Universität Rostock Traditio et Innovatio

# Three delivery methods of CRISPR/Cas9 to induce targeted mutations into centromeric histone H3 (CENH3) in carrots (*Daucus carota* L.)

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*“Never be so kind, you forget to be clever*

*Never be so clever, you forget to be kind”*

Taylor Swift

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eure Katharina

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# V. List of abbreviations

***µ***

µL ...... *microliter*
µM...... *micromolar*

***A***

*A. rhizogenes*...... *Agrobacterium rhizogenes*
*A. thaliana* ...... *Arabidopsis thaliana*
*A. tumefaciens* ...... *Agrobacterium tumefaciens*

***B***

bp ...... *base pair*

***C***

Cas ...... *CRISPR associated proteins*
CATD ...... *centromer-targeting domain*
CENH3 ...... *centromere specific histone 3*, *centromere-specific histone 3*
cfu ...... *colony forming unit*
CRISPR ...... *Clustered Regularly Interspaced Short Palindromic Repeats*
crRNAs ...... *CRISPR RNAs*

***D***

dCas9...... *deactivated Cas9*
DH ...... *doubled haploid*
*DMP*...... *DOMAIN OF UNKNOWN FUNCTION 679 membrane protein*
DNA ...... *deoxyribonucleic acid*
DSB ...... *double strand break*, *Double Strand Break*

***E***

eGFP ...... *enhanced green fluorescent protein*
EYFP...... *ehanced yellow fluorescent protein*

***G***

G1 phase...... *gap 1 phase*
*GFP* ...... *green fluorescent protein*
gRNA ...... *single guide RNA*
GWAS ...... *Genome-Wide Association Study*

***H***

h ...... *hour*
*H. bulbosum* ...... *Hordeum bulbosum*
*H. vulgare* ...... *Hordeum vulgare*
HDR ...... *Homologous Repair*
HFD ...... *histone fold domain*

***I***

indels ...... *insertions and deletions*

***K***

kb ...... *kilo base pair*

***L***

LB ..... *Left border*

***M***

min ..... *minutes*
mL ..... *milliliter*
*MTL* ..... *MATRILINEAL*

***N***

NCBI ..... *National Center for Biotechnology Information*
ng ..... *nanogramm*
NGT ..... *New Genomic Techniques, New Genomic Techniques*
NHEJ ..... *Non-Homologous End Joining*
*NLD* ..... *NOT LIKE DAD*
nt ..... *nucleotide*

***O***

ori ..... *origin of replication*

***P***

PAM ..... *protospacer adjacent motif*
PEG ..... *polyethylene glycol*
pegRNA ..... *prime editing gRNA*
*PLA1* ..... *pollen-specific phospholipase*

***Q***

QTL ..... *quantitative trait loci*

***R***

RB ..... *right border*
RGEN ..... *RNA Guided Endonucleases Technique*
Ri-plamsid ..... *root-inducing plasmid*
RNA ..... *ribonucleic acid*
RNAi ..... *RNA interference*
RNPs ..... *ribonucleoprotein complexes*
rpm ..... *rounds per minute*

***S***

S phase ..... *synthesis phase*
*S. pyogenes* ..... *Streptococcus pyogenes*
SDN ..... *Site Directed Nucleases*
spCas9 ..... *S. pyogenes Cas9*

***T***

$T_0$ ..... *primary transformants*
TALEN ..... *Transcription Activator-Like Effector Nuclease*
T-DNA ..... *transfer DNA*
Ti-plasmid ..... *tumor inducing plasmid*
tracrRNA ..... *trans-activating CRISPR RNAs*

***V***

vir ..... *virulence gene region*

***Z***

ZFN ........................................................................................................ *Zink-Finger Nucleases*

# 1 Introduction

## 1.1. Plant breeding methods are constantly evolving

Domestication of plants allowed for settlement of the human population ten thousand years ago (Doebley et al. 2006). Six thousand years later, breeding with favorable phenotypes has resulted in high yielding crops feeding billions of people. However, during the last decades the food sector has been challenged to find modern solutions to ensure food safety in the future. Climate change increases the occurrence of extreme weather events like droughts or flooding, calling for adapted crops that still produce constant yield under harsh and fast changing conditions. At the same time more and more agricultural land is lost to make space for urban developments, demanding intensification of plant cultivation. Increasing the productivity by the use of fertilizer and pesticides should be done cautiously to prevent the pollution of groundwater and disruption of sensitive ecosystems (Galloway et al. 2003; Billen et al. 2013). Improving plant breeding can help to harvest the full genetic potential of plants. The use of Mendelian genetics to modern genome-wide association studies (GWAS), not only enables the understanding of function and interaction of single genes, but also help to unlock the potential of natural variation for breeding purposes. Over the past 60 years, the plant breeding method of mutagenesis exploits the variation of genes by increasing the general mutation rates artificially via chemical compounds or by irritating pollen (Hartung and Schiemann 2014). New genomic techniques (NGTs) have been developed that circumvent the time and cost consuming need to screen those highly mutated lines by e.g. inducing targeted mutations in the gene of interest by leading DNA cutting enzymes (nucleases). That way adapted and high-yielding plant varieties might be developed in the near future that require little use of fertilizer or pesticides.

## 1.2. Plant breeding methods to induce targeted mutations

Side-directed nucleases (SDN) and mutagenesis are both breeding methods that induce breaks in the plant DNA. Those breaks are repaired by natural DNA repair mechanism like the non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR). The NHEJ pathway repairs double strand break (DSB) of the DNA by joining the DNA fragments via an enzymatic process and is the predominant and most efficient repair mechanism, active during all phases of the cell cycle (Asmamaw and Zawdie 2021). However, the NHEJ pathways is prone to errors during its repair, resulting in small insertion or deletions (indels) at the cleavage site that can lead to a frameshift or premature stop codon (Yang et al. 2020). While HDR is highly precise by repairing the DSB according to a template of homologues DNA, or synthetic templates, it is mostly active in the late S and G1 phases of the cell cycle and requires a high amount of template DNA to function efficiently (Liu et al. 2018). Methods are constantly developed to improve the low HDR efficiency to make use of this precise repair way (Riesenberg et al. 2023; Jin et al.

2024). The different SDN methods can be categorized in two major groups: The first group are modified nucleases and follow the principle of DNA-protein recognition e.g. meganucleases, zinc-finger nucleases (ZFN), transcription activator like effector nucleases (TALEN). In the second group RNA is used to guide the nucleases to the target region. The best-known RNA guided endonucleases techniques (RGEN) are CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-techniques like CRISPR/Cas9 (CRISPR associated protein 9).

## 1.3. How CRISPR/Cas9 leads to site-directed modifications

In bacteria and archaea the CRISPR-system is used to develop an adaptive immunity against invading viruses and plasmids by guiding their silencing via CRISPR RNAs (crRNAs) (Stan J. J. Brouns et al. 2008). The CRISPR-Cas -loci in those bacteria and archaea exists of an operon of *Cas* genes and a CRISPR array of identical repeats, in which the invader-DNA is intercalated, that encodes for the crRNA (Barrangou et al. 2007). By simplifying the divers and complex CRISPR-system, adaptive immunity is achieved by following the three steps of adaptation, expression and interference (Makarova et al. 2020). First adaptation is done by inserting short sequences of invading DNA into the CRISPR array as spacer sequences, followed of expression and maturation of crRNAs that exists of the spacer sequence and flanking repeats, in the last step interference with the foreign nucleic acids by Cas proteins that are bound to the sequence-specific crRNA (van der Oost et al. 2014; Doudna and Charpentier 2014). While different naturally occurring CRISPR-systems in bacteria and archaea are discovered (Makarova et al. 2020) and different Cas-proteins are used and modified (Mishra et al. 2024), the first genome editing was done using CRISPR/Cas9. In 2012 it was shown that in the CRISPR/Cas9 system from *Streptococcus pyogenes* (*S. pyogenes*) crRNAs are base paired to trans-activating CRISPR RNAs (tracrRNA) and built a two-RNA structure that directs Cas9 proteins to the invading nucleic acids from e.g. viruses where it induces double strand breaks (DSBs) in the targeted DNA sequence (fig.1a.)). The engineering of the dual tracrRNA:crRNA to one functional single guide RNA (gRNA) marked the starting point to exploit this system to induce side-specific modifications (fig.1b.)) (Jinek et al. 2012; Doudna and Charpentier 2014).

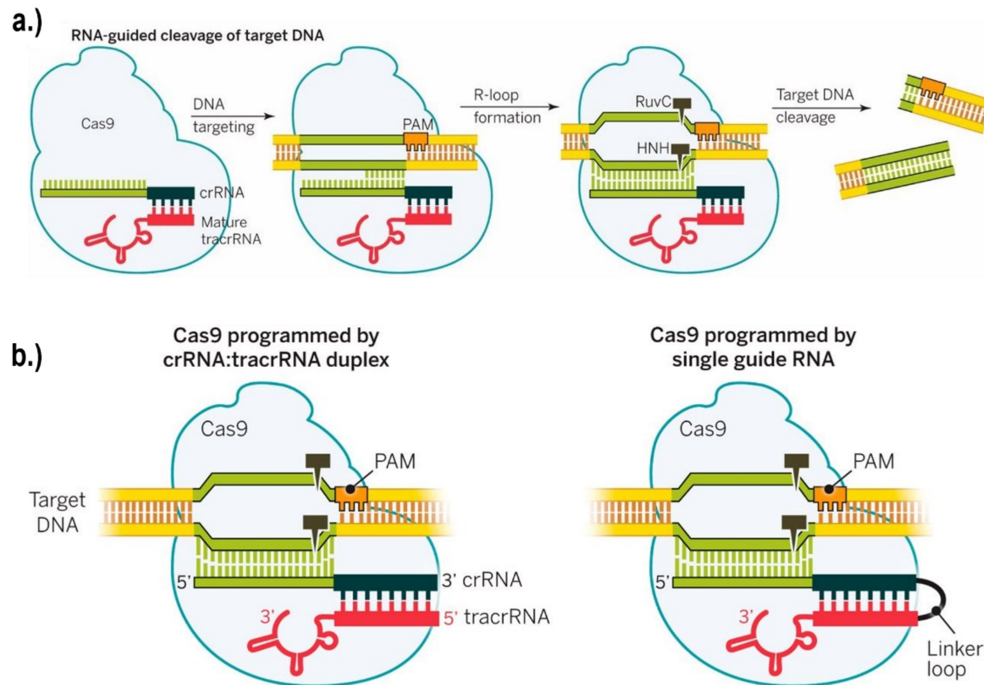


Figure 1: Biology of the CRISPR-Cas9 system. The Cas9 nuclease (blue) uses two catalytic centers (grey blades) to cleave the target site of a DNA sequence (gold) matching 20 nucleotides (nt) of the gRNA (green) next to a protospacer adjacent motif (PAM) (orange). The crRNA (black) and tracrRNA (red) built a complex that binds and stabilizes the Cas9 a.) natural DNA cleavage with the tracrRNA:crRNA duplex, b.) the crRNA:tracrRNA duplex was engineered to one gRNA without loss of function. Figure adjusted from (Doudna and Charpentier 2014).

Additionally to the base pairing of the crRNA sequence, a small short sequence adjacent to the target sequence is needed for target recognition (Jinek et al. 2012). This short sequence downstream to the cutting side is called protospacer adjacent motif (PAM). The PAM is approximately 2-5 base-pairs (bp) long and varies between different bacterial species. The term protospacer describes the 20 nucleotides (nt) long recognition site for the 5'end of the gRNA (Mojica et al. 2009; Jinek et al. 2012). For the most commonly used Cas, the Cas9 from *S. pyogenes* (spCas9), the PAM sequence is 5'-NGG-3' (with N being any nucleotide base) (Asmamaw and Zawdie 2021). After the Cas9 recognized the target site next to a PAM, it uses binding energy to melt DNA locally and utilizes the formation a RNA-DNA hybrid (Okafor et al. 2022). The non-target strand is displaced and builds a R- loop while the Cas9 undergoes a change in conformation, activating the two nuclease domains HNH (cleaving the complementary strand) and RuvC-like (cleaving the non-complementary strand which contains the PAM sequence) that leads to predominantly blunt-ended DSBs at three bps upstream to the PAM (Asmamaw and Zawdie 2021; Gong et al. 2018; S. Antony Ceasar et al. 2016). Modifications of the Cas9 protein allow for a variety of different applications exceeding the targeted induction of DSB. Mutating one of the Cas9 domains leads to nickase activity, while mutation of both deactivates dCas9 (Jinek et al. 2012). The dCas9 can be fused to other proteins, e.g. nucleobase deaminase enzymes, which leads to exchanging single bases without the need

for DSB repair (Rees and Liu 2018) or enhanced green fluorescent protein (eGFP) for live-cell imaging (Zhou et al. 2017; Khosravi et al. 2020) and many others. In prime editing a Cas9-nickase is fused with an engineered reverse transcriptase enzyme that interacts with an modified prime editing gRNA (pegRNA) which carries the repair template to encode the desired edit (Anzalone et al. 2019; Petrova and Smirnikhina 2023).

## 1.4. CRISPR/Cas9 as a plant breeding tool

In the last 10 years CRISPR/Cas9 was used to modify genes in a wide range of different crops to e.g. improve metabolic pathways, resistance to biotic and abiotic stress, yield or nutritional content and disease resistance (Mishra et al. 2024; Bhatta and Malla 2020). The system can be adapted to target different genes of interests easily and quickly and can be applied in every molecular biology laboratory (Holger Puchta 2017). The gene of interest can be screened via a variety of free-to-use online tools which do not only help to identify putative protospacer sequences next to a PAM, but also use algorithms to verify the structural integrity of planed gRNA (Concordet and Haeussler 2018; Varenyk et al. 2023). The 20nt long protospacer recognition side can be cloned into a variety of different CRISPR vectors to assemble the complete expression cassette for the adapted CRISPR/Cas9 system (Bhatta and Malla 2020). Different delivery methods are used to achieve CRISPR/Cas9 mediated genome editing, like stable integration of the expression cassette into the plant genome or transiently by infiltrating plant cells without cell walls (protoplasts) with ribonucleoprotein complexes (RNPs) from reassembled complexes of Cas9 and gRNA (Salvagnin et al. 2023; Bortesi and Fischer 2015). Since the early 1980s stable integration of transgenes is mediated by virulent *Agrobacteria* strains by making use of their naturally occurring integration of transfer DNA (T-DNA) from their tumor-inducing (Ti) or root-inducing (Ri) plasmids into host-cell genomes. Here the genes of the T-DNA induce neoplastic growth of the transformed tissue into either tumors (Ti-plasmid of *Agrobacterium tumefaciens* (*A. tumefaciens*) expressing nitrogen and carbon sources for the bacteria or hairy roots (Ri-plasmid from *Agrobacterium rhizogens* (*A. rhizogenes*) which provide a nutrient dense rhizosphere profiting the bacteria (Gelvin 2003; Tzvi Tzfira and Vitaly Citovsky 2006). Different regions on the Ti – or Ri- plasmids are involved during the T-DNA transfer. Two flanking regions of the T-DNA that are approximately 25 bp long play an important role in the excision and integration of the T-DNA (called left (LB) and right (RB) border), the virulence gene region (vir) encodes for proteins that initiate, process, mediate and integrate T-DNA into the nuclear genome of the plant, the conjugation gene region encodes proteins that regulate the transfer of the plasmid between bacteria, the origin of replication (ori) initiates the propagation of the genetic material (Rahman et al. 2024). Other virulence genes are located on the bacterial chromosomes and encode for membrane-bound proteins that are responsible for e.g. the chemotaxis of bacteria to recognize wound sites of plants for infection, or help in bacterial attachment to the cell (Rahman et al. 2024). By replacing the native T-DNA with the

genes of interest in the Ti- or Ri-plasmids of recombinant *Agrobacteria* strains, foreign genes can be introduced to produce transgenic plants (Hwang et al. 2017). Today, a wide variety of different agrobacteria strains, plasmids and protocols have been established and used to produce transgenic commercialized plants (Peter J. Christie 2004; Rahman et al. 2024). In a binary vector system, the T-DNA with its flanking border sequences is relocated from the around 200 kb big Ti-plasmid to a smaller plasmid that includes an antibiotic-resistance gene for easier handling (Lee and Gelvin 2008). Some protocols introduced a ternary vector which is an additional plasmid that overexpresses genes enhancing the virulence of *Agrobacterium* to allow T-DNA transfer into otherwise recalcitrant plants like maize (Anand et al. 2018). The stable integration of T-DNA encoding for a CRISPR/Cas9 cassette into the plant genome leads to *in vivo* expression of gRNA and Cas9 endonuclease (fig. 2A.), that build a gRNA:Cas9 complex inducing DSBs at the corresponding protospacer region and consequently mutations due to error prone NHEJ pathway (Metje-Sprink et al. 2019). The stable transformation of plants with a CRISPR/Cas9 expression cassette leads to a variety of transgenic primary transformants ($T_0$) with heritable modifications, however ongoing DSBs due to the Cas9 nuclease transgene result into new secondary mutations and creation of chimeric plants (Schiml and Puchta 2016; Zhang et al. 2014; Mao et al. 2013). Therefore the transgene must be segregated in later generations to have a non-transgenic but gene edited plant without ongoing Cas9 activity (Xu et al. 2015). In transient transformations, the coding genes for gRNA and Cas9 are not stably integrated into the plant genome. Here the unique ability of plant cells with removed cell walls (protoplasts) to uptake macromolecules like DNA, RNA or proteins is utilized (Panda et al. 2023). Polyethylene glycol (PEG) guided transfection can be used to transport preassembled Cas9:gRNA complexes into the plant nucleus (fig.2B.)). This method has been successfully used to produce transgene free genome edited plants without prior segregation of the Cas9 transgene to stop the ongoing DSBs induction (Metje-Sprink et al. 2019; Bortesi and Fischer 2015). The infiltration of protoplasts with RNPs have shown cleaved target sites shortly after transfection and rapid degradation by endogenous proteases, reducing the likelihood of secondary mutations due to limited Cas9 exposure (Woo et al. 2015).

