Expression of microRNAs can be fine-tuned by the CRISPR/Cas9 system in potato

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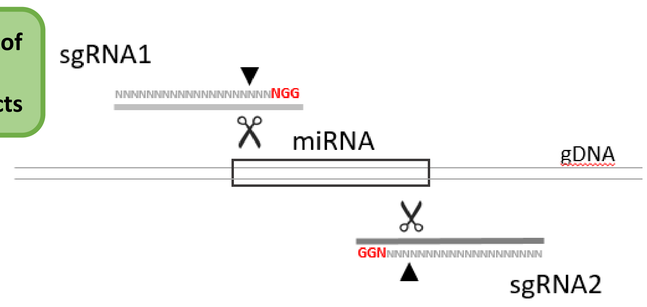
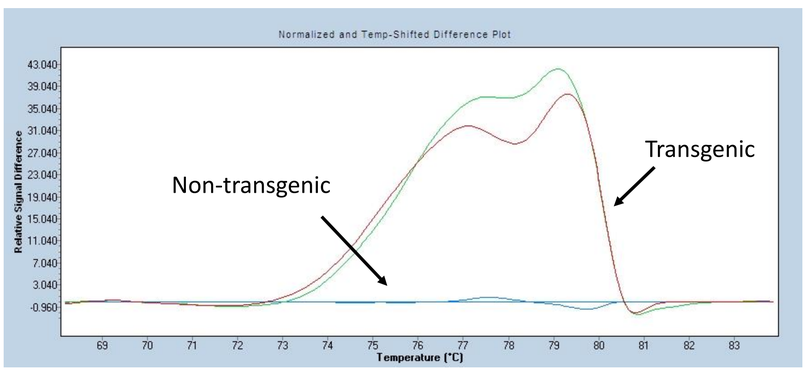
To date, CRISPR/Cas9-mediated microRNA editing has not been established in potato. However, there is a growing evidence that small noncoding RNAs can be targeted by CRISPR/Cas9 system in plants. The novel gene-editing strategy is a challenge yet worth accepting, due to the compelling robustness, specificity, and stability for the modification of microRNA expression. In potato, CRISPR/Cas9 technology was mostly used in combination with time-consuming *Agrobacterium*-mediated stable transformation. On the other hand, protoplasts transfection is a faster method, but protoplasts isolation and regeneration of transgenics remain bottlenecks. We established the complete pipeline for CRISPR-Cas9-mediated modulation of microRNA expression in potato, which considers pros and cons of both methods.

① Design and assembly of dual sgRNAs CRISPR/Cas9 constructs



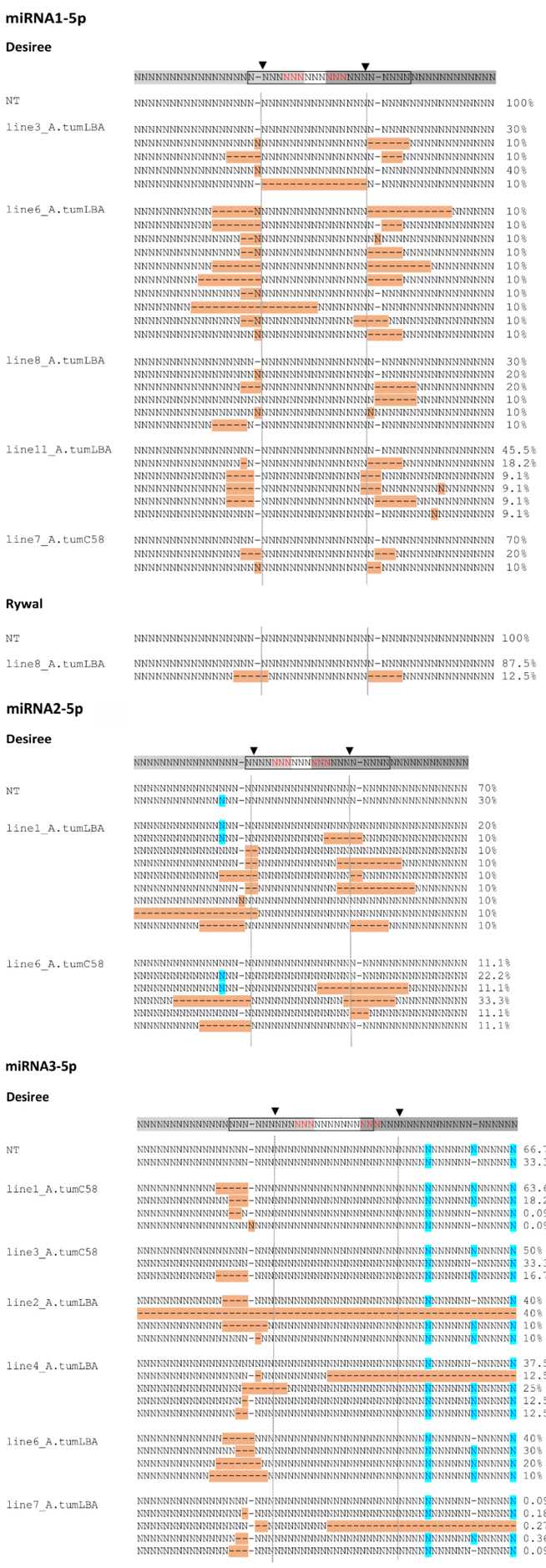
② Transient transfection of protoplast with constructs **HRM to select functional sgRNAs**



