Non-allelic gene reshuffling induced by CRISPR/cas9-mediated double strand breaks in a plant genome

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Unrepaired DNA double strand breaks (DSBs) are harmful events for the plant cell, leading to chromosome loss, gamete sterility or cell death. Organisms have developed several molecular repair mechanisms to correct these lesions, including homologous recombination (HR) and Non-Homologous End Joining (NHEJ). Nevertheless, repair of DSBs by these pathways, while being essential for the maintenance of genome integrity, may also be associated with ectopic recombination, gene conversion events and the more frequent NHEJ-induced insertion/deletion mutations that modify the original DNA sequences. In this study, we tested a CRISPR/Cas9 multiplexing vector system to the model species *Nicotiana benthamiana* to target the *PDS* (*Phytoene Desaturase*) gene. Among the usual effects of targeted mutagenesis detected, we also observed significant instances of **Non-Allelic Homologous Recombination (NAHR)** eliciting the reciprocal exchange of DNA stretches between paralogous genes present in the genome. These observations add further examples of gene reshuffling among the possible outcomes of CRISPR-based genome editing in plants. Moreover, they offer new sparks for the application of New Breeding Techniques (NBTs) and the investigation of genome topology through the effects of homologous recombination.

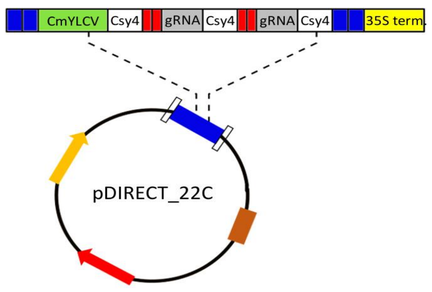
1. Rational and findings

A CRISPR/Cas9 experiment was set up in *Nicotiana benthamiana* with the purpose of testing a guide RNA (gRNA) multiplexing approach for the co-expression of two or more gRNAs. The system was successfully tested for the generation of controlled deletions by simultaneous cleavage of multiple target sites in the *Phytoene Desaturase* (*PDS*) gene. Regeneration of edited plants by somatic embryogenesis yielded the expected albino phenotype caused by putative disruption of proper PDS expression, with different severities ranging from partial (chimeric) mutants to full-albino individuals.

A *PDS* gene paralog (called B hereafter), made distinct from the main gene copy (A) by single nucleotide variants, was targeted as well. Unexpectedly, CRISPR-edited leaves exhibited A/B chimeric sequences at low frequency with A/B junctions being contiguous to gRNA targets, suggesting **homologous recombination between non-allelic sites induced by editing**.

2. Materials and Methods

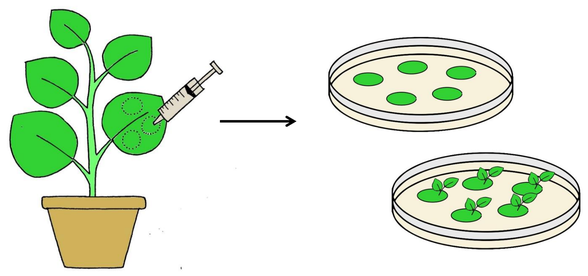
Cloning



Two gRNAs previously described (Nekrasov *et al.*, 2013; Upadhyay *et al.*, 2013) were used for CRISPR/Cas9 targeting of the *Phytoene Desaturase* (*PDS*) gene in *N. benthamiana*. They were assembled in a multiplexing array into the plasmid pDIRECT_22C using the protocol described in Čermák *et al.*, 2017.

The resultant construct was transformed into *Agrobacterium tumefaciens* strain LBA4404 for agroinfiltration-based transient gene expression in *N. benthamiana* as described in Bos *et al.*, 2010.

Plant transformation and somatic embryogenesis



Agroinfiltration of 4 weeks old *N. benthamiana* plants

***Transformed leaf discs* were collected for somatic embryogenesis**

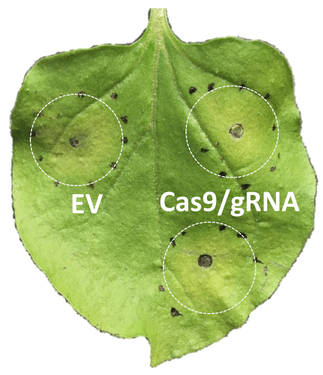
A. tumefaciens carrying Cas9 and the double-sgRNA expression construct was infiltrated into *N. benthamiana* leaves with a final OD600 of 0.5, 1.0 and 1.5. Empty plasmid pDIRECT_22C was used as negative control. Transformed leaf discs were both used for genomic DNA analysis and somatic embryogenesis.

Sequencing analysis of editing products

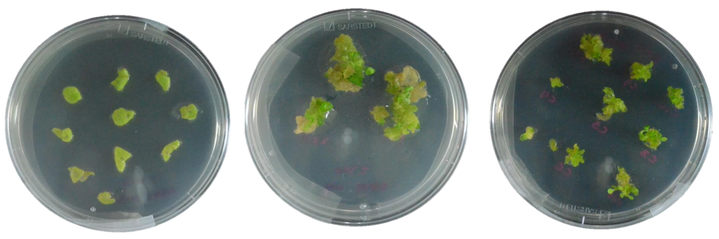
After genomic DNA extraction from leaf discs, PCR amplification was performed using primers flanking the target sites within the *PDS* gene. Samples were sequenced by Sanger and NGS approaches and mutation analysis was performed.