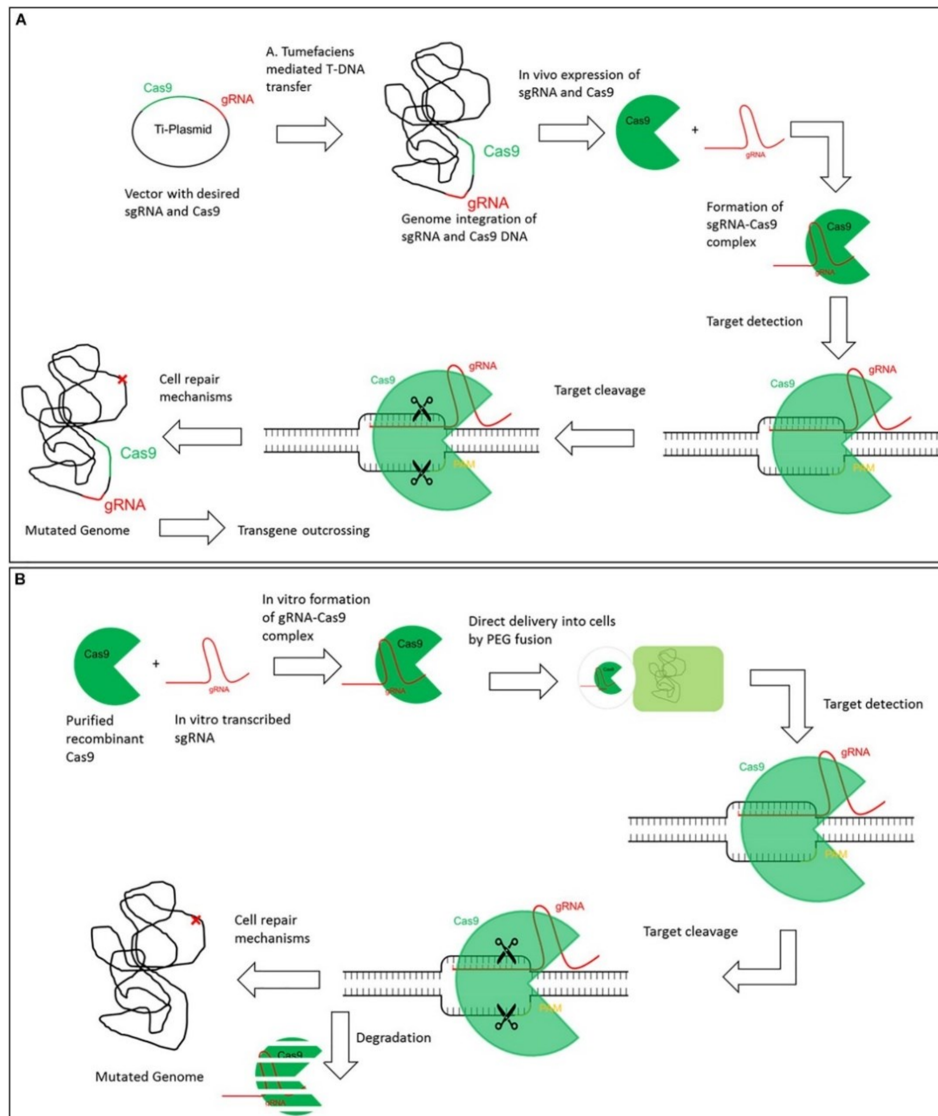


Figure 2: Illustration of the agrobacteria mediated stable integration of the CRISPR/Cas9 expression cassette into the plant genome and the transient delivery of preassembled gRNA:Cas9 complexes into plant protoplasts. In A) The endogenous T-DNA from the Ti-plasmid of *A. tumefaciens* is replaced with the CRISPR/Cas9 expression cassette. The bacterium transfers its T-DNA into the plant genome. The gRNA and Cas9 nuclease are transcribed and translated *in vivo* and build the active gRNA:Cas9 ribonucleoprotein-complex, that cleaves the DNA at the target side, creating a DSB that is repaired by internal cell repair mechanisms. The cleavage by Cas9 continues until the Cas9 transgene is segregated by outcrossing. In B) the gRNA:Cas9 ribonucleoprotein-complex is built *in vitro* and then delivered into protoplasts where it instantly starts to detect target sequences and induces DSBs. The RNP complex degrades quickly and can no longer induce DSBs. Figure from (Metje-Sprink et al. 2019).

Regardless of the applied delivery method of CRISPR/Cas9, a major bottleneck for the success is a functional transformation and regeneration protocol (Altpeter et al. 2016). Therefore, transforming plants that are known to survive and regenerate well from tissue culture is advantageous in novel CRISPR/Cas9 approaches.

## 1.5. Breeding of carrots (*Daucus carota*)

Carrot acts as a model system for tissue culture, with root tissue being one of the very first successful plant tissue cultures in 1936 first reported totipotency (Thorpe 2012; Steward et al. 1958). *Daucus carota* (*D. carota*) is a diploid (2n=2x=18) outcrossing and insect pollinated vegetable crop that belongs to the *Apiaceae* family (Simon 2021) and is high in β-carotene (provitamin A), vitamins B1 and C2, is a good source of dietary fiber and grown worldwide (Wally and Punja 2015). It is a biannual plant and flowers mostly in its second year of growth after being exposed to a period of cold temperature (vernalization). Additionally, to general breeding goals like yield, adaptability and tolerance to various stresses, breeding of carrots aims to improve e.g. the appearance and storage ability of the tap root or the carotenoid and anthocyanin content (Kalia et al. 2023). Selection breeding within different populations together with optimized cultivation conditions allowed for a profitable production of carrots. However, the heterozygosity within the varieties prevents the high level of uniformity that supports a market stable yield. Here hybrid breeding has highly improved the uniformity (Stein and Nothnagel 1995). Since the early 20th century, hybrid breeding has been adapted for a high number of different species like e.g. cereals, sunflowers and carrots. Here the crossing of two contrasting in-breed lines not only leads to heterozygous progeny ($F_1$ generation) with a uniform phenotype but also shows an enhancement of parental traits due to the positive effect of heterosis (Jagosz 2012). After six years of self-pollination 98 % homozygosity can be achieved, but the carrots suffer severely under inbreed depression (Li et al. 2013). Therefore, methods are exploited to fasten the production of complete homozygous parental lines without the need for continuous self-pollination to use in hybrid breeding.

## 1.6. Doubled haploid technology to produce parental lines for hybrid breeding

Haploid plants have the chromosome number of a gametophyte (in carrot 1n=1x=9) and doubled haploid (DH) plants are haploids after chromosome duplication. The doubled haploid technology aims to produce completely homozygous material from heterozygous plants without the need of generations of self-pollination (Germana 2011). Here haploid cells are regenerated into to a sterile haploid embryo which later becomes a fertile diploid plant - on its own or by treatment with colchicine or oryzalin that promote chromosome doubling by preventing cell division during mitosis (Hooghvorst et al. 2020; Chaikam et al. 2020; Pickett-Heaps 1967). While it can be difficult, tedious and time consuming to establish protocols for DH production, it allows to produce fully homozygous parental lines in two years compared to seven (Hussain and Franks 2019). In carrot, tissue culture of unfertilized ovules and anther cultures have been used to produce haploid and doubled haploid plants (Kielkowska et al. 2014). However, those *in vitro*

methods by tissue culture are not applicable to most genotypes, which calls for a universal tool to achieve DH lines for breeding purposes.

## 1.7. Haploid induction by side specific mutations

In recent years CRISPR/Cas9 was used to target different genes that are involved in fertilization, paternal chromosome elimination or chromosome segregation to establish robust *in vivo* haploid production by modified haploidy inducer lines (fig. 3.). In maize the haploid inducer line stock 6 and its derivates have widely been used in hybrid breeding to produce around 3 % maternal haploids (Meng et al. 2021). Maize chromosome elimination methods allow for high production of haploids in wheat and other cereals like barely, triticale, rye, or oats (Wędzony et al. 2009) or pearl millet (Inagaki 2003). The ability of this line to induce haploid progeny could be ascribed to 8 QTLs (quantitative trait loci), with changes to *qhir1* and *qhir8* affecting the haploid induction rate significantly (Prigge et al. 2012). Targeting the pollen-expressed gene *DMP* (*DOMAIN OF UNKNOWN FUNCTION 679 membrane protein*) of *qhir8* with two gRNAs increased the haploid induction rate by up to a 3-fold (Zhong et al. 2019). Homologous of *DMP* have been targeted in numerous dicot species with differences in the resulting haploid induction rate (Gawande et al. 2024; Wang et al. 2022a). Membrane proteins that are located to the plasma membrane of gametes, like the ones encoded by *DMP*, regulate the pairing and fusion between male and female gametes which is crucial for the successful double fertilization in plants (Takahashi et al. 2018).

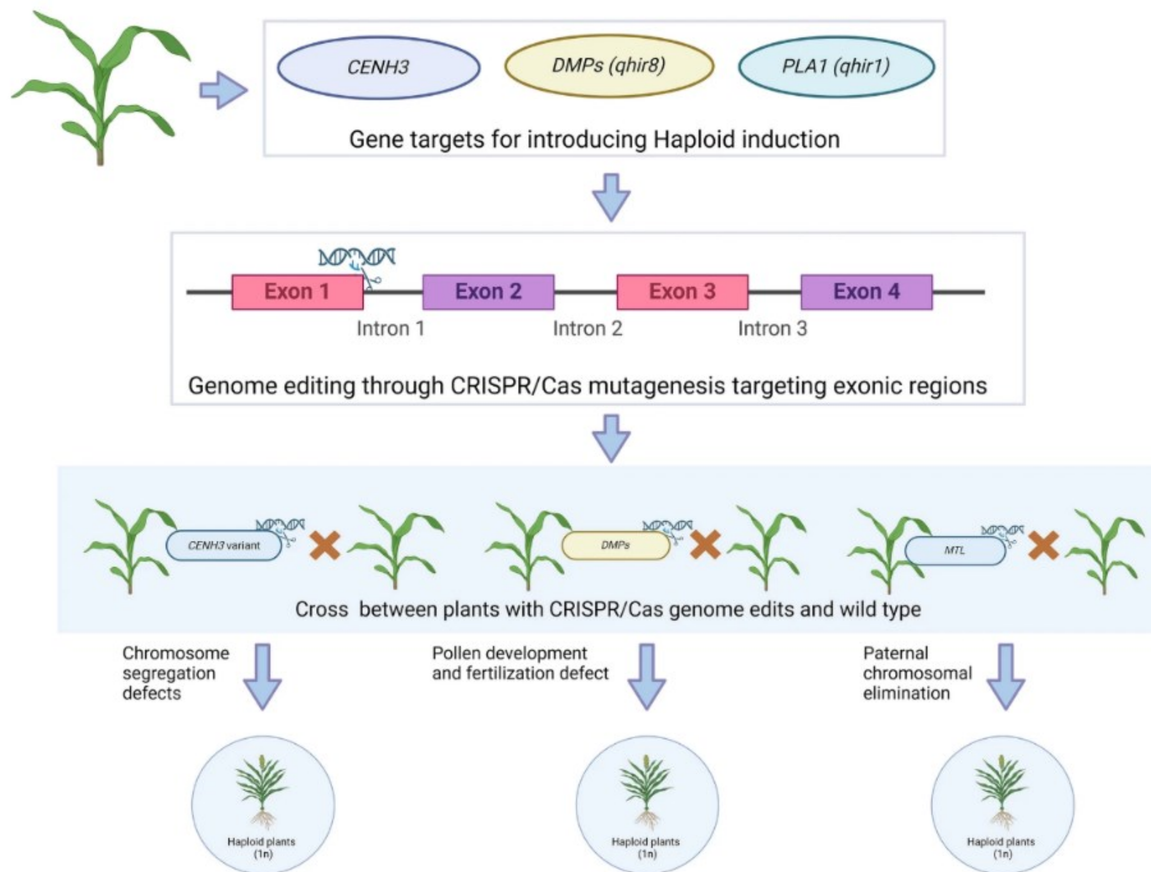


Figure 3: Genome editing strategies for haploid induction. CRISPR/Cas9 is used to mutate genes that are associated with haploid inducing effects. Crossing those mutated parental lines with wild type plants should lead to defects in chromosome segregation after fertilization (CENH3), defect in the development of pollen and their fertilization ability (DMPs) or the elimination of paternal chromosomes (PLA1). From (Gawande et al. 2024)

Targeting the QTL *qhir1*, also known as *PLA1* (*pollen-specific phospholipase*) or *NLD* (*NOT LIKE DAD*) or *MTL* (*MATRILINEAL*) has led to the elimination of parental chromosomes in the zygote and haploid plants in maize and other cereals like e.g. rice and wheat (Liu et al. 2017; Yao et al. 2018; Liu et al. 2020). Combining the mutated *DMP* and *PLA1* alleles increased the haploid induction rate of mutated haploid inducers to 6-10 % (Zhong et al. 2019). So far no homologous of *PLA1*, *NLD* or *MTL* have been found in dicots (Zhong et al. 2022). The segregation of chromosomes is manipulated by targeting the centromere specific histone 3 (CENH3), a protein of the kinetochore region of centromeres to which spindle microtubules attach to during cell divisions and that is highly conserved in different plant species.

## 1.8. Functional CENH3 is crucial for the proper segregation of chromosomes

In eucaryotic cells the DNA is organized into chromatin made of an array of nucleosomes, a basic unit of chromatin, and associated proteins. A nucleosome is formed by ~150 bp DNA and two copies of four core histones (H2A, H2B, H3 and H4) (Luger et al. 1997). Additionally, most eukaryotic species use variants

of those histones to attribute specific properties to certain genomic regions (Danhua Jiang and Frédéric Berger 2017). On of those genomic regions with specific histone variants are centromeres. Here histone chaperons load the histone variant CENH3, replacing the canonical histone H3 (Takeuchi et al. 2024). The histone variant CENH3 binds to a complex of over 100 structural and regulatory proteins, the kinetochore, which mediates the attachment of microtubule fibers of the spindle apparatus during cell divisions (Kursel and Malik 2016; Ines A. Drinnenberg et al. 2016) (fig. 4).

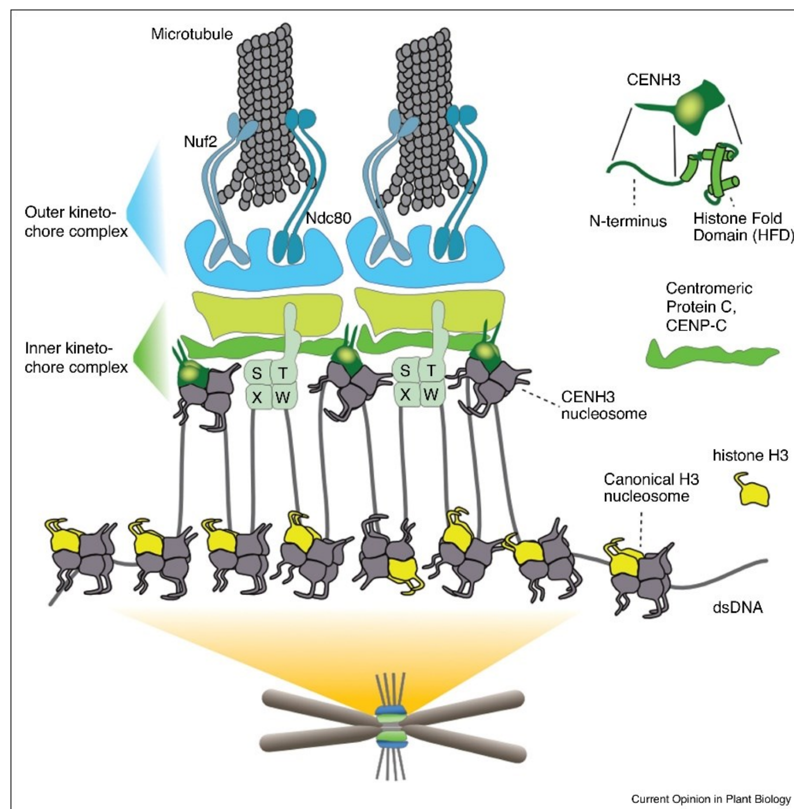


Figure 4: Model of a kinetochore. In the centromere the canonical histone H3 is replaced by CENH3, creatin a unique chromatin environment. CENH3 binds proteins of the inner kinetochore complex, while the outer kinetochore complex interacts with the microtubules of the spindle apparatus during mitosis and meiosis. From (Luca Comai et al. 2017)

The histone variant CENH3, like other histones, contains a C-terminal histone-fold domain (HFD) which mediates the histone-histone and histone-DNA interaction and an N-terminal tail that protrudes from the nucleosome and undergoes posttranslational modification like e.g. methylation, acetylation, phosphorylation and ubiquitination (Khorasanizadeh 2004). Within the HFD a specific CENH3 centromere-targeting domain (CATD) has been identified to be required for the centromere loading of CENH3 by histone chaperons (Takeuchi et al. 2024). While other histone H3 variants are highly conserved, CENH3 is species specific and evolves as quickly as centromeric DNA (Henikoff et al. 2001; Sanei et al. 2011). In 2010 Ravi and Chan (Ravi and Chan 2010) found that modifications of CENH3 led

to haploid progeny in *Arabidopsis thaliana* (*A. thaliana*), since then different modification have been done to understand and exploit CENH3 as a target to create haploid inducer lines.

## 1.9. Modifications of CENH3 produce haploid inducer lines

Native *CENH3* was knocked out in *A. thaliana* via mutagenesis (creating the null mutant with *cenh3-1)* and replaced with transgenes encoding recombinant CENH3 variants (Ravi and Chan 2010) (fig. 5).

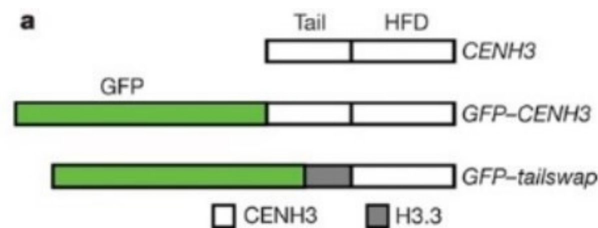


Figure 5: Wild type and modified CENH3 variants used in *A. thaliana*. A full knocked out of native *CENH3* was replaced with transgenes encoding recombinant CENH3 either fused to GFP (*GFP-CENH3*) or tailswap of the native N-terminal tail with GFP and conventional histone H3.3 (*GFP-tailswap*). From (Ravi and Chan 2010)

The *cenh3-1* mutation leads to an embryo-lethal phenotype, but complementing *cenh3-1* with *GFP-CENH3* created plants with normal phenotype and complementing with *GFP-tailswap* created plants with normal mitotic divisions but mostly male sterile plants and only 60-70 % fertility when pollinated with a wild type plant indicates possible defects in meiosis. Further, up to 95 % of fertilized ovules have been apported early in their development and 10 of the 16 viable off springs presented only wild type *CENH3* showing uniparental genome elimination of the transgenic parent expressing GFP-tailswap CENH3 (Ravi and Chan 2010). Functional segregation of chromosomes during mitosis, but semi-sterile plants with distorted chromosome segregation after meiosis have also been seen in *A. thaliana* with either N-terminal tail modifications in *cenh3* null variants by fusion or full or partial replacement with green fluorescent protein (GFP) (Ravi et al. 2011; Ravi and Chan 2010) and enhanced yellow fluorescent protein (EYFP) or in *A. thaliana* with low levels of fully functional CENH3 via RNA interference (RNAi) (Lermontova et al. 2011). Therefore, only the C-terminal HFD of CENH3 is required for the deposition and function of somatic centromeres during mitoses, however the N-terminal tail seems to play an important role during loading of CENH3 onto centromeres in pre-meiotic cells (Schubert et al. 2014). Genomic surveillance mechanism are expected to remove flawed CENH3 at fertilization (Marimuthu et al. 2021) or from pre-meiotic cells followed by a post-divisional reloading after the first or second meiotic divisions that does not accept imperfect CENH3 (Schubert et al. 2014). Further it is believed, that meiotic – contrary to mitotic - divisions can occur with very little CENH3 content (Lermontova et al. 2011; Ravi et al. 2011). So far, there is a consensus that the modifications of CENH3 cause weak centromeres in the gametophytes due to little

CENH3 loading, resulting in the genome elimination when those chromosomes compete with the wild type during embryonic mitosis (Naish and Henderson 2024) (fig. 6).

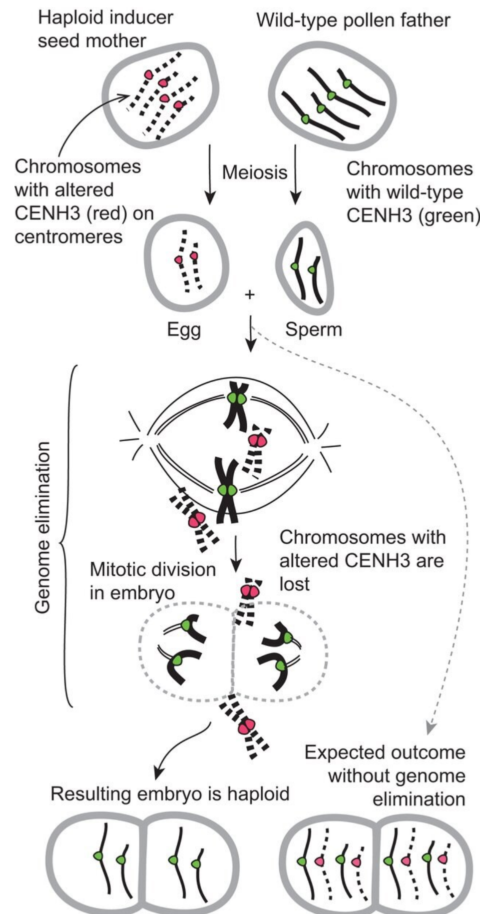


Figure 6: Genome elimination induced by modification of CENH3. The native CENH3 is altered in the haploid inducer line, still allowing for mitotic divisions but unstable and eliminated during first mitotic cell divisions when wild type CENH3 is present. From (Comai 2014)

Different theories exist on why missegregation of the chromosomes with weak centromeres occur in the early embryo. Spindle fibers cannot attach properly onto the barely present kinetochore (Karimi-Ashtiyani et al. 2015), the decreased amount of functional CENH3 within the centromeres prolongs the time the cells spend in the prometaphase/metaphase (Capitao et al. 2021) or due to asynchronous movement of chromosomes with weak centromeres during partition (Lermontova et al. 2013).

