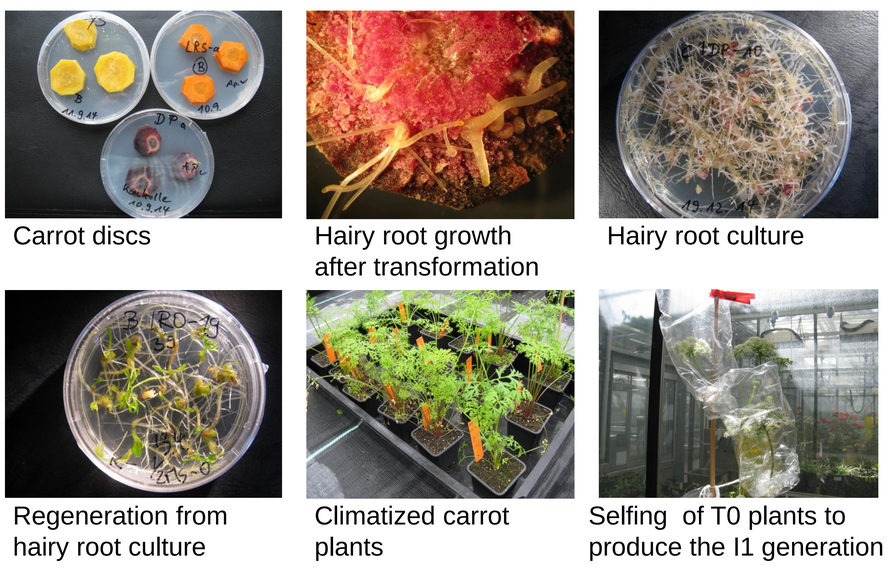

Fig. 1 Regeneration of hairy roots and transformed carrot plants

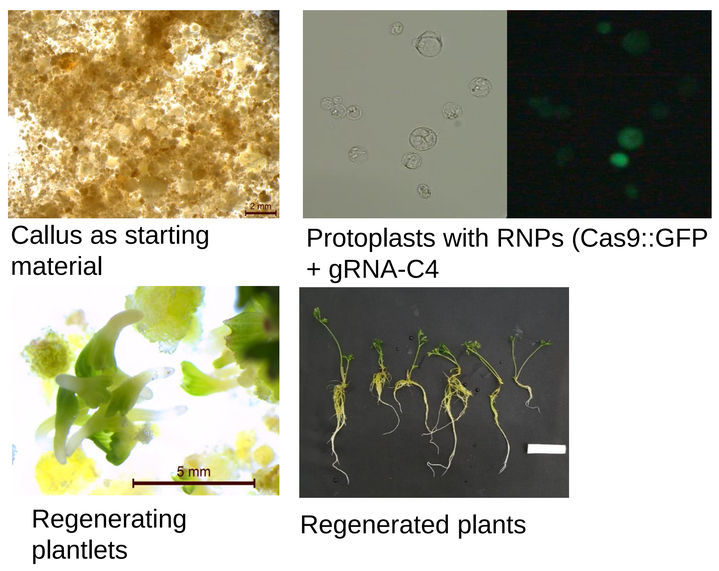

Fig. 2 Carrot protoplast isolation and transformation

Table 2 Mutation analysis of inbreeding progeny 19.032 by Illumina Amplicon-sequencing

Plant	No. of seq. total	No. of seq. with mutations	%	No. of seq. with insertion T	%	No. of seq. with deletion T	%	No. of other sequences	%
19.032-1	2726	2555	93,7	1258	49,2	1187	46,4	110	4,4
19.032-2	5717	5330	93,2	3368	63,2	1712	32,1	250	4,7
19.032-3	3530	3334	94,4	3034	91,0	161	4,8	139	4,2
19.032-4	2761	2519	91,2	1616	64,2	792	31,4	111	4,4
19.032-5	1252	1173	93,7	664	56,6	366	31,2	143	12,2
19.032-6	1585	1479	93,3	1356	91,7	56	3,8	67	4,5
19.032-7	2007	1894	94,4	989	52,2	543	28,7	362	19,1
19.032-9	2507	2321	92,6	1233	53,1	670	28,9	418	18,0
19.032-11	5786	3289	56,8	2901	88,2	177	5,4	211	6,4
19.032-14	1370	898	65,5	455	50,7	361	40,2	82	9,1
T0-Parent C4/YS-21-4	539	343	63,6	169	49,3	154	44,9	20	5,8

5'- TAGGGAGATTAGCTTCT- ACC**TGG** -3' (WT)
5'- TAGGGAGATTAGCTTCT**T**ACC**TGG** -3' (Insertion T)
5'- TAGGGAGATTAGCTTC - -ACC**TGG** -3' (Deletion T)

Analysis of regenerated plants (hairy root system)

All 74 transformed T0 plants were analyzed by PCR, and their expected transgenic genotype was confirmed. Flowering T0 plants were used to generate the I1 generation by self-pollination (to get homozygous mutants) and crosses with wild type carrots (to resolve chimeric structures in T1 individuals). Amplicon sequencing showed mutations in C3 and C4 targets of all T0 genotypes. Appearantly homozygous mutants could be obtained for target C4 in I1 progeny 19.032 (**Table 2**). Some of the plants (marked in yellow) probably have also lost their transgene(s). Next crosses (to induce putatively haploids) are possible in fall 2019.

Protoplast transformation and regeneration

A protocol for the DNA-free transformation of carrot protoplasts and the plant regeneration have been implemented successfully (**Fig. 2**). A PEG-guided transformation with RNPs (ribonucleoproteins) with gRNA for target C4 and GFP-tagged Cas9 was performed with protoplasts of 'Blanche', and callus with compromised growth was obtained. About 150 plantlets were regenerated, but all so far analyzed plants did not show any mutations within *DcCENH3*.

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