3. CRISPR/Cas9-induced albino phenotypes

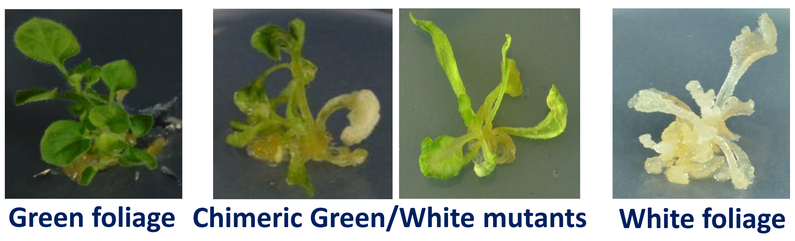
In Vivo Cultures of N. benthamiana



Agro-Infiltrated leaves



Transformed Leaf disks | ***Callus* formation** | **Plantlet regeneration**

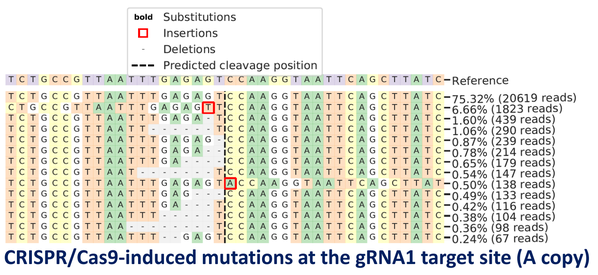


	Green foliage	Chimeric Green/White mutants	White foliage
Primary Shoots			
Frecuency %	**27%**	**68%**	**5%**

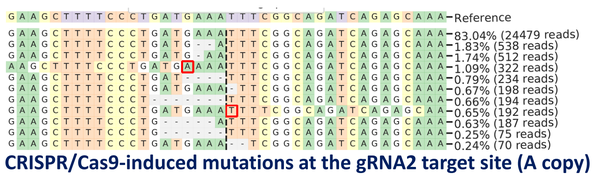
4. *In silico* sequence analysis and data interpretation

The analysis of sequencing data from PCR amplification products revealed CRISPR/Cas9-mediated editing marks in the A copy of the *PDS* gene. These included small insertions/deletions (a) as well as a large deletion (~6000 bp) with defined boundaries resulting from the loss of the DNA fragment ranging from the gRNA1 to the gRNA2 target site followed by end joining (b).

(a)

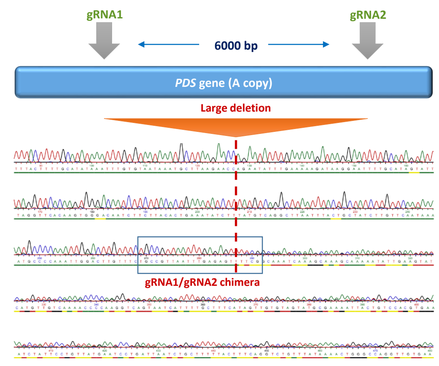


CRISPR/Cas9-induced mutations at the gRNA1 target site (A copy)

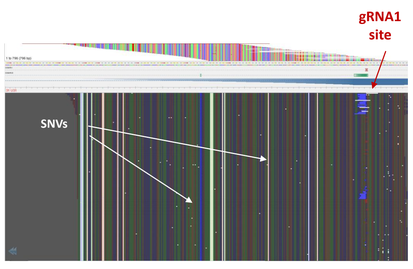


CRISPR/Cas9-induced mutations at the gRNA2 target site (A copy)

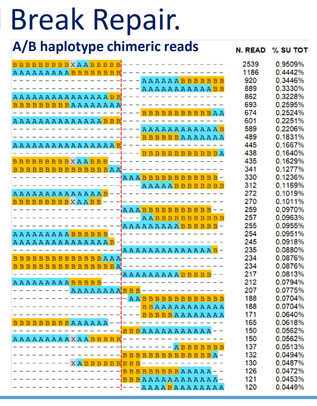
(b)

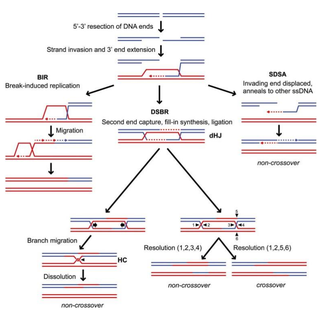


Chimeric NGS reads exhibiting stretches of DNA from the A and B genes suggest Non-Allelic Homologous Recombination (NAHR) compatible with the non-crossover resolution model of Double Strand Break Repair.



Reads from the B paralog aligned to the A copy showing the single nucleotide variants (SNVs) that define the A and B haplotypes

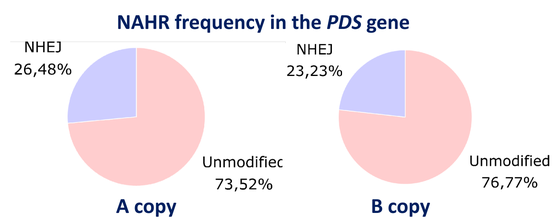




From Symington *et al.*, Genetics 2014

5. Conclusions

Besides the multiple-site mutations commonly reported during DSB repair (deletions, insertions and substitutions), Non-Allelic Homologous Recombination (NAHR) was detected, although at a much lower frequency, between DNA sequences from two *PDS* paralog genes. We believe that the different chromosomal arrangements shown in this work via CRISPR/cas9 are highly relevant in the development of NBTs for precise genome editing.



6. Acknowledgments

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