The role of CENH3 in genome elimination not only leads to haploidy inducing effects when replaced with transgenic homologous but was identified to play a major role in long applied *in vivo* breeding methods for wide hybridization. In cultivated barley, haploid production is the result of inter-specific hybridization between *Hordeum vulgare* (*H. vulgare*) as the mother and wild *Hordeum bulbosum* (*H. bulbosum*) as the father and is the first known uniparental genome elimination by hybridization established in the early

1970s (Kasha and Kao 1970). During early embryogenesis, the *H. bulbosum* chromosomes are missegrated and consequently lost due to missing of loading of CENH3 onto *H. bulbosum* chromosomes, possibly due cell cycle asynchrony (Sanei et al. 2011). This method allows to produce a large number of haploid embryos in most genotypes and is applied in different breeding programs.

## 1.10. Modifications of CENH3 for genome elimination in different crops

With promising results to create haploidy inducing lines in *A. thaliana*, implementation in crop species is of high interest for plant breeding purposes. Targeting CENH3 to produce DH breeding lines is currently tested for different crop species (e.g. tomato, rice, banana, barley, tobacco, soybean, sugar beet, sweet potato, different cucurbits and carrot) (Tek *et al.*, 2015, Britt and Kuppu, 2016). Mutants created by mutagenesis led to the successful generation of a small percentage of haploid progeny in tomato and rice (Op den Camp, R. H. M. et al.) as well as in the cucurbits cucumber, melon and watermelon (van Dun *et al.*, 2017) which have been patented. Overall three different approaches of disrupting the CENH3 function have been identified (Wang et al. 2019). The editing of the N-terminal tail of CENH3, secondly editing of the CATD and third the replacement of CENH3 with a homologue. Changing the structure of CENH3 by fusion to GFP or YEP to the N-terminal tails in maize (Feng et al. 2020), similar to the tail swap approach in *A. thaliana* (Ravi and Chan 2010; Kelliher et al. 2016). Heterozygous knock-out of endogenous CENH3 in maize lead to haploid inducing effect by dilution of functional CENH3 during postmeiotic cell divisions prior to the formation of gametes (Wang et al. 2021). Minimizing functional CENH3 by RNAi approaches also lead to weak centromeres with haploidy inducing properties (Gao et al. 2020) same applies for inducing alterations of the protein structure by alternating in-frame mutations (Kuppu et al. 2020). A complete knock-out of endogenous CENH3 with following complementation with either homologous CENH3 (Maheshwari et al. 2015) or altered chimeric CENH3 (Ravi and Chan 2010) also successfully worked to induce haploid progeny.

The high number of possible CENH3 modifications shows the necessity for versatile tools to induce precise editing of the *CENH3*, and which are quick to adjust for new target sides.

## 1.11. Aim of this work

This work aims to identify the advantages and disadvantages of the stable transformation via *A. tumefaciens* or *A. rhizogenes* and the transgene-free method using preassembled RNPs to target CENH3 in *D. carota* to produce haploid inducing lines for the accelerated breeding of carrots.

# 2 Material and Methods

## 2.1. Cloning

### 2.1.1. Cloning of the pEn-Chimera T7 expression vector

The pEn-ChimeraT7 expression vector is used to express gRNA for in vitro testing and protoplasts transformation. It is based on the pEn-Chimera vector gifted from Holger Puchta (Addgene plasmid # 61432; http://n2t.net/addgene:61432 ; RRID:Addgene_61432). The *Bsa*I cloning sites have been added to the respective protospacer sequences (sense: 5‘ GAGAGG -protospacer; anti-sense: 5‘ AAAC-reverse complement protospacer - CC). The oligonucleotides have been ordered at metabion.com (metabion international AG; Planegg/Steinkirchen; Germany). Annealing of the oligonucleotides was done by incubating 50 µM of each oligonucleotide in 50 µL $H_20$ at 95 °C for 5 min and followed by 20 min at room temperature. For the ligation into the pEn-Chimera T7 expression vector, 2 µL of the linearized pEn-Chimera T7 vector (5 ng/µL) was incubated with 3 µL of annealed oligonucleotides, 1 µL T4 ligase and 5 µL 2x Quick Ligase Buffer at room temperature for 1 h. Followed by transformation in DH5α according to the standard freeze thaw protocol and plated onto LB-Amp100 plates (Lennox Broth (LB) with 100 µg/mL Ampicillin) for selection. After growing the bacteria over night at 37°C, single colonies have been transferred in liquid LB media and grew over night at 37°C on a shaker (180 rpm).

Plasmid isolation from *E. coli* DH5α grown in 5 ml LB-media was done using the GeneJET Plasmid-Miniprep-Kit (ThermoFisher Scientific, Waltham, USA) according to protocol. Isolated plasmid DNA (pDNA) from three clones for each target have been Sanger sequenced (Genewiz, an Azenta Life Science Company, Leipzig, Germany) using the M13 forward primer. Cyro-cultures have been prepared by mixing 3 mL of the bacteria culture with 3 mL 30 % sterile Glycerin and kept at -80°C.

### 2.1.2. Cloning of the pDE-Cas9 expression vector

For the stable transformation of carrot, the Crispr/Cas 9 expression cassette of binary expression vector pDE-Cas9 was modified to the target sides Cr3 and Cr4 according to protocol (Schiml et al. 2017).

Table 1 : List of plasmids.

| Plasmid | Bacteria | use |
|---|---|---|
| pDE-Cas9::Cr3 | *A. tumefaciens* LBA4404pSB1<br>*E. coli* DH5α | Stable plant transformation |
| pDE-Cas9::Cr4 | *A. rhizogenes* ATCC 15834<br>*E. coli* DH5α | Stable plant transformation |
| pEn-Chimera T7::Cr3 | *E. coli* DH5α | Expression gRNA |
| pEn-Chimera T7::Cr4 | *E. coli* DH5α | Expression gRNA |

Table 2 List of primer

| Name | Sequence | use |
|---|---|---|
| CR4L_fw | ATGTACGGCACCTTTGTAACCT | Amplify Cr4 and Cr3 target site |
| CR4L-rev | AGTGTGTTTCTTCCAGAGCCAA | Amplify Cr4 and Cr3 target site |
| CR4k_fw | TGTCTACTTGTGAATTCAGTTGG | Amplify Cr4 and Cr3 target site |
| CR4k_rev | AGTGTGTTTCTTCCAGAGCCAA | Amplify Cr4 and Cr3 target site |
| M13_fw | GTAAAACGACGGCCAG | Amplify insert from pEN-Chimera T7 vector |
| M13_rev | CAGGAAACAGCTATGAC | Amplify insert from pEN-Chimera T7 vector |
| dcCENH3/1_fw | AGGACAGCGGGAACGAAAAT | Amplify genomic CENH3 for cleavage assay |
| dcCENH3/1_rev | TCATACCACAATATCCCG | Amplify genomic CENH3 for cleavage assay |
| Cas9_fw | TCCTCAAAGAGCACCCTGTT | Amplify Cas9 transgene |
| Cas9_rev | TAGAGAATCCACCGGTCTGC | Amplify Cas9 transgene |

## 2.2. Protospacer design and testing of the gRNAs

### 2.2.1. Protospacer design

The protospacer for Cr3 and Cr4 have been designed by screening the HFD coding region of *CENH3* for putative PAM (NGG) sites.

### 2.2.2. Preparation of gRNA and testing them *in vitro*

Starting with generating the template for T7 RNA polymerase. Here 10 µL 5x Q5-Buffer; 1 µL dNTPs (10 mM), 0.5 µL M13_fw and M13_rev (50 µM) each; 100 ng plasmid DNA, 0.1 µL Q5 have been filled up with $ddH_20$ to 50 µL. In a thermocycler annealing was done at 54°C for 30 sec., elongation at 72 °C for 45 sec, for a total of 40 cycles. The PCR product was purified via the GeneJET PCR Purification Kit (ThermoFisher) according to the manufacturer protocol. For the T7-Polymerase reaction, all chemicals thaw on ice and have been pipetted at room temperature. Here 10 µL T7 Transcription Buffer (5x), 10 µL NTPs (10 mM), 1 µg DNA, 1.25 µL RiboLock, 30 U T7 RNA-Polymerase have been filled up with $H_20$ (DEPC-treated) to 50 µL and incubated at 37 °C for 2 h. DNA residue was digested by DNaseI treatment. Here 2 µL DNase I was added to every 50 µL sample and incubated at 37 °C for 2 hrs. The treatment was blocked by adding 2 µL 0.5 EDTA (pH 8) followed by incubation at 65 °C for 10 min. The RNA was cleaned via the GeneJET RNA Purification Kit according to the manufacturer protocol and stored at –80°C. Genomic DNA was amplified from *D. carota* Yellowstone via CR4L_fw and CR4L_rev primer (369 bp). The PCR product was purified via GeneJET PCR Purification Kit (ThermoFisher, Darmstadt,

Germany), cloned into pGEM®-T Easy Vector (Promega, Madison, USA) according to the manufacturers protocol and transformed into *E. coli* DH5α according to standard freeze-thaw protocol. Transformed *E. coli* DH5α were plated on LB Amp100 plates (20 g /L LB (Lennox), 15 g/L agar, autoclaved and add 100 µg/mL ampicillin after cooling down) and grown overnight at 37°C. Colonies were picked and grown overnight at 37 °C in liquid LB-media, pDNA from three clones for each target have been isolated via Thermo Fisher GeneJET Plasmid Miniprep Kit (ThermoFisher, Darmstadt, Germany) and sanger sequenced using the M13 forward primer (Genewiz, an Azenta Life Science Company, Leipzig, Germany). Cyro cultures have been prepared by mixing 3 mL of the bacteria culture with 3 mL 30 % sterile Glycerin and kept at -80°C.

### 2.2.3. Cleavage assay

The final concentrations are 500 nM purified SpCas9-Nuclease (provided by J. Metje-Sprink); 500 nM gRNA; 900 ng DNA; 10 µL NEB Buffer 3 10 x; up to 100 µL $H_20$ (DEPC-treated) (100 mM NaCl; 50 mM Tris-HCl; 10 mM $MGCl_2$; 1 mM DTT, pH 7.9). First the DEPC-treated water and NEB Buffer 3 10 x are mixed, SpCas9-Nuclease and gRNA are added and preincubated for 15 min at room temperature. The DNA sample is added to the mix and incubated at 37°C, 50 µL of each sample is taken after 4 hrs. The reaction was stopped by adding 1 µL proteinase K (400 U/µL) (NEB, #P8107S) and incubated for 15 min at room temperature. Add 6X DNA Loading Dye (ThermoFisher, Darmstadt, Germany), and run on 1.5 % agaroses gel at 100 V.

Table 3: list of used target sequences. The PAM is bold and underlined.

| Name | Sequence | Position | Region |
|---|---|---|---|
| Cr3 | ATAATAATTGTTAACAGGTT***<u>AGG</u>*** | Exon 5 | HFD |
| Cr4 | TAGGGAGATTAGCTTCTACC***<u>TGG</u>*** | Exon 5 | HFD |

## 2.3. Plant material and transformation

### 2.3.1. Transformation of carrot discs with *A. rhizogenes*

The *D. carota* cultivars Deep Purple and Yellowstone have been planted in the greenhouse and transformed with *A. rhizogenes* and checked for mutations as described in (Dunemann et al. 2019).

### 2.3.2. Transformation of carrot callus culture with *A. tumefaciens*

Seeds of *D. carota* (var Rotin und Nantaise) were sterilized by washing them for 15 min in 70 % EtOH, followed by 20 min in 5 % NaOCl with a droplet of Tween 20, shake continuously and wash with sterile $H_20$ until foam build up stops. Sterilized seeds are given on germination media (for 1 L: 2.15 g Murashige

& Skoog Medium (Micro and Macro elements; Duchefa 0221), 3.164 g Gamborg B5 vitamin mixture (Duchefa G0415), 15 g sucrose, 7.5 g plant agar (Duchefa); filled up to 1 L with aqua dest, pH 6; autoclave). The germination was done at 24 °C and moved from dark to light after three days. After 7-14 days, segments of shoots, petioles, hypocotyls and cotyledons were plated onto CB5D4P medium (For 1 L: 3.164 g Gamborg B5 medium including vitamins (Duchefa G0210), 20 g saccharose, 250 mg Casein hydrolysate (Sigma A-2427), 4 g Phytagel, filled up to 1 L, pH 5.7, autoclave, add 2 µM sterile 2,4-D). Callus is induced at 24 °C in the dark for two weeks. Freshly formed callus were transferred onto new plates every two weeks, after three months the callus can be used for transformation. Inoculation and co-culture of *A. tumefaciens* and carrot callus was done according to an adjusted protocol (Otto et al. 2015). The callus was inoculated in 1.5 mL CC media with 5 x $10^7$ colony forming unit (cfu) aliquots of *A. tumefaciens* in each well of a six well cell culture dish and incubated at 65 rpm and 21° C in the dark. After two days *A. tumefaciens* was removed by washing with 0.4 mL CAst media (for 1 L: 3.164 g Gamborg B5 medium including vitamins (Duchefa G0210), 20 g saccharose, 250 mg Casein hydrolysate (Sigma A-2427); filled up to 1 L with aqua dest, pH 6; autoclaved. After cooling down, 200 mg/mL Timentin (Tim), 25 mg/ml Phosphinothricin (PPT) and 500 µM Acetosyringone were added), after washing, the callus was kept in 1.1 mL CAst media at 65 rpm and 24 °C in the dark. The media was replaced every week for a total of three weeks. For selection and regeneration of the callus was plated onto CSR media (for 1 L: 2.15 g Murashige & Skoog Medium (Micro and Macro elements; Duchefa 0221), 3.164 g Gamborg B5 vitamin mixture (Duchefa G0415), 15 g sucrose, 4 g pythagel; filled up to 1 L with aqua dest, pH 6; autoclave. After cooling down, 200 mg/mL Timentin, and 25 mg/L Phosphinothricin were added) and kept at 24 °C under light. After three weeks, sprouts were transferred onto fresh CSR media, after five weeks the young carrots plants have been transferred into soil and acclimated under a domed plant tray.

DNA was isolated from leaves using the innuPREP Plant DNA Kit (IST Innuscreen GmbH, Germany), the Cr4 target side has been amplified via PCR using the CR4L_fw and CR4_L-rev primer pair (369 bp) and Sanger sequenced at Genewiz/Azenta (Leipzig, Germany) according to their guidelines using CR4L-fw as sequencing primer.

### 2.3.3. Transformation of carrot protoplasts with RNPs

Protoplasts have been isolated from the callus of petioles of *D. carota* var. Blanche according to (Grzebelus et al. 2012). Here isolated protoplasts have been resuspended at 2x$10^5$ $mL^{-1}$ in MMG solution (4 mM MES pH 5.7, 0.4 M mannitol; 15 mM $MGCl_2$; sterilized by filtration) at room temperature; the RNP complexes were preassembled by incubating 1 µg gRNA with 1 µg spCas9::GFP (prepared by Dr. Janina Metje-Sprink as described in (Ishii et al. 2019)) in 1 µL 10 x NEB 3 Buffer filled up to 10 µL sterile $H_2O$. After 15 min in the dark at room temperature, 100 µL of the protoplast solution is added and gently mixed,

then 110 µL of PEG solution (40 % PEG 6000, 0.2 M mannitol, 100 mM $CaCl_2$; gently mix in rotating wheel and sterilize by filtration) is added. After 15 min at room temperature, 450 µL W5 solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCL, filled with $H_2O$ up to 100 mL and sterilized by filtration) is added and mixed in by inverting the tube gently. The samples are then centrifuged at 100 xg for 5 min at room temperature, the supernatant is carefully removed and the protoplasts resuspended in 1 mL CPW solution (For 1L: 27.2 mg $KH_2PO_4$; 101 mg $KNO_3$; 1480 mg $CaCl_2x2H_2O$; 246 mg $MgSO_4$ x 7 $H_2O$; 0.16 mg KI; 0.025 mg $CuSO_4$; 130 g mannitol; 3 g PVP; pH 5.8 with KOH, filled up to 1L; autoclave) and centrifuged again at 100 xg for 5 min. The protoplasts are diluted in CPW media and adjusted to 8 x $10^5$ $ml^{-1}$. For regeneration the protocol was followed for half of the sample. Carrot embryos were placed on solid CSR+Tim250 media for further grow. The other half was kept in CPW for 24 hrs., after checking for GFP signals in the protoplasts under a fluorescence microscope, gDNA was isolated using innuPREP Plant DNA Kit. The target region was amplified with the CR4L primer pair, cleaned up using GeneJET PCR Purification Kit (ThermoFisher, Darmstadt, Germany) according to the manufacturer protocol, normalized to 20 ng/µL and Amplicon sequenced at Genewiz/Azenta (Leipzig, Germany). The raw fastq files were analyzed using the Galaxy JKI server as described in (Salvagnin et al. 2023).

### 2.3.4. Regeneration from meristem

Leaf axils from greenhouse plants were dissected and washed for 15 min in 70 % EtOH, followed by 20 min in 5 % NaOCl with a droplet of Tween 20, shacked continuously and washed with sterile $H_20$ until foam build up stops. Dead plant tissue was removed, and the meristem was placed on CB5D4P + Tim250 (250 mg/mL Timentin) to induce callus. After around 4 weeks, formed callus is plated on CSR+Tim250 for regeneration. DNA was isolated from regenerated plants and the Cr4 target was amplified using the Cr4L-primer pair, and Amplicon sequenced at Genewiz/Azenta (Leipzig, Germany).

## 2.4. Cytogenetics

Three different methods have been used to fixate and visualize carrot chromosomes. For showing mitotic metaphases, flowerpots with *D. carota* var. Yellowstone were placed on sand for a week to facilitate harvesting of fresh root tips. The preparation of root tips was done according to (Plath et al. 2022), preparation and the immunostaining against CENH3 according to (Dunemann et al. 2014) and (Dunemann et al. 2019).

# 3 Results

## 3.1. Inducing mutations inside CENH3 of carrot using CRISPR/Cas9

The aim in this study was to induce mutations inside the genomic DNA coding for CENH3 in carrot via CRISPR/Cas and compare three different transformation systems. Potential effects of the induced mutations were screened by analyzing the CENH3 phenotype.

The Crispr/Cas9 system was introduced stably into the carrot genome by *A. rhizogenes* and *A. tumefaciens* guided transformation. For non-transgenic lines, preassembled RNPs have been introduced into the cytoplasm of carrot protoplasts via PEG guided transformation.

The gRNAs were designed by screening the CENH3 sequence of *D. carota* for PAM sites within the functional and highly conserved HFD. The two gRNAs gCr3 and gCr4, both targeting the HFD, have been chosen to induce mutations within CENH3 (fig. 7).

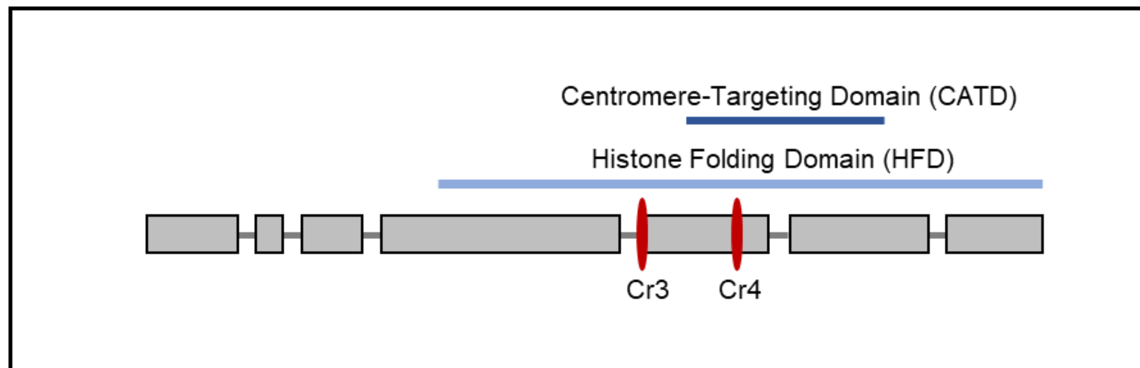


Figure 7: Representation of the genomic CENH3 sequence. The total reading frame exists of seven exons. Both target regions are placed on exon four. The regions translating for the Histone folding domain (HFD) including the highly conserved centromere-targeting domain (CATD) are shown above the exon structure.

### 3.1.1. Screening for possible off-target sides

Before introducing Crispr/Cas9 *in vivo*, the two gRNAs gCr3 and gCr4 have been tested *in silico* for their likelihood to induce unwanted off-targets.

Putative off-target events were predicted by blasting the Cr3 and Cr4 protospacer sequence against the whole transcriptome of *D. carota* via NCBI (Genome assembly ASM162521v1) short sequence search (National Center for Biotechnology Information, Maryland, USA). Here, only the match within the targeted *CENH3* transcript was found for the protospacer region of both gRNAs. To further verify this result, the online tool cas-OFFinder (rgenome.net, BAE Lab, Seol National University College of Medicine, South Korea) was used and up to three theoretical mismatches between protospacer and putative off-target side

have been allowed for. This search addressed potential binding of the gRNAs to similar, but not identical, regions to the protospacer (used reference genome: PRJNA268187). No putative off-targets sides with one or two mismatches have been found (tab. 4). However, five possible off-target sides for gCr3 and one for gCr4 have been identified with three possible mismatches between protospacer and genomic DNA.

Table 4: Estimated off-target sides of the gRNAs gCR3 and gCR4 using online tool cas-OFFinder. The search included sides that have up to three mismatches to the respective protospacer.

| gRNA | Number of allowed mismatches | Number of putative off-target sites |
|---|---|---|
| gCr3 | 0-2 | 0 |
| | 3 | 5 |
| gCr4 | 0-2 | 0 |
| | 3 | 1 |

### 3.1.2. Both gRNAs function *in vitro*

The functionality of the gRNAs was tested via an *in vitro* cleavage assay of gDNA fragments. Here a 1344 bp long fragment of *CENH3* covering both target regions was incubated with complexes of preassembled gRNA and spCas9 protein (RNP::gCr3 and RNP::gCr4 respectively). The assay of both RNP complexes lead to the expected products after an incubation period of 4 hrs. (fig 8). Cleavage by RNP::gCr3 resulted in 457 bp and 887 bp long fragments, while cleavage by RNP::gCr4 resulted in 476 bp and 868 bp long fragments. Both gRNAs functioned properly *in vitro* and could be used for transformation of *D. carota.*

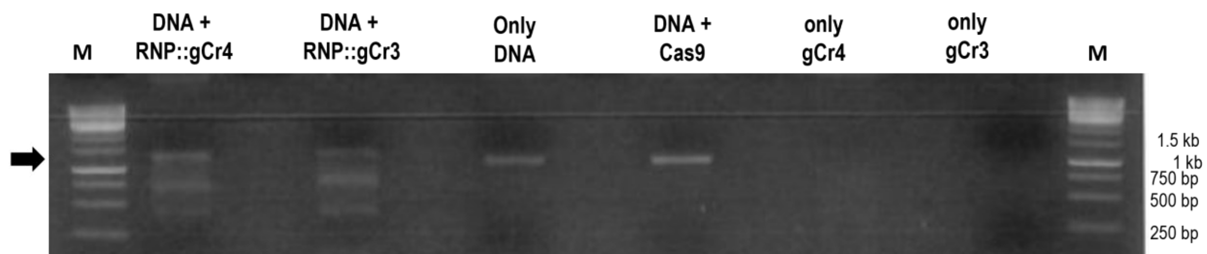


Figure 8: Cleavage assay to test the functionality of the two gRNAs gCr3 and gCr4. A 1344 bp long amplicon of *CENH3* was incubated with the RNP complexes RNP::gCr4 or RNP::gCr3 for four hrs. Used controls: Only *CENH3* amplicon and buffer, *CENH3* amplicon together with spCas9 and buffer, only gCr3 or gCr4 and buffer. The arrow indicates the expected band size of the uncleaved *CENH3* amplicon at 1344 bp. Marker is the GeneRuler 1kb Thermo, 1.5% Agarose Gel

## 3.2. Transformation of carrot via *Agrobacterium tumefaciens* leads to transgenic plants with mutated *CENH3* in the Cr3 target side

*A. tumefaciens* was used for stable transformation of *D. carota*. The expression cassette for gRNA and spCas9 was introduced into the plant genome by inoculating callus cultures.

### 3.2.1. Testing different plant tissues for their regeneration capability

The successful production of genetically modified plants depends not only on the transformation methods or the induced modification but highly on the ability to regenerate transformants also. Therefore, the regeneration capacity of callus cultures obtained from the base of shoots (just above the hypocotyl), petioles and cotyledons of young *D. carota* plantlets (varieties Nantaise 2 and Rotin) have been tested for their ability to regenerate prior to transformation.

The plant tissue of two weeks old plantlets was placed on callus inducing media for three months. After one month on regeneration media, the callus samples (1.5 to 2 cm in diameter) have been divided according to their number of developing embryos and categorized into three groups (no embryos; less than five embryos; or more than six embryos) (fig. 9).

Figure 9: Samples of callus cultures from *D. carota* with embryos at different developing stages. The first developing group of callus samples shows no embryo development, the second group exists of samples with less than five developing embryos and callus samples showing at least six developing embryos are within the third group.

In total, 109 callus samples of Nantaise 2 and 156 callus samples of Rotin have been categorized. Only one petri dish of callus obtained from shoots of Nantaise 2 survived, which resulted in a smaller sample size. Additionally, to the small sample size for this plant tissue, no callus sample showed the development of embryos in callus originating from shoots of Nantaise 2 (fig. 10). Most callus samples originating from petioles and cotyledons also showed no embryos, followed by the percentage of samples presenting only a small number of up to five embryos. Around 20 % of the samples from Nantaise 2 showed a higher degree of developing embryos, with cotyledons as starting material appearing slightly better. Overall, most callus samples originating from Rotin showed a high number of developing embryos regardless their tissue of origin. However, almost as many samples showed no embryonal development at all, being categorized as “no embryo”. Only a small percentage was categorized in the intermediate group of five or less developing embryos per callus sample.

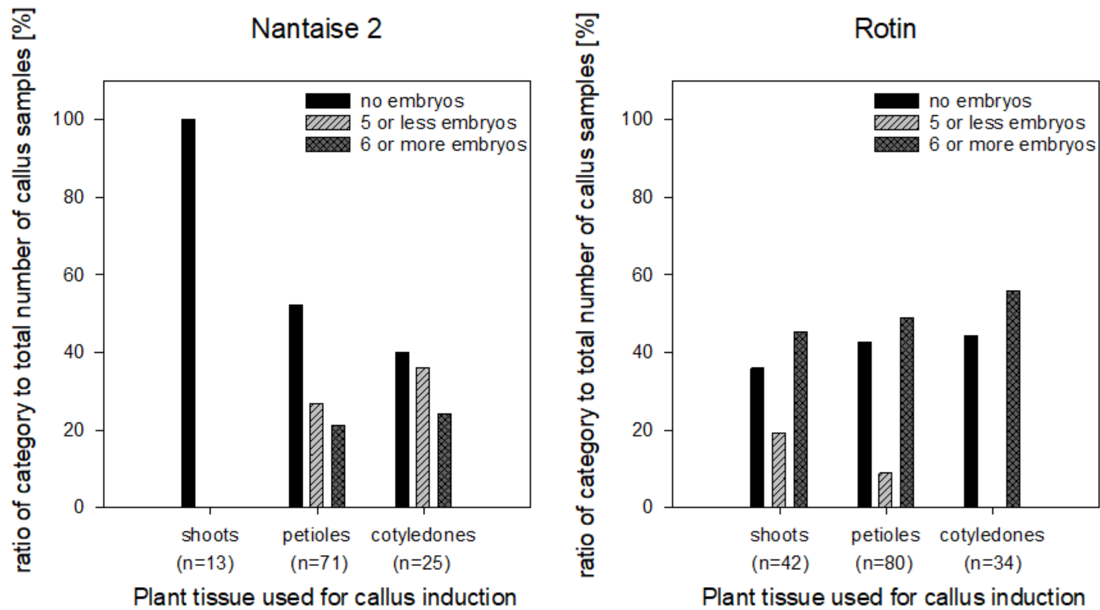


Figure 10: Percentage of callus samples produced from shoots, petioles or cotyledons of the *D. carota* cultivars Nantaise 2 and Rotin in regard of their category to show "no embryos", "five or less embryos" of "six or more embryos".

In this study the cultivar Rotin has produced more callus samples with a higher degree of presenting embryos compared to Nantaise 2. Embryos grew on regeneration media for 12 weeks and 18 plantlets have been transferred into soil to test for possible differences in their phenotype. Two of those 18 plants did not grow in soil. Both non-growing plantlets originated from the same callus sample, produced from shoots of Nantaise 2. The other 16 plants showed a normal phenotype (shown exemplary in fig. 11). Here all callus samples (except from shoots of Nantaise 2) led to regenerated plants with normal phenotype. However, cotyledons provided a bigger surface compared to shoots and petioles and can easily be cut into multiple pieces, each piece developing into callus culture. Since they also showed most callus samples within the "six or more embryo" category (Rotin), further experiments used cotyledons to produce callus for transformation experiments.

Figure 11: Examples of plants regenerated from callus of different plant tissue. Here regenerated plants of the Nantaise 2 cultivar plants regenerated from hypocotyl and cotyledons originated from the Rotin cultivar.

### 3.2.2. Mutations induced in Cr3 by inoculation of callus cultures with *A. tumefaciens*

Callus cultures from cotyledons of the Rotin and Nantaise2 cultivars were inoculated with *A. tumefaciens* carrying the expression vector pDE-Cas9::Cr3 for two days. Multicellular aggregates were visible in selective media 17 days after inoculation (fig. 8a.) and c.)). The inoculated callus from Rotin showed a higher number of embryos 44 days after inoculation than callus from Nantaise 2 (fig. 12b.) and d.)).

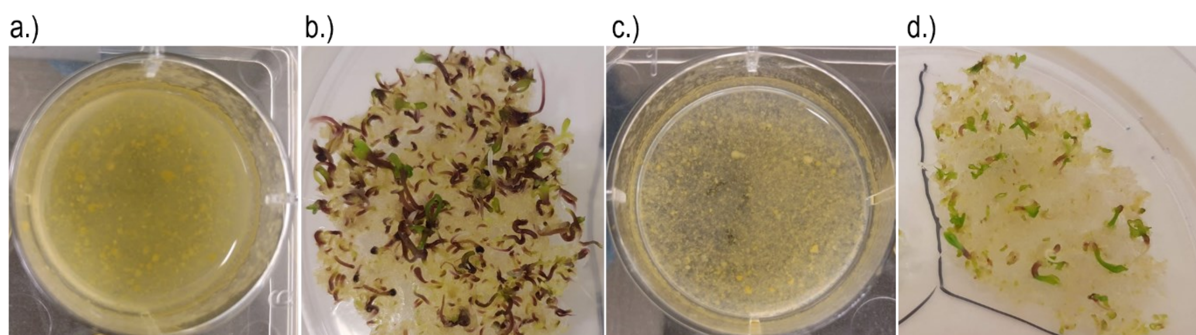


Figure 12: Regeneration of *D. carota* callus of Rotin and Nataise 2 cultivars after inoculation and co-culture with *A. tumefaciens* carrying the expression vector pDE-Cas9::Cr3. Callus from Rotin is shown in a.) and b.) and Naintaise 2 in c) and d.). In a.) and c.) callus cultures in liquid selection and regeneration media 17 days after inoculation are shown. In b.) and d.) regeneration on solid selection and regeneration media 44 days after inoculation.

At this time of the experiment the decision was made to stop working on target Cr3 and to focus on Cr4 instead. This was decided since Cr4 targets the centromere-targeting domain (CATD) which is especially crucial for the function of CENH3. Nevertheless, 17 plantlets have been tested for transgenicity via PCR.

From those 17 plants, eight plants carried transgenes. To find possible mutations, the Cr3 region of two transgenic and four non-transgenic plants was amplified via PCR and sent for Sanger sequencing. The transgenic samples showed variations in their chromatography compared to the non-transgenic wild type samples (fig. 13).

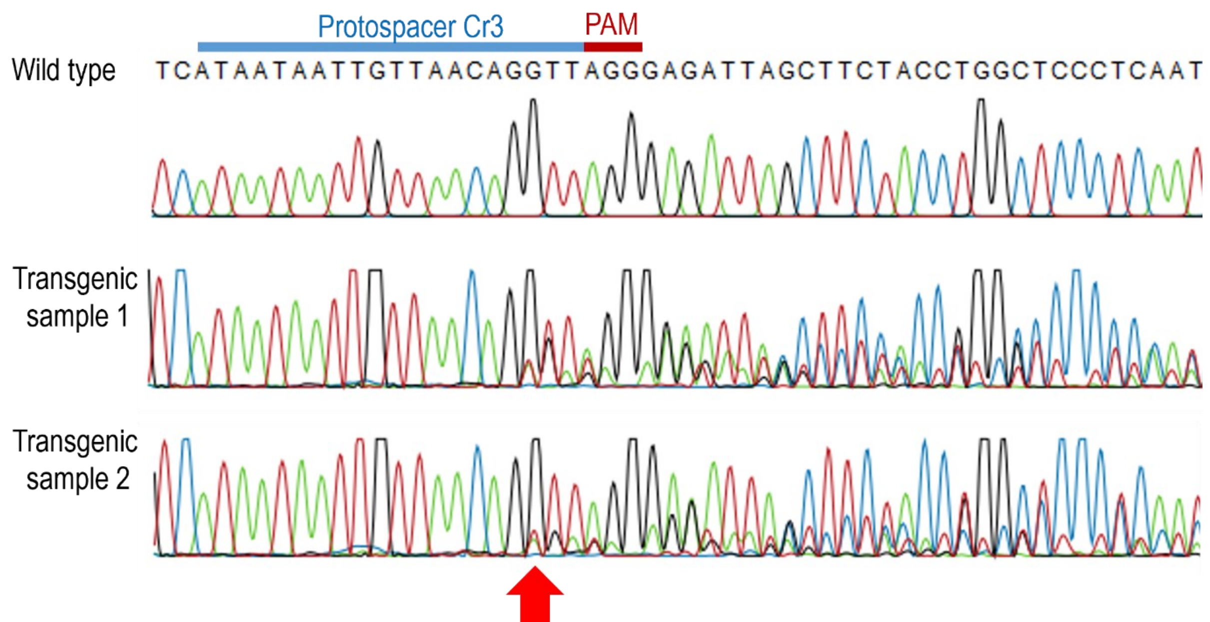


Figure 13: Chromatogram of sequenced amplicons of the Cr3 target region on transgenic and wild type *D. carota* plantlets. The signal intensity is given in relative fluorescence units and plotted over time (time correlates with the base positions), the color of the peaks shows the detected label of each nucleotide (red=thymine; blue=cysteine; green=adenine and black=guanine). The protospacer region Cr3 as well as the PAM is shown, the red arrow marks the position three bps upstream of the PAM.

The chromatogram is the result of Sanger sequencing and shows the detected peaks of signal intensity from labeled bases of the amplicons of Cr3. While some background is visible within the complete protospacer Cr3 region, most overlaying signals start to appear three base pairs (bps) upstream of the PAM. The red colored peak three bps upstream of the PAM indicates a change from only detecting the signal for guanine at this position to also detecting the signal for thymine (marked by the red arrow in fig. 13). Downstream from there the detected signals clearly overlay and indicate a frameshift and/or other mutations within the amplicons of the Cr3 region. While no definite assertion about the type of mutations within the Cr3 region can be given, the shift of signals at three bps upstream of the PAM strongly indicate that the introduction of an CRISPR/Cas9 expression cassette via *A. tumefaciens* led to mutations within the target sequence Cr3 in *CENH3* of *D. carota*.

## 3.3. Transformation of carrot lateral roots via *Agrobacterium rhizogenes* leads to transgenic plants with mutated *CENH3* in Cr4

Transformation of the *D. carota* cultivars Yellowstone and Deep Purple was done by *A. rhizogenes* to produce mutated hairy root cultures. Those cultures have been used for phenotypical characterization of CENH3 modified in the Cr4 target. The results from chapter 3.2 have partly been published (Dunemann et al. 2019).

### 3.3.1. Mutated *CENH3* in transgenic hairy root cultures

The initial transformation via *A. rhizogenes* carrying the expression vector pDe-Cas9::Cr4 and first screening of transformed hairy roots was done by the working group of Dr. Dunemann with support of Dr. Thorben Sprink. An initial screening took place to identify highly mutated hairy root lines (Dunemann et al. 2019). According to the results of the initial screening a subset of four hairy root lines was chosen for further analysis (C4/YS-11; C4/YS-21; C4/DP-12 and C4/DP-21). Here C4/YS-11 and C4/YS-21 originated from transformed Yellowstone (YS) cultivars while C4/DP-12 and C4/DP-21 originated from the Deep Purple (DP) cultivar.

A high-resolution-melt (HRM) of three technical and three biological replicants of the preselected subset of four transgenic plants lines (C4/YS-11; C4/YS-21; C4/DP-12 and C4/DP-21) and their respective wild type (YS and DP) was done (fig. 14).

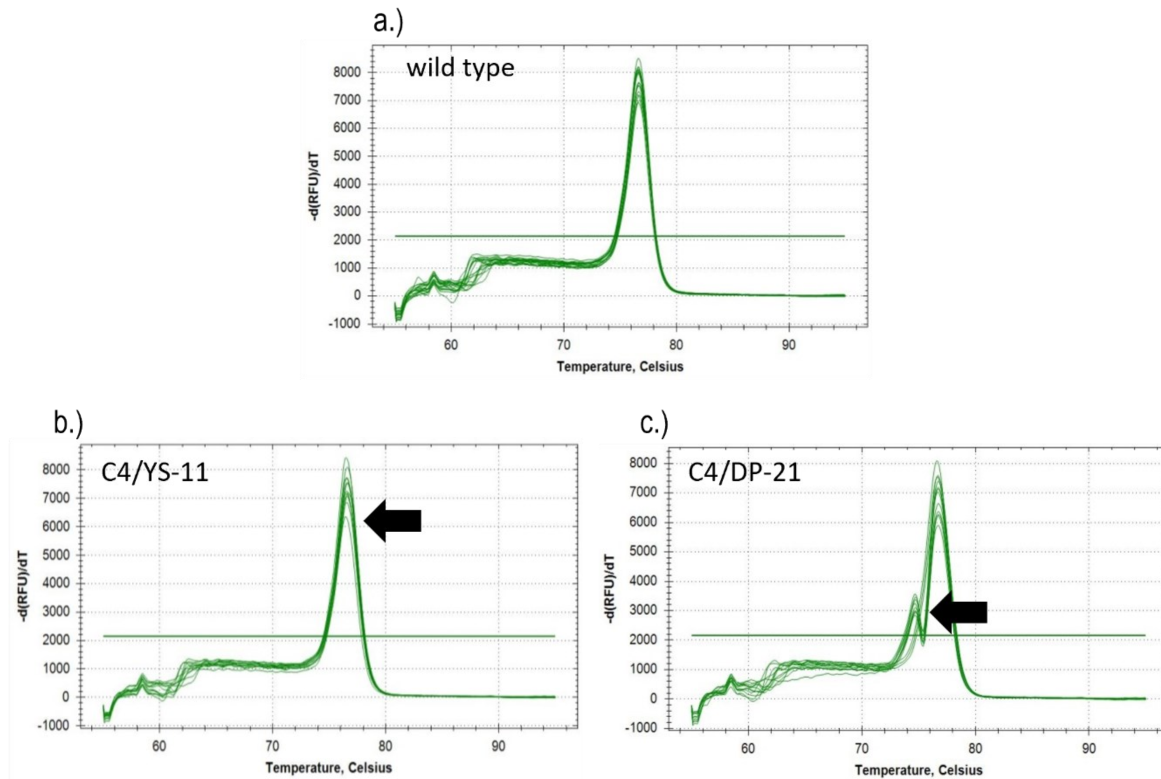


Figure 14: Melting curves of a 100 bp long amplicon surrounding Cr4. Shown is the change of relative fluorescence [RFU] with time [T] ([d(RFU)dt]) in relation to temperature [C°] for (a) the wild type controls, (b) and the transgenic lines C4/DP-21 and (c) C4/YS-11.

A shifting peak along the melting curves shows variations within the 100 bp long amplicon surrounding Cr4. In the combined wild type control of DP and YS cultivars, slight changes of fluorescence are visible along the time axes, but the melting temperatures of the individual amplicons overlayed (fig. 14a.)). Adding melting curves from the transgenic line C4/DP-12, led to a shifted peak of multiple amplicons of -2 °C (fig. 14b.)). The wild type samples plotted together with the amplicons of the transgenic line C4/YS- 11 showed one single melting curve peaking at a slightly lower temperature compared to the wild type control (fig. 14c.)). For C4/YS-21 and C4/DP-21 no difference was seen between the melting curve of the wild type control and the transgenic lines.

After the pre-screen of transgenic lines by HRM, single amplicons of the Cr4 target region have been singularized by cloning them into a T-vector system. Six to seven plasmids carrying singled out amplicons of Cr4, amplified from three biological replicas of C4/YS-11, C4/YS-21, C4/DP-12 and C4/DP-21 each, have been Sanger sequenced. The result showed a high number of different mutations within the target region (tab. 5).

Table 5: Result of Sanger sequencing vector containing single amplicons of Cr4 of the transgenic lines C4/DP-12; C4/DP-21; C4/YS-21 and C4/YS-11. All lines were tested via three biological replicas, the number of sequenced cloned of each replica is given as n. Mutations are marked red. The frequency of the different variants in the CR4 sequence is shown in percentage of n sequences. The PAM is underlined.

| Wild type | | | | | |
|---|---|---|---|---|---|
| | GTTAGGGAGATTAGCTTC-T-ACCTGG | | | GTTAGGGAGATTAGCTTCT--ACCTGG | |
| C4/DP-12 | | [%*] | C4/DP-21 | | [%*] |
| n=6 | GTTAGGGAGATTAGCTTC-T**T**ACCTGG | 50 | n=7 | GTTAGGGAGATTAGCTTCT--ACCTGG | 85.7 |
| | GTTAGGGAGATTAGCTTC-T**G**ACCTGG | 33.3 | | GTTAGGGAGATTAGCTTCT**G**-ACCTGG | 14.3 |
| | G**GCT**GGG**GAGG**TAG**G**TT**TG**T**TT**CCTGG | 16.6 | n=7 | GTTAGGGAGATTAGCTTCT--ACCTGG | 71.4 |
| n=7 | GTTAGGGAGATTAGCTT-----CCTGG | 42.9 | | GTTAGGGAGATTAGCTTCT**T**-ACCTGG | 28.6 |
| | GTTAGGGAGATTAGCTTC-T-ACCTGG | 42.9 | n=6 | GTTAGGGAGATTAGCTTCT--ACCTGG | 33.3 |
| | GTTAGGGAGATTAGCTTC-T**C**-CCTGG | 14.3 | | GTTAGGG**G**GATTAGCTTCT--ACCTGG | 16.6 |
| n=6 | GTTAGGGAGATTAGCTTC-T**T**ACCTGG | 50 | | GTTAGGGAGATTAGCTT**T**T**GG**ACCTGG | 16.6 |
| | GTTAGGGAGATTAGCTTC-T**C**-CCTGG | 16.6 | | GTTAGGGAGATTAGCTTCT**T**-ACCTGG | 16.6 |
| | GTTAGGGAGATTAGCTT-----CCTGG | 16.6 | | GTTAGGGAGATTAGCTTCT**C**-ACCTGG | 16.6 |
| | GTTAGGGAGATTAGCTTC-T**G**ACCTGG | 16.6 | | | |
| Wild type | | | | | |
| | GTTAGGGAGATTAGCTTCT-ACCTGG | | | GTTAGGGAGATTAGCTTC-TACCTGG | |
| C4/YS-11 | | [%*] | C4/YS-21 | | [%*] |
| n=7 | GTTAGGGAGATTAGCTTCT-ACCTGG | 85.7 | n=7 | GTTAGGGAGATTAGCTTC-TACCTGG | 100 |
| | GTTAGGG**G**GATTAGCTTCT-ACCTGG | 14.3 | n=6 | GTTAGGGAGATTAGCTTC-TACCTGG | 66.7 |
| n=6 | GTTAGGGAGATTAGCTTCT-ACCTGG | 50 | | GTTAGGGAGATTAGCTTC**T**TACCTGG | 33.3 |
| | GTTAGGGAGATTAGCTTCT**C**-CCTGG | 16.6 | n=6 | GTTAGGGAGATTAGCTTC-TACCTGG | 83.3 |
| | GTTAGGGAGATTAGCTT----CCTGG | 16.6 | | GTTAGGG-G-----------CCTGG | 16.6 |
| | GTTAGGGAGATTAGCTTCT**T**ACCTGG | 16.6 | | | |
| n=6 | GTTAGGGAGATTAGCTTCT-ACCTGG | 100 | | | |

* Percentage of different sequences detected in one biological replicon

Between all replicas, 19 amplicons from C4/DP-12 were sequenced in total. Of those 19 sequences, three (15.8 %) were wild type. In nine sequences, one base was inserted (thymine (T) or guanine (G)) three base pairs upstream of the PAM, which shifted the stop codon TGA 25 bp downstream of the PAM into the reading frame. In two sequences, adenine (A) was substituted with cytosine (C). This replacement leads the amino acid change of tyrosine, with a hydrophobic side chain, to serine, with an uncharged side chain. In three cases, the triplet translating for tyrosine was deleted. In one sequence, twelve substitutions over an area of 21 bps upstream of the PAM were detected. Overall, the 19 sequenced amplicons of Cr4 from the three biological replicas of C4/DP-12 showed six different variations. For C4/DP-21, 20 amplicons were sequenced in total and 65 % were wild type. In one sequence, adenine was substituted with guanine 15 bp upstream of the PAM, changing glutamic acid with a negatively charged side chain to glycine, which has a single hydrogen atom as its side chain instead of the typical carbon side chain. In five sequences the insertion of a single base pair moves the stop codon TGA within the reading frame. In one sequence, three bps are inserted (TGG). They are outside the reading frame, resulting in changing tyrosine, which has hydrophobic traits, with the also hydrophobic leucine and aspartic acid, which has a negatively charged side chain. The variety in the Cr4 region within the three replicas of C4/YS-11 was screened by sequencing 19 amplicons, of which 15 (79 %) were wild type. Adenine was substituted with guanine in one sequence, changing glutamic acid to glycine 15 bp upstream of the PAM. Cytosine substituted adenine three bp upstream of the PAM, changing tyrosine to serine. In one sequence, the triplet coding for tyrosine was deleted. From the 19 clones sequenced for C4/YS-21, 89.5 % were wild type. Only two amplicons showed mutations. Here, thymine was inserted four bp upstream of the PAM once and twelve bp have been deleted in the other, starting two bp upstream of the PAM, interrupted by a single guanine. The relation of counted events to the total number of sequences (fig. 15), shows that deletions occurred over a broad area while insertions (mainly thymine) were most common three bps upstream to the PAM.

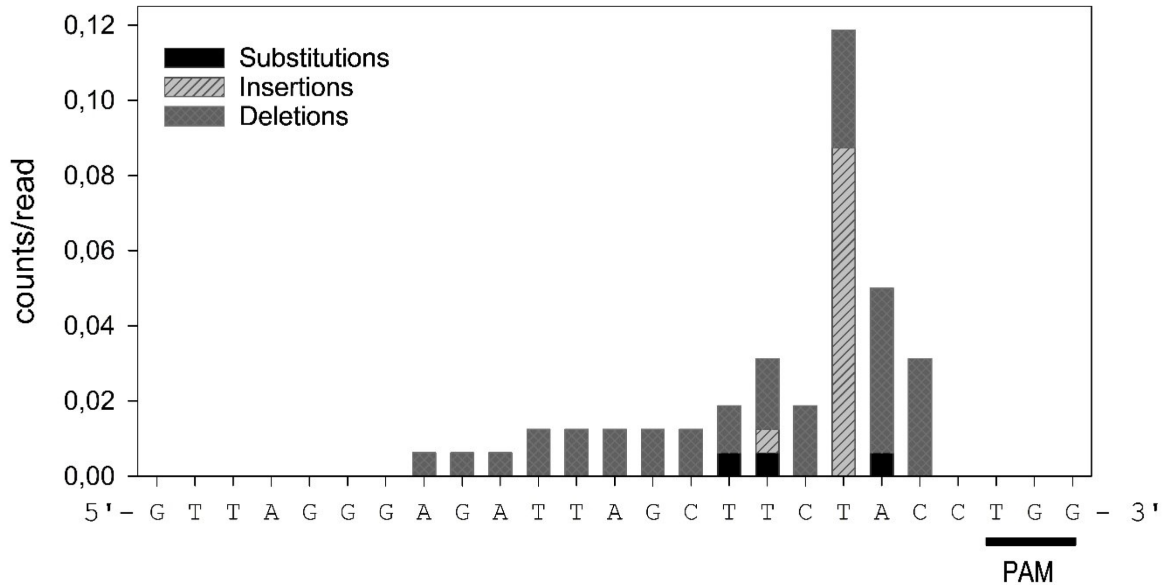


Figure 15: Relation of counted events to the total number of sequences as counts/reads showing single events along Cr4 of 77 single amplicons from four different transgenic lines.

The transformation of the two *D. carota* cultivars Yellowstone and Deep Purple with *A. rhizogenes* carrying the expression vector pDE-Cas9:Cr4 led to hairy root lines with a highly mutated Cr4 region.

## 3.3.2. Accumulation and qualification of CENH3 by immunostaining

Specific immunostaining against CENH3 in mutated root tip cells was done to find putative changes in the accumulation of CENH3. Polyclonal antibodies corresponding to the N-terminus of CENH3 and a Cy3-conjugated anti-rabbit IgG as the secondary antibody was used for staining. The carrot has 18 chromosomes (2n=2x) (fig. 16a.)). Staining CENH3 in condensed chromosomes during metaphase showed the location of CENH3 within the centromeric region of DAPI-stained chromosomes (fig. 16b.)). Staining interphase nuclei of mutated lines was used to quantify the difference in the signal strength between wild type root tips and some of transgenic lines (fig. 16c.)d.)).

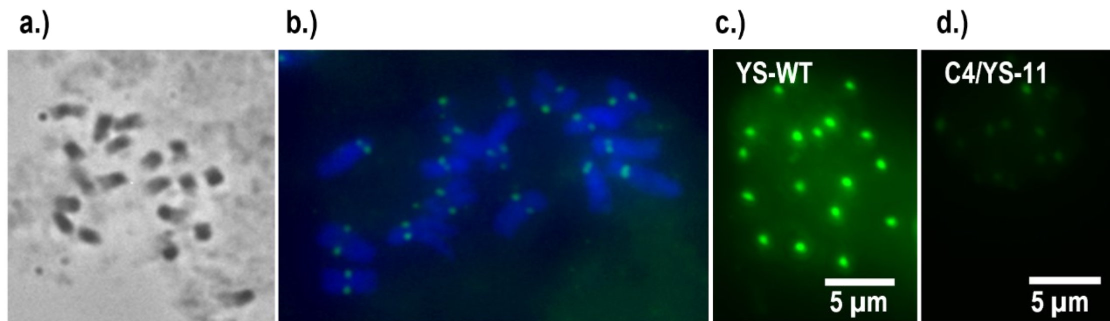


Figure 16: Unstained and stained carrot chromosomes (2n=2x=18). a.) unstained metaphase chromosomes, b.) metaphase chromosomes stained by DAPI and carrot specific antibodies against CENH3 and stained interphase nuclei of c.) wild type and d.) mutated root tips of the C4/YS-11 hairy root culture.

The saturation of the green fluorescence signals from stained CENH3 was measured as mean grey value of single pictures taken with fixed parameters and calculated via the open-source program ImageJ to identify differences in signal strength of stained CENH3. The different mean grey values from 50 nuclei of the root tips of four transgenic and mutated lines have been compared to 50 nuclei of their respective wild type (fig. 17).

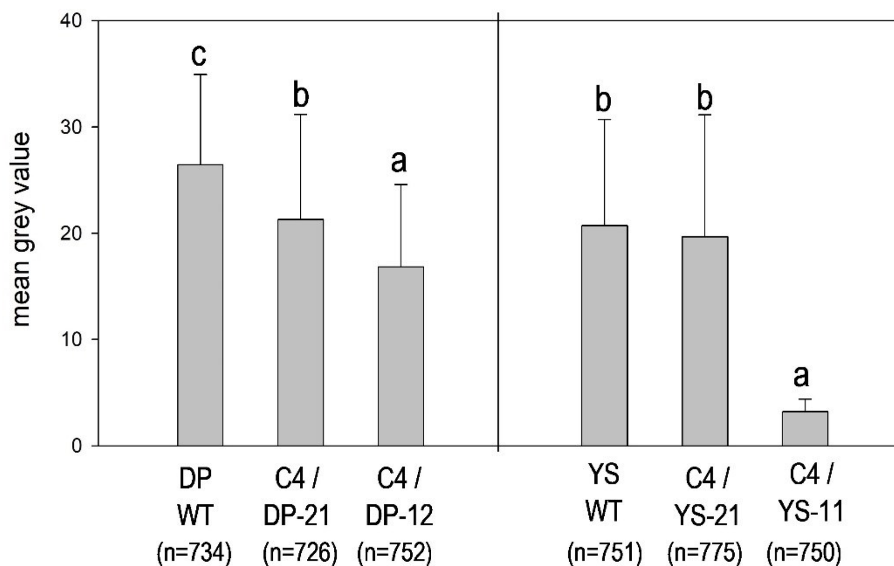


Figure 17: Mean grey value showing the saturation of the green fluorescence signals from stained CENH3 in the two cultivars Deep Purple and Yellowstone. The data is shown with the standard deviation and homogenic subgroups in their respective cultivar showing significant difference within their mean grey value and consequently their signal strength. n shows the total number of measured signals.

The saturation of stained wild type nuclei of DP showed significant differences to both transgenic and mutated nuclei (tab. 6). The dispersion of measured mean grey values are shown by the standard deviation. The saturation of stained CENH3 in roots tips of C4/DP-21 was 30.2 % and the one of C4/DP-12 was 39.9 % lower than of the wild type sample. While the saturation in wild type root tips of the

Yellowstone cultivar do not differ significantly from C4/YS-21, the signals from stained CENH3 in C4/YS-11 was noticeable low with 80.2 % lower mean grey value than the wild type control (see also fig. 16c.) and d.)). Here also the dispersion of measured mean grey value was comparably low shown as seen by the smallest standard deviation.

Table 6: Results of the Dunn-Test comparing the mean grey values of the wild type and each mutated line pair wise. The top number shows the z test statistic, the lower number shows the p-value. A p-value ≤ alpha /2 shows a significant difference between the pair. (a) The data taken from YS-lines have an overall chi-square of 1406.62, df = 2, p-value = 0 in the rank sum test. (b) The mean grey value from the DP cultivars had to be squared for normal distributions of the data. The rank sum test showed a chi-square of 451.33, df = 2, and a p-value of 0.

(a)

| | DP wild type | C4/DP-12 |
|---|---|---|
| C4/DP-12 | 20.11 | |
| | 0.0* | |
| C4/DP-21 | 16.02 | -4.26 |
| | 0.0* | 0.0* |

alpha = 0.05
Reject H0 if p ≤ alpha/2

(b)

| | C4/YS-11 | C4/YS-21 |
|---|---|---|
| C4/YS-21 | -32.02 | |
| | 0.0* | |
| YS wild type | -33.01 | -1.25 |
| | 0.0* | 0.32 |

alpha = 0.05
Reject H0 if p ≤ alpha/2

The comparison of the signal strength showed a measurable effect on the mutated lines (except C4/YS-21). Callus cultures of the analyzed hairy root cultures have been regenerated into plants. However, C4/YS -11 plantlets not survived. Sanger sequencing of two plants that originated from C4/YS-21 showed an overlay of sequencing indicating a genetic mosaic within the Cr4 target region similar to the sequencing results of the hairy root lines.

## 3.4. Fixation of mutations and dispending of chimeric genotypes

While some effect in the accumulation of CENH3 in the centromeric region of transgenic carrots could be seen, the apparent on-going activity of Cas9 resulted in plants with various gen variants of the target region within *CENH3* including secondary mutations. Linking the effect on the accumulation of CENH3 to certain genotypes would be crucial to identify promising breeding lines that could later be used as putative haploid inducer lines due to their compromised CENH3.

### 3.4.1. Fixation via the germline

Self-crossing of mutated lines of the Yellowstone cultivar is done to use the germline to dispend the different gene variants. Since Deep Purple cultivars have shown to be male sterile and no plants could be regenerated from the C4/YS-11 hairy root line, two plants originating from C4/YS-21 could be used for self-crossing (C4/YS-21-1 and C4/YS-21-4). To have some more plant material C4/23-4, which has been

regenerated from hairy root line C4/YS-23 was included. The hairy root line C4/YS-23 was obtained from the same preliminary transgenic root as C4/YS-21.

The self-crossing of C4/YS-21-1 resulted to one seed which was sown, C4/YS-21-4 resulted in 56 seeds and C4/YS-23-4 to four seeds. Amplicon sequencing of ten progenies resulting from the selfing of C4/YS-21-4 was performed and gave insight into the ratio of mutation events within the Cr4 target side of the first selfing generation ($S_1$) (fig. 18).

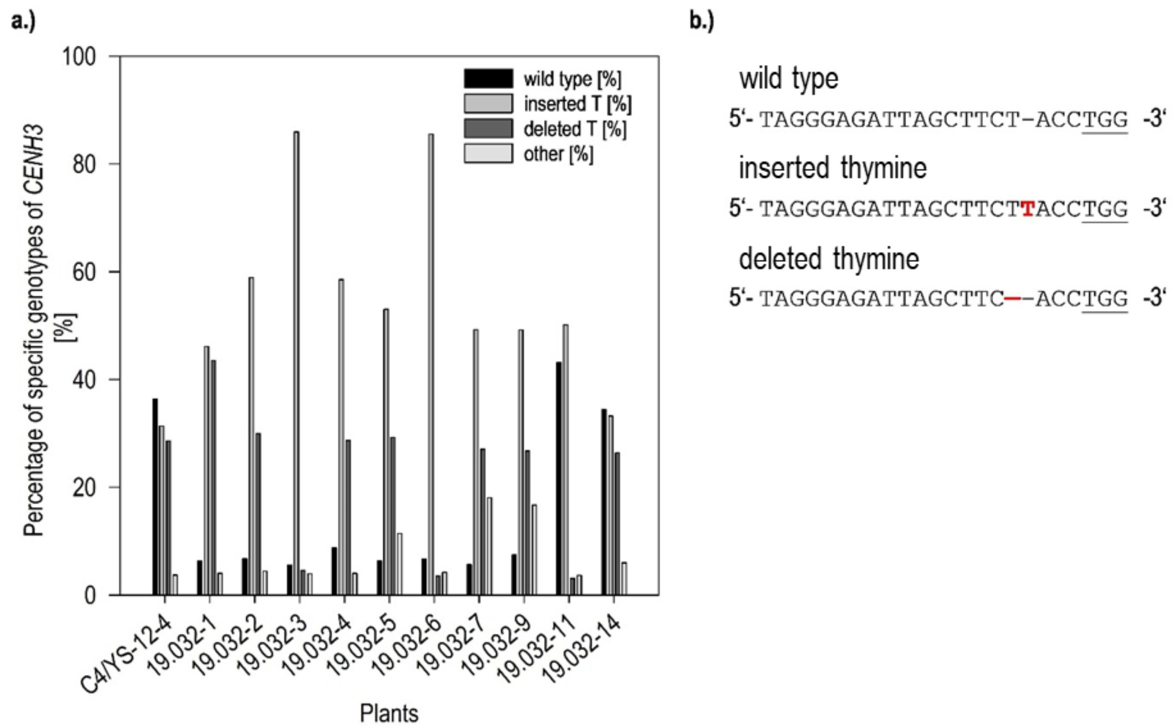


Figure 18: Mutations in the Cr4 target site within the first selfing generation ($S_1$) of transgenic and mutated plant. a.) Percentage of different variants within the Cr4 target side seen in amplicon sequencing; b.) main genotypes of the Cr4 target sites

In the first selfing generation of transgenic carrot plants, the most common mutation was the insertion or deletion of thymidine within Cr4. However, in all plants some wildtype *CENH3* remained as well as other gene variants. Since the gametes carry only one gene variant of *CENH3* it was expected that passing the germline would minimize the genomic mosaic of different *CENH3* variants. Since the possibility occurred that compromised CENH3 expressed from certain *CENH3* variants could be lost during meiosis due to intercellular competition between wild type and compromised CENH3, meristem culture was used to regenerate plantlets from single mutated cells to dissolve the genetic mosaic structure of Cr4 variants.

### 3.4.2. Via regeneration from meristem tissue

Since the germline did not dissolve the genomic mosaic in mutated carrot lines, meristem tissue of leaf axis taken from plants growing in the greenhouse was regenerated over a callus-phase to dissolve the chimeric genotypes (fig. 19).

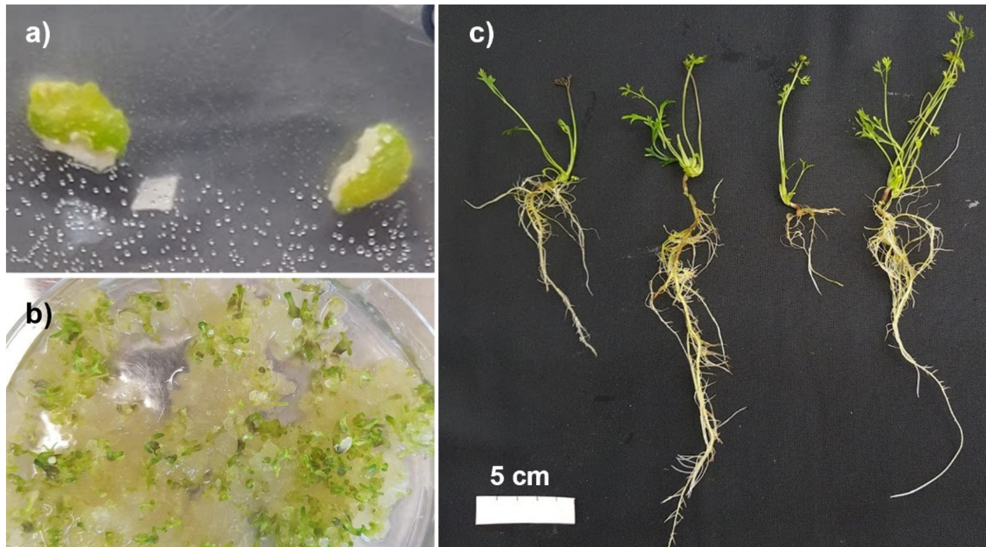


Figure 19: Plants regenerated from meristem tissue via callus culture. a) meristem prepared from the leaf axis of greenhouse carrot plants starts to form callus b) embryos and young plantlets growing from the callus culture after 47 days on regeneration media and c) regenerated plants.

All leaves of C4/YS-21 have been cut for rejuvenation, preventing the preparation of meristem tissue of the leaf axis from this explant. Greenhouse plants originating from the mutagenic hairy root lines C4/YS-23 (plant C4/YS-23-13) and C4/YS-22 (C4/YS-22-2) have been used. The meristem samples could be sterilized and converted into *in vitro* culture successfully. The meristem developed callus on 2,4-D containing media. After 47 days on regeneration medium, single embryos have been separated. Some plantlets have been transferred into soil and all showed a normal phenotype.

The Cr4 target side was amplified from two plantlets of each origin via PCR and Sanger sequenced to see if the regeneration from meristem tissue broke up the genomic mosaic structure of mutated *CENH3* (fig. 20).

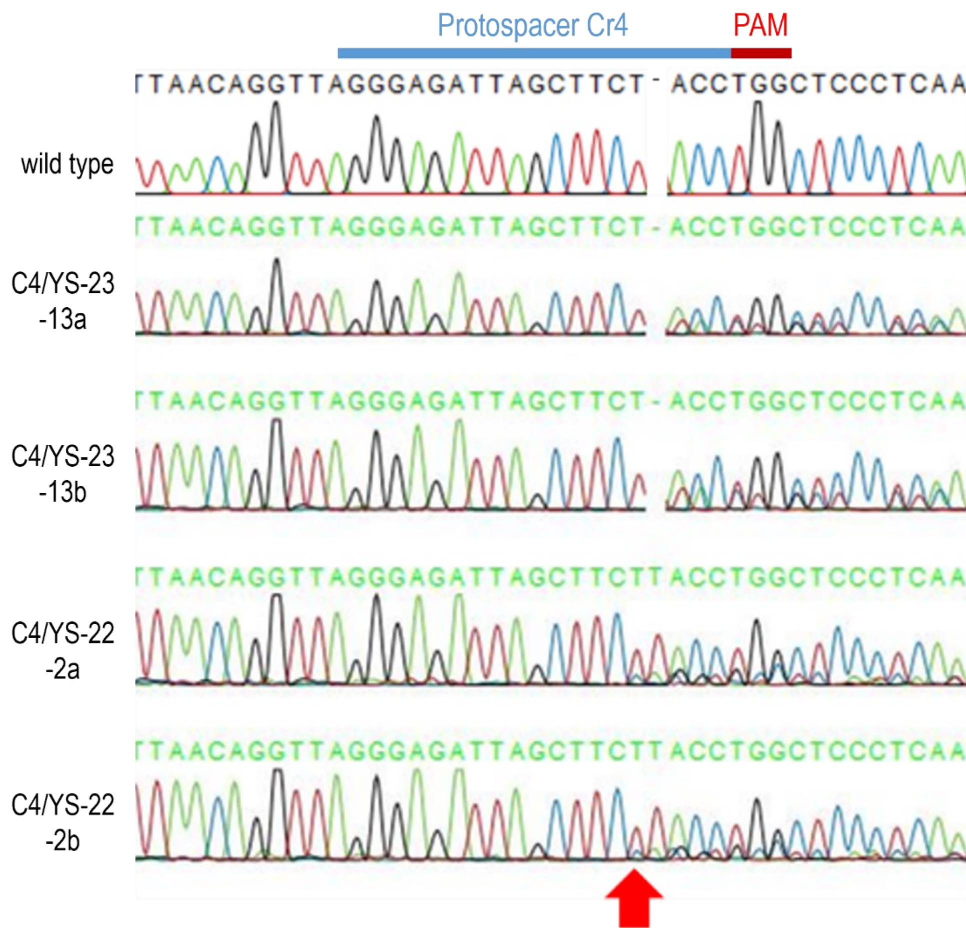


Figure 20: Chromatogram of sequenced amplicons of the Cr4 target region from carrot plants regenerated from meristem of mutated plants compared to wild type. The signal intensity is given in relative fluorescence units and plotted over time (time correlates with the base positions), The color of the peaks shows the detected label of each nucleotide (red=thymidine; blue=cysteine; green=adenine and black=guanine). The protospacer region Cr3 as well as the PAM is marked, the red arrow marks the position three bps upstream of the PAM.

The chromatogram is the result of Sanger sequencing and shows the detected peaks of signal intensity from labeled bases of the amplicons of Cr4. The chromatograms of the two plants regenerated of the meristem culture of C4/YS-23-13 differ from the plants regenerated from C4/YS-22. Both plants from the meristem of C4/YS-23-13 start showing an overlay of signals starting three bps upstream to the PAM, however the signal intensity for the wild type signal is stronger. The two plants regenerated from meristem of C4/YS-22 have a stronger signal for an inserted thymidine at three bp upstream to the PAM than the signal for wild type. That indicates that the mixture of Cr4 amplicons have more amplicons with an inserted thymidine than the wild type variant. Since the information obtained from Sanger sequencing amplicons of the target region is limited and lacks insight, the next-generation sequencing (NGS) technique of amplicon sequencing was used to quantify the different *CENH3* variants within C4/YS-23-13a and C4/YS-22-2b.

Amplicon sequencing provides single reads for each amplicon in one PCR sample to identify insertions, deletions (INDEL) and substitutions in the Cr4 region. All raw data was quality trimmed to a quality score of 30 and mapped against the wild type variant via BWA-MEM (Burrows-Wheeler Aligner) aligner via the Galaxy server at JKI (Julius Kühn – Institute, Quedlinburg, Germany). For C4/YS-23-13a 46215 utilizable sequences could be mapped against the wild type sequence of Cr4 (tab. 7). Here 40794 (88.3 %) reads showed the wild type sequence, the most common INDEL was the insertion of thymine three bps of the PAM (TGG) with 5324 reads showing that variant, which is corresponding to the signal shift at the same position seen in the chromatogram of the Sanger sequencing. Additionally, some reads with inserted thymine showed additional substations along the Cr4 region. Deletion of adenine three bps of the PAM was the second most common INDEL (124 reads), followed by the occurrence of deletion of adenine as seen in 124 reads. All single bp insertions lead to a frame shift of the coding region. In three reads, five bp were missing 12 bp upstream of the PAM.

Table 7: Amplicon sequencing of Cr4 of plants regenerated from meristem culture of transgenic C4/YS-23-13. n gives the number of reads for each event. The PAM is underlined.

| C4/YS-23-13a | | n |
|---|---|---|
| Utilizable sequences | | 46215 |
| Wild type | TAGGGAGATTAGCTTCT-ACCTGG | 40794 |
| Insertion T three bp upstream of PAM | | |
| | TAGGGAGATTAGCTTCT**T**ACCTGG | 5324 |
| | TAGGG**T**GATTAGCTTCT**T**ACCTGG | 2 |
| | TAGGGAG**T**TTAGCTTCT**T**ACCTGG | 2 |
| | TAGGGAGATTAGCTTCT**T**ACCTG**T** | 2 |
| Deletions | | |
| | TAGGGAGATTAGCTTCT-**-**CCTGG | 124 |
| | TAGG**-----**TAGCTTCT-ACCTGG | 3 |
| Substitutions | | |
| | **nnnnnnnn**T**nnnnnnn**T-**nnnnnn** | 169 |

Amplicon sequencing of the Cr4 region from C4/YS-22-2a resulted in 57148 sequences that were mapped against the wild type sequence (tab. 8). Here the mutation rate is 95 % with more reads with a thymine insertion three bp upstream of the PAM (51050) than wild type reads (2865). This result corresponds to the result from the Sanger sequencing where the peak of the signal for thymidine was higher than the signal for the wild type. In 392 reads additional substitutions could be seen additionally to the insertion of thymidine three bp upstream of the PAM. In 2796 reads, a deletion of the triplet coding for tyrosine was deleted one bp upstream of the PAM. In 12 reads with the deletion additional substitution took place.

Table 8: Amplicon sequencing of Cr4 of plants regenerated from meristem culture of transgenic C4/YS-22-2. n gives the number of read for each event. The PAM is underlined.

| C4/YS-22-2a | | n |
|---|---|---|
| Utilizable sequences | | 57148 |
| Wild type | | |
| | TAGGGAGATTAGCTTC-TACCTGG | 2865 |
| Deletion two bp upstream of PAM | | |
| | TAGGGAGATTAGCTT----CCTGG | 2784 |
| | TnnnnAGnTTAGCTT----CCTnG | 12 |
| Insertion T | | |
| | TAGGGAGATTAGCTTCTTACCTGG | 51050 |
| | nnnnnnnnnnnnnnnnTTnnnnnn | 392 |
| Other | | |
| | TAGGGAGATTAG---C-TACCTGG | 2 |

The Amplicon sequencing gave a deep insight into the different mutations within the Cr4 target side of the plants regenerated from meristem cultures of transgenic and mutated plants. Both plants remained genetic mosaics within Cr4. While most reads of C4/YS -22-2 showed the insertions of thymidine, the number of reads with the deletion of the triplet coding for tyrosine was almost identical to wild type reads.

The range and quantity of single nucleotide polymorphisms (SNPs) within the 392 reads of the Cr4 amplicon of C4/YS-22-2 that showed additional mutations to the insertion of thymidine three bps upstream of the PAM (fig. 22).

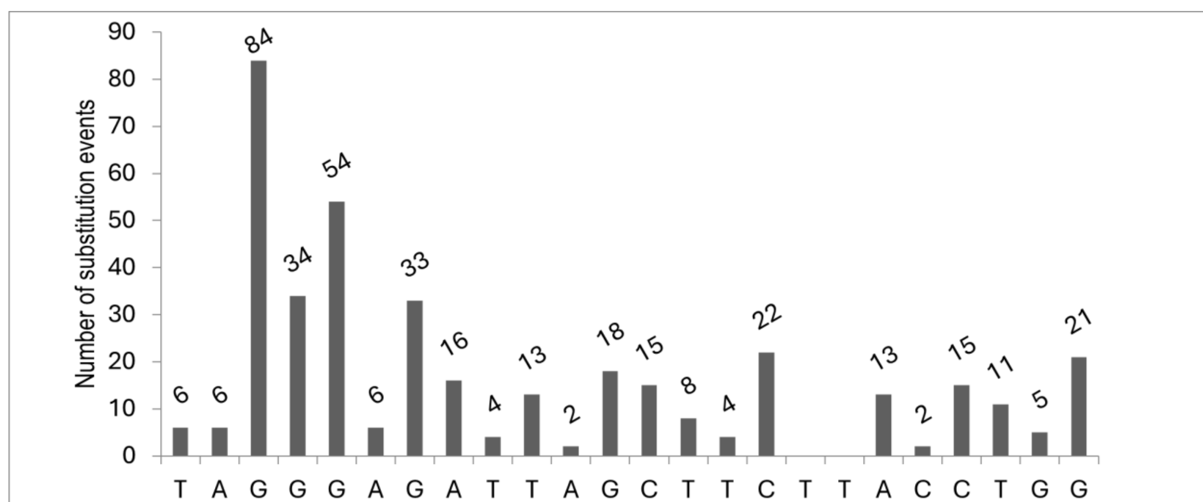


Figure 21 Frequency of substitution events within the Cr4 target of mutated plants. The amplicon sequencing of plants regenerated from transgenic C4/YS-22 showed 392 reads with inserted thymidine three bps upstream to the PAM with different substitutions along the target region, shown as number of substitution events for each base.

Substitutions of single bp range over the complete target region and the PAM. The only position without any substitutions is downstream next to the inserted thymine. To find if some nucleotides have been substituted more often than others, the number of reads in which certain nucleotides have been substituted and what nucleotide replaced them is shown (fig. 22a.)).

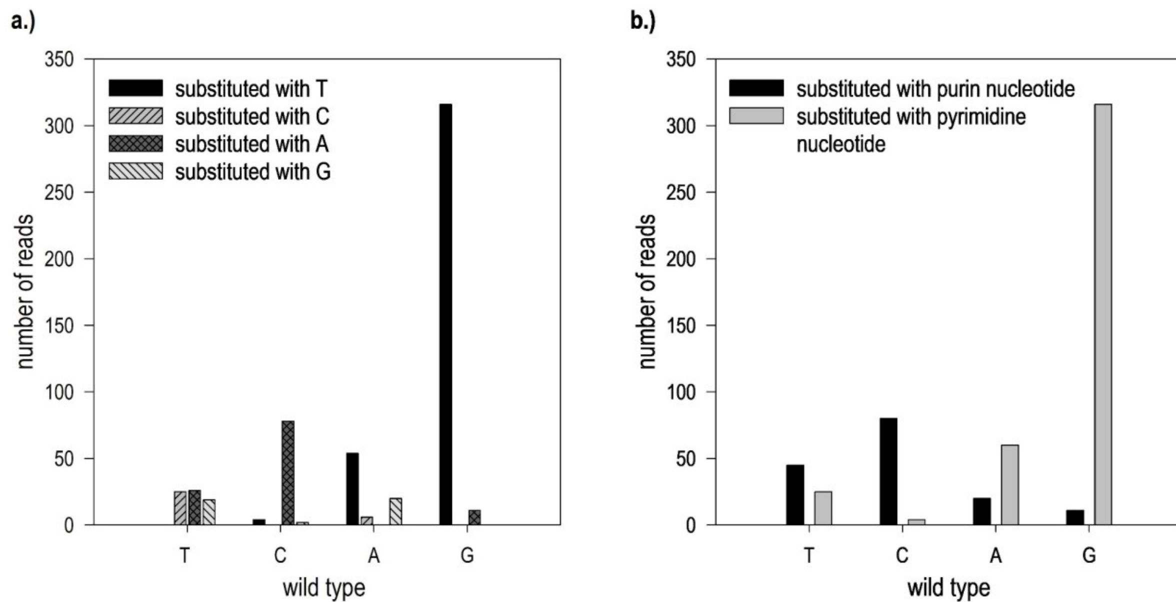


Figure 22: Substitutions according to nucleotide and type of nucleobase. a.) what nucleotides substituted different wild type nucleotides in a total of 392 reads and in b.) substitutions of different nucleotides with or purin or pyrimidine nucleotide.

In total guanine was substituted most often (89 %) and replaced with thymine in 240 reads and with adenine in nine reads. Adenine was substituted in 43 reads, in 30 reads by thymine, six in reads by cytosine and in seven reads by guanine. Cytosine was substituted in 54 reads, mainly by adenine, in four reads by thymine and in two reads by guanine. Thymine was substituted equally by cytosine (15 reads), adenine (16 reads) and guanine (15 reads). When distinguishing between the chemical properties of the four nucleotides, the nucleotides of the pyrimidine group have been substituted by nucleotides with purine bases in 83.3 % of substitutions (fig. 22b.)). In 93.6 %, the nucleotides with purine bases were substituted by thymine.

The genetic mosaic of transgenic carrot lines could not be dissolved via the germline nor regeneration of meristem culture. The stable transformation leads to constant expression of CRISPR/Cas9 which cuts until the target region is mutated and is prone to cut even with some mismatches between gRNA and target region. With multiple gene variants of the Cr4 region in *CENH3* occurring mutations cannot be linked to changes in the accumulation of CENH3 in the centromere. Also, what kind of *CENH3* variant will be expressed in the gametes is unpredictable in those plants, making them unfit candidates to find a reliable haploid inducer line. Therefore, transgene free plants have been produced by infiltrating carrot

protoplasts with preassembled ribonucleoproteins (RNPs) built by the gRNA for Cr4 and the spCas9 nuclease that have been tagged with green fluorescent protein (GFP).

## 3.5. Mutations in carrot protoplasts by infiltration with RNPs

The CRISPR/Cas9 complex was no longer stably integrated into the plant genome but reassembled *in vitro* and introduced as RNPs into carrot protoplasts via polyethylene glycol (PEG) mediated transformation. This method circumferences the need to segregate the CRISPR/Cas9 expression cassette of transgenic plants for mutated *D. carota* plants without ongoing CRISPR/Cas9 activity. Additionally previous results showed the importance of using the right carrot cultivar, transforming the male sterile Deep Purple via *A. rhizogenes* lead to problems in self-crossing experiments. Addressing the breeding goal of creating an haploidy introducing parental line which genome is meant to be eliminated during the first cell division of fused gametes the *D. carota* cultivar Blanche was chosen for protoplast infiltration. The white tap root color of the Blanche cultivar is a dominate trait. Therefore, crossing mutated Blanche cultivars with compromised centromeres with wild type carrots with a colored tap root could make the color of the tap root of the progeny an indication of successful uniparental genome elimination of mutated Blanche. Prior to infiltration of the protoplasts, a protocol for producing viable protoplasts from Blanche was tested.

### 3.5.1. Establishing isolation and regeneration of carrot protoplasts

First callus cultures have been produced by placing pieces of cotyledons of young Blanche plantlets on callus inducing media containing 2,4-D. Then $1.05 \times 10^7$ viable protoplasts have been isolated from one gram of callus culture and regenerated (fig. 23). Viable protoplasts were collected and appeared round and pale (fig. 23a.)). After three days, first cell divisions were visible. After 22 days, first microcalli formed. After eight to nine weeks, embryos started to grow from callus (fig. 23b.)). After around 12 weeks plantlets developed (fig. 23c.)). The plantlets have been transferred into the greenhouse and showed a normal phenotype (fig.23d.)). The development of fertile flowers could not be determined due to time restriction.

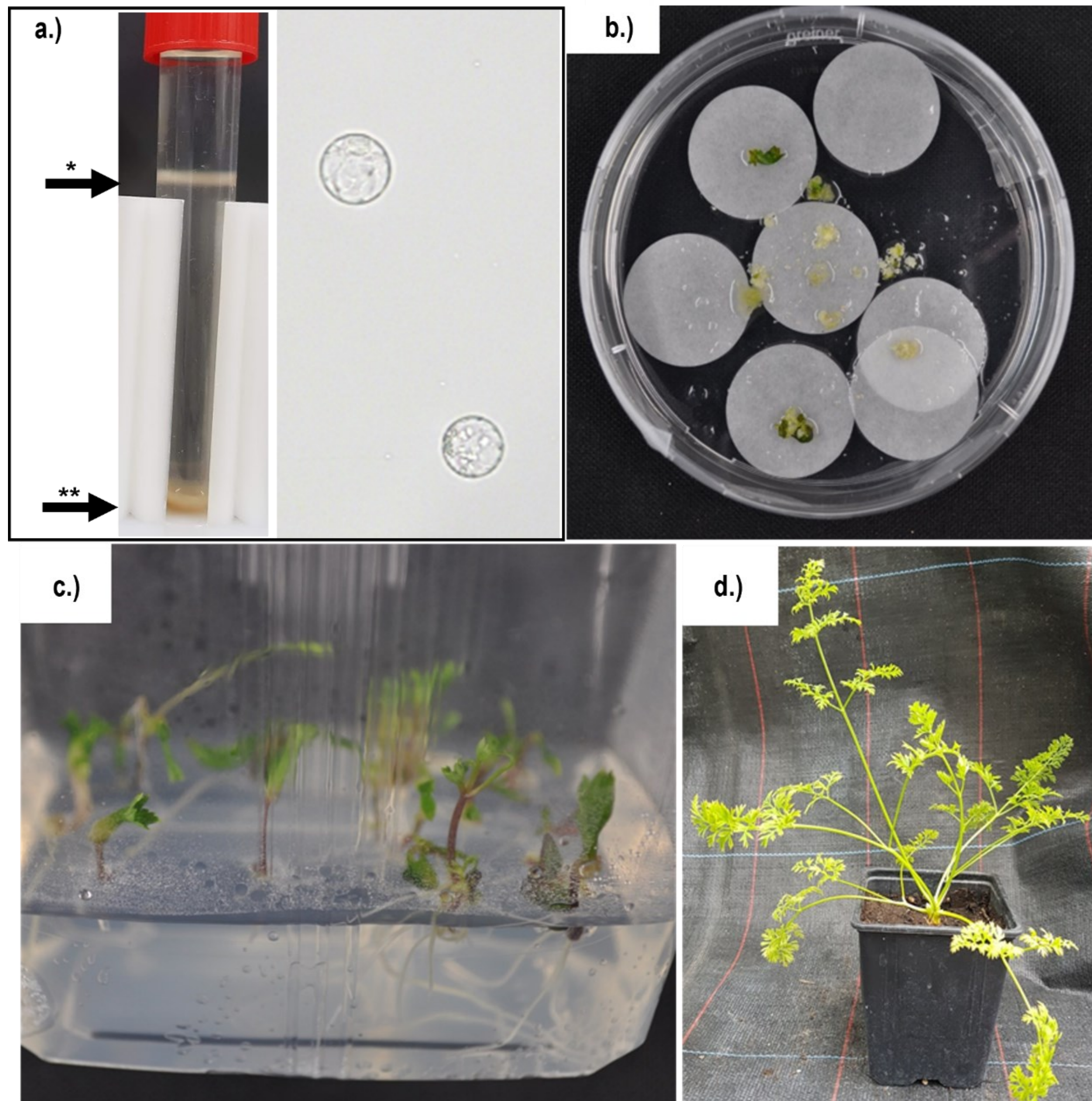


Figure 23: Isolation and regeneration of protoplasts from the Blanche cultivar. a.) Viable protoplasts (*) were separated from dead protoplasts and other plant tissue (**), b.), embryos start to develop, c.) plantlets *in vitro*, c.) plants transferred into greenhouse.

The protocol for isolating and regenerating viable carrot protoplasts has been established successfully. Now carrot protoplasts will be infiltrated with the RNP complex targeting Cr4.

### 3.5.2. Mutations in carrot protoplasts by infiltration with RNPs

The infiltration of protoplasts with the tagged RNPs have been documented 2 and 20 hrs. after infiltration (fig. 24 and 25). After 2 hrs. protoplast that have been infiltrated with the RNPs complex and the control that has only be infiltrated with spCas9::GFP fusion protein emitted the green fluorescence of GFP (fig. 24a.)c.)). The controls with only gRNA or with neither spCas9::GFP nor gRNA showed only self-fluorescence of protoplasts but no GFP signal.

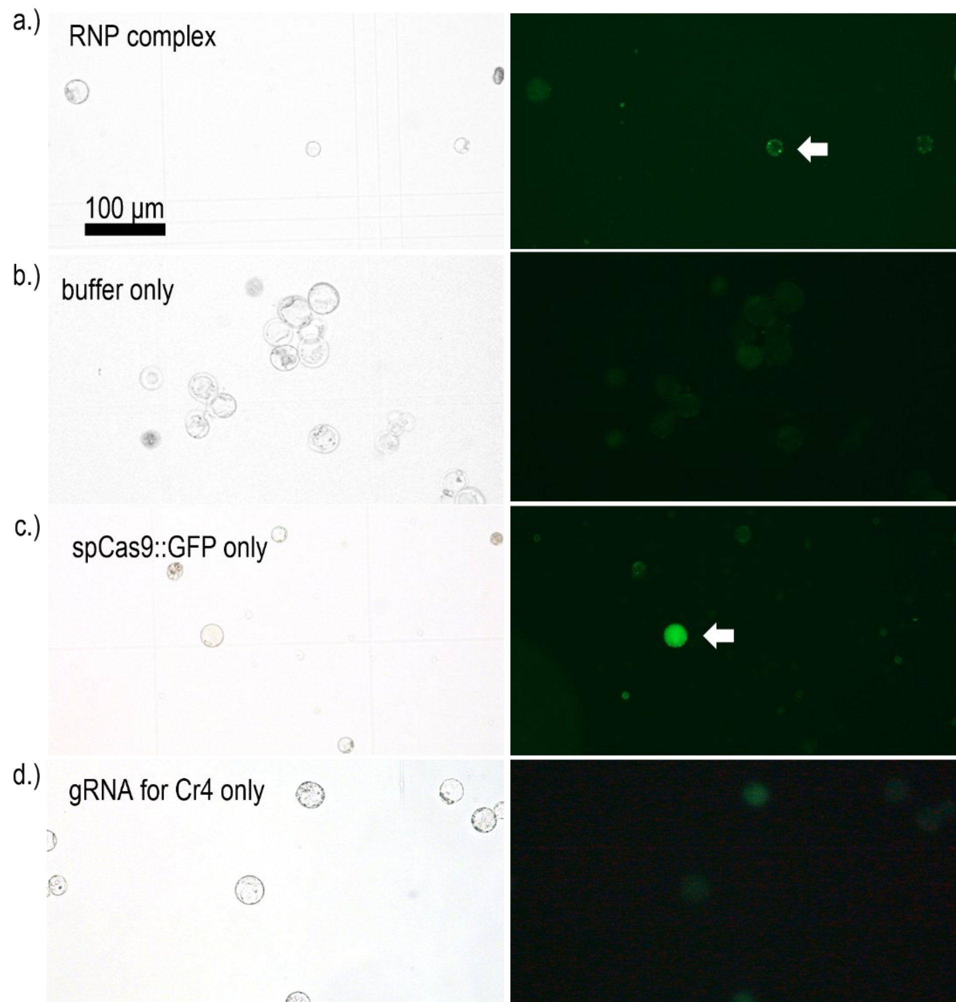


Figure 24: Carrot protoplasts **2 hrs**. after infiltration with the RNP complex targeting Cr4.Left under light field and right under fluorescence. In a.) the arrow shows protoplasts showing GFP signal after infiltration with the RNP complex, b.) control with protoplasts only in buffer, c.) only infiltrated with spCas9::GFP and d.) only infiltrated with gRNA for Cr4.

After 20 hrs. first cell divisions could be seen under light field microscopy (fig. 25c.)d.). The protoplasts infiltrated with RNPs and with only spCas9::GFP still emit the green fluorescence of GFP (fig. 25a.)b.).

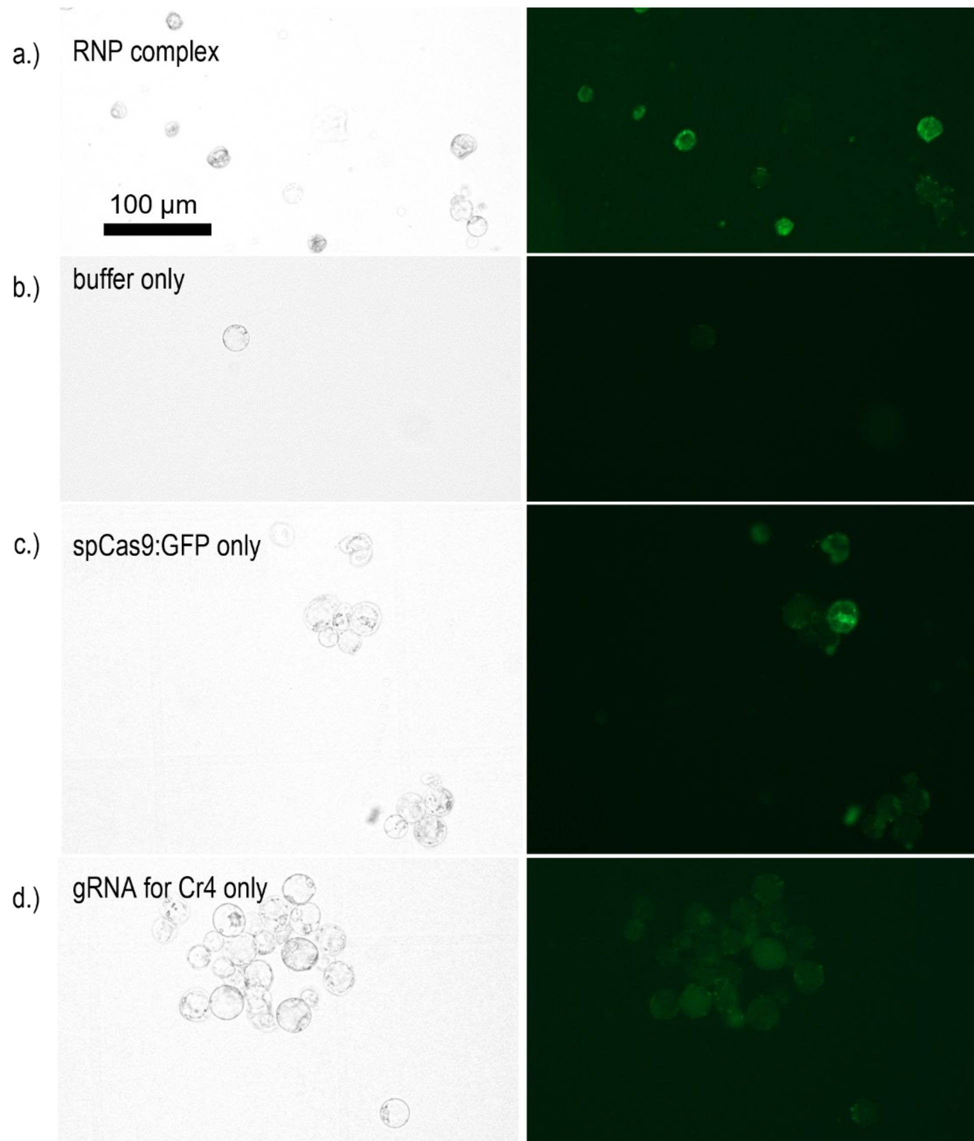


Figure 25: Carrot protoplasts **20 hrs**. after infiltration with the RNP complex targeting Cr4.Left under light field and right under fluorescence. In a.) infiltration with the RNP complex, b.) control with protoplasts only in buffer, c.) only infiltrated with spCas9::GFP and d.) only infiltrated with gRNA for Cr4.

Since the infiltration of protoplast with tagged RNP complexes seemed to be successful with protoplasts omitting the GFP signal visualized by fluorescent microscopy, the Cr4 target side of protoplast infiltrated with RNP complexes and protoplasts of the buffer control was amplified via PCR and amplicon sequenced (tab. 10 and 11).

The amplicon sequencing of the amplified Cr4 target region of protoplasts infiltrated with the RNP complexes resulted in 88643 utilizable sequences (tab. 9). The vast majority of sequences are wild type (88325). However, in 430 sequences thymidine is inserted 3 bp upstream of the PAM and in 29 reads the triplet coding for leucine was deleted. The insertion of thymidine at this exact location was seen throughout all mutated lines, the deletion of the triplet at this position also occurred in a high percentage of reads in

hairy root lines (C4/DP12 and C4/YS11) and also in YS-22-2 (regenerated from meristem of mutated plants).

Table 9: Results from amplicon sequencing of the Cr4 target region in protoplasts infiltrated with RNP complexes. n gives the number of reads of different variants of the Cr4 region. The PAM is underlined.

| | n |
|---|---|
| Utilizable sequences | 88643 |
| Wild type | |
| GTTAGGGAGATTAGCTTCT-ACCTGG | 88325 |
| INDELs | |
| GTTAGGGAGATTAGCTT----CCTGG | 29 |
| GTTAGGGAGATTAGCTTCT**T**ACCTGG | 430 |

The amplicon sequencing of the amplified Cr4 target region of untreated protoplasts resulted in 50374 utilizable sequences. While only wild type sequences were expected, 10 reads showed the insertion of thymidine 3 bp upstream of the PAM (tab.10).

Table 10: Results from amplicon sequencing of the Cr4 target region in protoplasts of the untreated control. n gives the number of reads of different variants of the Cr4 region. The PAM is underlined.

| | n |
|---|---|
| Utilizable sequences | 50374 |
| Wild type | |
| GTTAGGGAGATTAGCTTCT-ACCTGG | 50364 |
| INDELs | |
| GTTAGGGAGATTAGCTTCT**T**ACCTGG | 10 |

# 4 Discussion

All three delivery methods have successfully induced multiple targeted mutations in *CENH3*. The effect of the mutations was shown in chances of the accumulation of CENH3 in mutated hairy root lines, creating small centromeres in some lines.

## 4.1 Pre-assessment of gRNAs to test for its functionality while minimizing putative off-target events

A well-functioning gRNA must fold correctly to interact with the Cas9 endonuclease to build functional RNPs and further only bind to the defined target sites. The ability of gRNA to attach to DNA sequences despite mismatches was seen in multiple studies, e.g. a systematic review compared off-target events in 468 studies with 6’416 potential off target sequences (Modrzejewski et al. 2020); and found off-target events in 3 % of tested genomic sequences with up to three mismatches. Therefore, when screening the plant genome for possible unwanted cleavage sites, sequences with three mismatches to the protospacer should be considered. The quality of the *in silico* pre-screen to find possible target sides as well as to identify putative off-target sites depends highly on the quality of the reference genome. The genome of more and more crop species is published (https://www.plabipd.de/pubplant_main.html) to allow screening for possible target and off-target sites. In the first published study using CRISPR/Cas9 in carrot Cas9 cleaved DNA sequences that showed one mismatch between the gRNA and the target gene sequence producing off-target events (Klimek-Chodacka et al. 2018). Screening of the carrot genome identified no possible off-target sides for the two gRNAs gCr4 and gCr3. Since cleavage can occur in sequences that mismatch the gRNA in up to three bps, the screen was widen to screen for possible off-targets with up to three mismatches and showed five possible off-target sides for gCr3 within non-coding DNA but none for gCr4. While unwanted editing by gCr3 in non-coding DNA could occur, a possible influence on gene expression is not expected. While Cas9 might cleave at unpredicted sides, the off-target activity of Cas9 does not increase background mutations as seen within soma-clonal variations due to stress induced by other *in vitro* methods (Li et al. 2019; Tang et al. 2018; Young et al. 2019). Furthermore, EFSA (European Food Safety Authority) does not consider possible risks due to off-target events to exceed the risk of naturally occurring mutations or mutations induced by conventional breeding methods (Mullins et al. 2022). However, a gRNA with a low likelihood for off-target activity should be chosen to utilize the benefits of the sequence-specificity of CRISPR/Cas9.

Genomic carrot DNA was incubated with gRNA/Cas9 complexes to test their functionality to cleave the target site. This method allows to quickly test the RNPs prior to *in vivo* applications (Mehravar et al. 2019). The efficiency to cut the target region is influenced by the concentration and structure of the RNPs, as

well as features of the target DNA (Javaid and Choi 2021). Here the *in vitro* digestion of *CENH3* amplicons showed cleavage by both RNP complexes. The pre-assessment of both gRNAs showed their ability to lead Cas9 to cleave the target site within *CENH3 in vitro* with low to no putative off-target activity.

## 4.2. The great regeneration ability of carrot

One of the major limitations of using CRISPR/Cas9 in plants is establishing protocols for the *in vitro* regeneration of plants through tissue culture (Wang et al. 2022b). in this study, plants were successfully regenerated from callus of different plant tissues of two carrot cultivars. While all used cultivars showed regeneration by tissue culture, some differences could be seen between the tested cultivars Nantaise 2 and Rotin but close to none between the used plant tissues. The Rotin cultivar showed more embryogenic regeneration than Nantaise. Differences between genotypes to produce and regenerate from callus is widely seen not only in carrot (Rabiei et al. 2010) but in other plant species like rice (Lee et al. 2002), barley (Tanasienko et al. 2009) and tomato (Chaudhry et al. 2007). This shows the influence of single genotypes on the regeneration ability. While the differences between Nantaise 2 and Rotin have been minor and both, in some cases *in vitro* culture is only available in certain genotypes, e.g. in ryegrass (*Lolium perenne*) (Grogg et al. 2022). Not only was high regeneration achieved, but somatic embryogenesis occurred in all (but one) callus samples. The only callus not producing embryos was callus from shoots of Nantaise 2. Here the sample size was compromised and only one petri dish survived. Interestingly the Rotin samples also showed rather callus samples with either no embryos or a vast number of them. While not all molecular mechanism behind the regeneration of plants from callus are understood yet, it was found that regenerating callus release different volatile compounds (VOCs) like e.g. cyclohexane that might trigger further regeneration in callus samples in close proximity (Yang et al. 2022). In most plants regeneration is not achieved via somatic embryogenesis that directly creates rooted shots directly, but via organogenesis. Achieving rooted plants via organogenesis requires multiple steps by first inducing the growth of shoots on cytokinin rich media and later rooting on auxin rich media (Lee et al. 2024). However, carrots tend to directly built somatic embryos from callus in plant hormone free media (Fujimura 2014). This saves time and resources for regenerating plants and shows why carrot is a great model organism to test molecular plant breeding methods that require *in vitro* regeneration.

## 4.3. Best methods to identify mutations

This study shows how methods to identify mutations induced by CRISPR/Cas9 have evolved. The high-resolution melt (HRM) method can give a fast overlook if there are any mutations within the target side and use pooled samples (Thomas et al. 2014). Most molecular laboratories are equipped with real time PCR cyclers that are needed to perform and analyze HRM, but the results have not been that precise in identifying the exact mutations in $T_0$ of transgenic carrot. In this study the amplicons of the target region

did show abbreviations within their melting curves in some mutated lines, but not in all. In other studies, HRM was used successfully to compare the melting curves of mutated $T_0$ plants with their respective $T_1$ to check if mutations have been inherited (Li et al. 2018). Detecting mutations via Sanger sequencing does give a more detailed insight into the genotype but often requires external service provider. The Sanger sequencing of amplicons directly amplified from the plants showed the exact position of mutations induced within Cr3 and Cr4, but the overlay of signals prevented a clear assertion of the different genotypes and exact mutation events. The mixture of amplicons has then been separated by cloning into vectors prior to Sanger sequencing. While this method did allow to see the exact mutations of different gene variants in single plants, it is very cost and time consuming and lacks depth about the occurrence of the different gene variations. The Next Generation Sequencing method amplicon sequencing does mostly require external service provider but gave information on gene variants and their frequency within the plants. The comparably high cost of amplicon sequencing compared to Sanger sequencing can be overcome by pooling samples via e.g. tagged primer (Salvagnin et al. 2023). To first use sanger sequencing of amplicon mixtures to identify mutated plants and follow that up with NGS Amplicon sequencing was most insightful and resourceful and became the preferred method to sequence carrot plants.

## 4.4. Mutations in *CENH3* by CRISPR/Cas9

In all amplicon sequenced plants, the same reoccurring insertion of thymine was observed at the expected position three bp upstream to the PAM. Since this is also seen in other studies, it is believed that DNA repair enzymes might favor thymine insertion, or that Cas9 tends to make staggered end in presence of thymine rather than blunt ends (Allen et al. 2018). The other reoccurring mutation is the deletion of a triplet within the reading frame. Unlike the insertion of thymine this is not as preliminary seen in other CRISPR/Cas9 studies. It reinforces the assumption of a non-random NHEJ repair that is influenced by the protospacer sequence or the PAM  (Kutubuddin A. Molla and Yinong Yang 2020). The general influence of the targeted sequences has been utilized in other studies to predict possible mutations by programmable tools, making Genome Editing by CRISPR/Cas9 more plannable (Molla et al. 2022). A high number of secondary mutations were seen in amplicon sequenced plants that have been regenerated from meristem showing the ongoing Cas9 activity and the ability of the gRNA to recognize the target region even after mutation by inserted thymine. Here most substitutions have been transversion from purine to pyridine nucleotides and vice versa. This contradicts findings stating a higher transition to transversion frequency (Beura et al. 2022), and shows the versatility of different cell repair mechanism that they are not completely understood yet.

## 4.5. Changes to the accumulation of CENH3 in centromeres of mutated hairy root lines

In this study, immunostaining against CENH3 in hairy root lines showed weaker signals in some mutated and transgenic lines than in the wild type controls. This corresponds with findings in *A. thaliana*, in which mutated lines with incorrect CENH3 folding indicated a substantial decrease of CENH3 accumulation that creates smaller centromeres with weaker signals (Capitao et al. 2021). Plants regenerated from the carrot harry root lines with the weakest signal did not survive, possibly since the amount of functional CENH3 was under the threshold needed for proper mitosis (Lermontova et al. 2011). If the functionality of CENH3 is too severely compromised, the kinetochore cannot bind to CENH3 of loaded centromeres, preventing the microtubules of the spindle apparatus from attaching. The changes in accumulation of CENH3 could not be clearly linked to one specific phenotype since all hairy root cultures have been chimeric with multiple mutations within the Cr4 target. The most common mutation was a knock-out mutation by insertion of thymine, but also in frame mutations that could have changed the functionality of the CATD region, which is crucial for the localization of the kinetochore protein complex and wild type *CENH3*. In maize the quantitative reduction of CENH3 by a heterozygous knock-out mutation diluted the available CENH3 in the diploid plant allowing the plant to grow with functional mitosis (Wang et al. 2021). In the same study, the gametophyte carrying the allele with the knocked-out *CENH3* variant had to rely on CENH3 residue from the sporophyte for haploid cell divisions preceding the formation of sperm and egg cells. Crossing those maize plants with wild type plants resulted in haploid progeny, with eliminated chromosomes of the mutated parents (Wang et al. 2021). The chimeric genotype of CENH3 in the carrot plants of this study prevents to predict possible *CENH3* variants in the gametophyte. Additional secondary knock-out events of *CENH3* due to ongoing Cas9 activity, might decrease the amount of sporophytic CENH3 residue further. Therefore, while the effect of the mutations in Cr4 are visible due to smaller centromeres in mutated lines, the mutation should be fixed and secondary mutations prevented to achieve valuable results on any haploidy inducing effect.

## 4.6. The pros and cons of stable transformation via *A. rhizogenes* or *A. tumefaciens*

The carrot was successfully transformed to express a functional CRISPR/Cas9 expression cassette that induced mutations within *CENH3*. The wild type *A. rhizogenes* strain ATCC 15834 was used to transform carrots and caused hairy root disease producing hairy root cultures. Generally hairy root cultures are predominantly used to produce metabolites used for cosmetics, functional foods, preservatives, additives, and pharmaceuticals (Hu and Du 2006). Here their rapid growth and easy preparation helped screening a high number of root tips for a possible effect of the mutations induced within *CENH3.* The root tips of

hairy roots have long been used for cytogenetic analysis (Aird et al. 1988). However, the expression of *A. rhizogenes* T-DNA genes also effects the production of secondary metabolites and can lead to an altered phenotype of regenerated plants, including wrinkled leaf, shortened internodes or altered flower morphology (Sarkar et al. 2018). This makes it difficult to ascribe changes in the phenotype of carrots, especially of the flowers, to induced mutations within *CENH3*. Therefore, while hairy roots have been a great way to quickly assess possible effects of mutated *CENH3* via immunostaining of root tips, further regeneration and assessment of possible effects as a haploid inducer could be ambiguous. Considering the timeframe to prepare, transform and regenerate carrots, the *A. tumefaciens* approach appeared to be the better choice. The transformation of carrot callus with the unarmed *A. tumefaciens* strain did require establishing callus cultures beforehand, but the transformation with *A. rhizogenes* needed formidable taproots for which carrots needed to grow for multiple weeks/months in the greenhouse. The time spent in the greenhouse exceeded the time needed to generate callus cultures used for transformation via *A. tumefaciens*. After transformation, the transformed callus only spent a short time on selection media before regeneration started, while hairy root cultures had to undergo a callus phase preliminary to carrot regeneration. In this study transformation using *A. tumefaciens* showed to be faster with a lower risk of abnormal phenotypes in regenerated carrot plants. Regardless of the Agrobacteria used for stable transformation, the integration of the CRISPR/Cas9 expression cassette into the plant genome leads to ongoing Cas9 activity known to produce chimeric $T_0$ plants with secondary mutations (Feng et al. 2014). Here all transgenic carrots have been chimeric showing a variety of mutations within the respective target sides, while in some studies e.g. a $T_0$ with homozygous or bi-allelic mutations could be achieved in chicory (Salvagnin et al. 2023), or an 49 % chimeric $T_0$ in tomato (Zhang et al. 2020). The considerable small number of tested carrots might be the reason to not have found a plant that was mutated in both alleles, but it cannot be excluded that carrots without wild type CENH3 could not undergo mitosis since the kinetochore complex can no longer be loaded onto centromeres. Taking the high number of secondary mutations into account, the CRISPR/Cas9 have been actively cleaved the Cr4 target region, but wild type sequences remained in all analyzed plants. There is a possibility that cells with homozygous or bi-allelic mutations within Cr4 could not divide anymore, but only cells with remaining wild type could. However, this theory requires further investigation without ongoing Cas9 activity. Different approaches can inhibit or prevent the ongoing cleavage by Cas9. The target sequence must be mutated in a way that prevents the gRNA from recognizing it any longer. Another method is deactivation of the Cas9 via e.g. specific gRNAs that include a photocleavable moiety that allows for light-mediated control over the Cas9 activity (Zou et al. 2021), or self-restriction CRISPR/Cas9 systems in which the target sequence is inserted at both sides of the Cas9 promoter resulting to stop its transcriptions after cleavage by Cas9 (Wang et al. 2020), or other methods. However, methods to inhibit the cleavage by Cas9 are still under development

and not widely established yet. The most common approach is the segregation of the Cas9 transgene by selfing. While this ensures to stop any Cas9 cleavage, it presents a time-consuming obstacle. It is especially time consuming in biannual, cross-pollinated species that require a cold period to induce blooming like carrot. Furthermore, the selfing did not dissolve the chimeric structures within the first selfing generation of carrot. The need of additional selfing generations to segregate the transgenes is not uncommon. In tobacco *(Nicotiana benthamiana)* for example, 50 % of the $T_2$ generation have segregated the Cas9 transgene (Song et al. 2022) calling for at least one additional selfing generation of the transformed carrots. Nevertheless, even with active Cas9 mutated alleles can be fixated via the germline, as long as the gRNA can no longer recognize the mutated target side. However, with the single thymine insertion as the most common mutation, the gRNA is likely to still recognize the target even with one mismatch (Klimek-Chodacka et al. 2018; Song et al. 2022). A different approach to try to dissolve the chimeric structures is via regeneration from meristem culture, as done in mutagenesis based breeding (Jankowicz-Cieslak et al. 2012). The regeneration of plants from the meristem of leaf axils from chimeric carrot plants might circumvent the possible selection of only wild type gametes. The regenerated carrots showed similarities in their mutations depending on the plants from which the meristem sample was taken from. However, they were still not homozygous, nor heterozygous mutated or carried bi-allelic mutations but remained chimeric in their *CENH3* genotype. Both tested plants showed secondary mutations alongside the insertion of thymine three bp upstream of the PAM. While some dissolvement appeared to have been achieved due to the distinguishable character between plants with different origin meristem culture, the ongoing Cas9 activity can still change their genotype over time as long as the gRNA can recognize the target sequence (Salvagnin et al. 2023). The chimeric structure due to the long exposure to Cas9 and its ongoing activity could not be dissolved via the germline nor by regeneration from meristem culture.

## 4.7. The transient transformation as an alternative for stable transformation to mediate CRISPR/Cas9 in carrot

Protoplasts have been transfected with preassembled RNPs to circumvent the need for segregation of Cas9 to stop ongoing DSBs within *CENH3*. Mutated plants regenerated from protoplasts are shown to be generally bi-allelic or homozygous mutated (Meyer et al. 2022). Pooling all transformed protoplasts and amplicon sequencing them showed that 0.3 % of reads have been mutated and either knock-out events or deletion of the triplet that codes for tyrosine. The achieved 0.3 % of mutated reads is comparable to achieved rates of 0.19 % and 0.92 % in banana that have been transfected with RNPs (Wu et al. 2020). However, much higher rates were achieved in other studies. In apple for example 6.9 % of protoplasts have been mutated (Malnoy et al. 2016), in maize 6.5 % (Sant'Ana et al. 2020) or even 44 % in tobacco

(Woo et al. 2015). While only a small percentage of the sampled protoplasts have been mutated, there have not been multiple mutations seen in the single reads, but either the same knock-out event that is predominant in the stable transformation or the deletion of the triplet also seen reoccurring in the stable transformants. With this method no secondary mutations occurred. However, the small mutation rate in the pooled protoplasts requires regenerating a higher of plants to find some that carry mutations in *CENH3*. While the regeneration capacity of carrots is enormous, the need to analyze this number of plants is not applicable. Other applications of transfecting carrot protoplasts with a vector carrying an expression cassette for CRISPR/Cas9 achieved a much higher mutation rate of e.g. 14.7 % with a base editing approach targeting the HFD region of *CENH3* (Meyer et al. 2022). Using a vector to express CRISPR/Cas9 can increase the mutation rate due to longer exposition to active RNPs complexes compared to transfecting protoplasts with preassembled RNPs. However, the transfection with plasmids bares the risk of unwanted integrating of transgenes. In in chicory 39 % of regenerated shoots carried copies of the SpCas9 ORF (Cankar et al. 2021) after infection of protoplasts with plasmids. Other studies were also able to find DNA fragments of the transformation plasmid within regenerates (Kim et al. 2014; Braatz et al. 2017). The efficiency of using preassembled RNPs to target *CENH3* in carrot might be increased by adjusting the protocol e.g. add additional transfection time, increase the RNP concentration or apply changes to the Cas9/gRNA ratio or PEG concentration (Sant'Ana et al. 2020; Sandhya et al. 2020; Wada et al. 2020). Transformed protoplasts without secondary mutations could further be utilized to test the theory if cells with homozygous or bi-allelic mutations within Cr4 are not able to divide anymore and to produce plants with genotypes that still can.

## 4.8. Conclusion and outlook

The mutations in Cr4 targeting the CATD region lowered the accumulation of CENH3. While most chimeric plants showed normal growth, homozygous or biallelic knock-out mutations could prevent mitosis as seen in the difficulty to regenerate the hairy root line with the lowest CENH3 accumulation. The ongoing cleavage by Cas9 leads to multiple secondary mutations and to an unpredictable genotype of meiotic cells. Therefore, applying the transgene-free transformation of protoplasts via RNPs can be used to test the effect of the different *CENH3* genotypes and their effect of mitoses, meiosis and possible haploidy inducing effect. The overall fast adaptability of CRISPR/Cas9 and the great regeneration of carrot shown in this study could allow test more different targets within *CENH3*. For example multiplexing of target region to imitate the tailswap approach seen in *A. thaliana* (Ravi and Chan 2010), or even a prime editing to exchange single amino acids within the HFD (Meyer et al. 2023). This study showed the versability to use CRISPR/Cas9 in carrot. Hairy roots are a great tool for cytogenetic studies, but they need mature taproots of carrots grown for multiple weeks prior to transformation and possible effects of mutations on the phenotype are difficult to identify due to effects of the virulence genes of the T-DNA of *A. rhizogenes*

that trigger hairy root growth. Stable transformation via disarmed *A. tumefaciens* can lead to a high number of transgenic carrot plants with unchanged phenotype, but the Cas9 transgene must be segregated to prevent ongoing Cas9 activity. Transforming of protoplasts circumvents the necessity to segregate the Cas9 transgene, but the low number of mutated protoplasts in this study would mean regenerating an excessive number of carrots to find plants carrying mutations, what might change by an improved transformation protocol or different target. While this study was not able to produce plants to test as potential haploid inducer effect, it did establish a resourceful workflow with insights about the advantages and disadvantages of different transformation techniques to apply CRISPR/Cas9 in carrot.

# 5 Summary

Modern challenges like climate change, growing population and decreasing arable land call for fast plant breeding methods to produce adapted crops that still produce constant yield under harsh and fast changing conditions. The New Genomic Technique (NGT) CRISPR/Cas9 induces site specific modifications in plants to e.g. improve metabolic pathways, resistance to biotic and abiotic stress, yield or nutritional content and disease resistance. Different delivery methods can be used to induce CRISPR/Cas9 mediated genome editing, like stable integration of the expression cassette into the plant genome via *Agrobacteria* or transiently by infiltrating plant cells without cell walls (protoplasts) with reassembled complexes of Cas9 and gRNA. All delivery methods require functional and efficient transformation and regeneration protocols, not available for all plants. Carrot (*Daucus carota*) acts as a model system for tissue culture while being a versatile crop of economic interest. The predominant breeding method of carrots is hybrid breeding that requires parental in-breed lines. However, the methods to produce those parental lines require either multiple years of self-pollination or specific genotypes to regenerate plants from haploid plant tissue. Therefore, a genotype independent and fast method to produce homozygous breeding lines is needed. In recent years CRISPR/Cas9 was used to target different genes that are involved in fertilization, paternal chromosome elimination or chromosome segregation to establish robust *in vivo* haploid production by modified haploidy inducer lines. The segregation of chromosomes is manipulated by targeting the centromere specific histone 3 (CENH3), a protein of the kinetochore region of centromeres to which spindle microtubules attach to during cell divisions and that is highly conserved in different plant species. Here *Daucus carota* was stably transformed via *Agrobacteria tumefaciens* and *Agrobacteria rhizogenes* for *in vivo* expression of CRISPR/Cas9 as well as transgene-free by infiltrating protoplasts using preassembled RNPs targeting CENH3. The high number of transgenic plants showed a chimeric genotype of *CENH3* with the post dominant mutation being single insertion of thymine leading to a knock-out of *CENH3.* The accumulation of CENH3 in some mutated lines was lower than in the wild type as seen by immunostaining of transgenic carrot rootlets, but ongoing Cas9 activity created secondary mutations even after self-pollination or regeneration from mutated meristem culture. Transforming of protoplasts created a low number of mutated protoplasts but without secondary mutations. While this study was not able to produce plants to test as potential haploid inducer effect, it did establish a resourceful workflow with insights about the advantages and disadvantages of different transformation techniques to apply CRISPR/Cas9 in carrot.

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# V. Declaration of independence

I herewith declare that I wrote and composed the presented PhD thesis independently. I did not use any other sources, figures or resources than the ones stated in the references.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne, außer die von mir angegebenen, Hilfsmittel angefertigt habe. Alle genutzten Quellen und Zitate sind als solche gekennzeichnet.

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Katharina Unkel

# VI. List of publications

**Peer reviewed:**

Purnhagen K.; Ambrogio Y.; Bartsch D.; Eriksson D.; Jorasch P.; Kahrmann J.; Kardung M.; Molitorisová A.; Monaco A.; Nanda AK.; Romeis J.; Rostoks N.; Unkel K.; Schneider XT. (2023) Options for regulating new genomic techniques for plants in the European Union. Nat Plants. 2023 Dec;9(12):1958-1961. doi: 10.1038/s41477-023-01570-2.

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**Book chapter:**

Unkel, K; Sprink, T. (2018): Accelerated Breeding of Carrots (*Daucus carota*) through the New Plant Breeding Method of Uniparental Genome Elimination by Haploid Technology: A Blur between Breeding and Regulation. In C. Dürnberger, S. Pfeilmeier, S. Schleissing (editors): Genome Editing in Agriculture. Between Precaution and Responsibility. TTN-Studien Band 7. Nomos, Baden-Baden.

**Talks:**

Unkel, K.; Romeis, J.: Anforderungen an eine sichere Zulassung- mit schweizerischem Blickwinkel, GeneBEcon Science Policy Symposium, 23rd October, Berlin, Germany

Unkel, K.; Bartsch, D. (2024): Biosafety Data Requirements. Risk Assessment under GeneBEcons' regulatory options. Midterm symposium, GeneBEcon Midterm Symposium, 20. February 2024, Brussels, Belgium

Unkel, K.; Sprink, T (2022): Uniparental genome elimination in carrot (Daucus carota L.) by targeting CENH3 via CRISPR/Cas9, 9th meeting of the IOBC-WPRS working group "Modern Biotechnology in Integrated Plant Production",28-30 September 2022, Berlin, Germany

Unkel, K.; Sprink, T (2022): Side-effects on the genetic level-results of off-target analysis. Chic-Project Stakeholder Symposium 21-22 September 2022, Brussels, Belgium

Unkel, K.; Sprink, T.; Dunemann, F. (2019): Accelerating the breeding of carrots (Daucus carota L.) via CRISPR/Cas9, International Plant Science Conference 2019, 15.-19. September, Rostock, Germany

Unkel, K. (2018): Using CRISPR/Cas9 to induce targeted modifications of centromeric histone H3 (CENH3) in carrots (*Daucus carota*)", Elevator Pitch, Plant2030 Progress seminar, 05.-07. February (2018), Potsdam, Germany

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# VII. Curriculum vitae