High resolution melting (HRM) analysis following protoplasts transfection is fast and efficient screening method for testing functionality of designed sgRNAs

Melting curves of transgenic plants differ from the one of the control, suggesting the presence of mutations in the gDNA isolated from transfected protoplasts.

③ Stable transformation of potato using functional sgRNAs **Selection of transgenic lines with desired mutations by sequencing**



miRNA-editing using dual sgRNA constructs results in different types of mutations

We obtained several Desiree transgenic lines with different types of mutations between transgenic lines but also in different alleles of the same plant. The most abundant type of mutations were short deletion, but we also identified 1-nt insertions (T or G) and longer deletions. Next to sequences, the percentage of amplicons (obtained by amplifying miRNA coding and adjacent regions by PCR) with the particular type of mutations is shown. miRNA (boxed), sgRNA1 (light grey), sgRNA2 (dark grey), PAM motif (red), theoretical cutting site (arrowhead and dotted line). Allelic variations are shown in blue, mutations are shown in orange.

④ Selection of transgenic lines with desired mutations according to the miRNA abundance by qPCR

Genotype	miRNA	A. tum. strain	Line	Growth	Sickle-shaped leaves	Genotypisation: No. of mutated amplicons	Genotypisation: Mutation types	miRNA abundance	Insert in the genomic DNA	Cas9 expression
Desiree	3	C58pMP90	1	Very fast	No	11/11	Short deletions	**0.12**	Yes	1.55
Desiree	3	C58pMP90	4	Slow	No	0/10		**1.27**	No	Under LOD
Desiree	3	C58pMP90	7	Medium	No	0/10		**0.25**	No	Under LOQ
Desiree	3	C58pMP90	8	Medium	No	0/11		**0.18**	No	Under LOD
Desiree	3	LBA44040	2	Slow	No	10/10	Long and short deletions	**0.06**	Yes	1.83
Desiree	3	LBA44040	3	Medium	No	0/10		**2.64**	No	Under LOD
Desiree	3	LBA44040	4	Very fast	No	5/8	Short deletions	**0.29**	Yes	1.05
Desiree	3	LBA44040	6	Fast	No	10/10	Short deletions	**0.07**	Yes	2.42
Desiree	3	LBA44040	7	Fast	No	9/10	Long and short deletions	**0.26**	Yes	2.59
Desiree	1	C58pMP90	5	Fast	No	0/10		**0.26**	No	Under LOD
Desiree	1	C58pMP90	6	Fast	No	NA		**0.36**	Yes	1.90
Desiree	1	C58pMP90	7	Medium	No	3/10	Short deletions	**0.30**	Yes	0.62
Desiree	1	C58pMP90	8	Slow/medium	No	0/7		**0.56**	No	Under LOD
Desiree	1	C58pMP90	10	Slow	No	0/6		**0.73**	No	Under LOD
Desiree	1	C58pMP90	11	Fast	No	0/10		**0.72**	No	Under LOD
Desiree	1	LBA44040	1	Fast	No	NA		**0.36**	NA	Under LOD
Desiree	1	LBA44041	3	Fast	Yes	7/10	Short deletions and insertio	**0.33**	Yes	1.73
Desiree	1	LBA44042	6	Very fast	Yes	10/10	Short deletions	**0.18**	Yes	0.94
Desiree	1	LBA44043	8	Medium	No	7/10	Short deletions and insertio	**0.43**	Partially	0.17
Desiree	1	LBA44044	9	Slow	No	0/11		**2.21**	Partially	Under LOD
Desiree	1	LBA44045	11	Fast	No	6/11	Short deletions and indels	**0.67**	Yes	0.21
Desiree	2	C58pMP90	1	Slow	No	NA		**2.47**	No	0.06
Desiree	2	C58pMP90	2	Very fast	No	NA		**0.78**	No	Under LOQ
Desiree	2	C58pMP90	6	Fast	No	6/9	Short deletions	**0.17**	Yes	1.01
Desiree	2	C58pMP90	8	Fast	No	0/11		**2.09**	No	Under LOD
Desiree	2	C58pMP90	12	Fast	No	0/12		**0.92**	No	Under LOD
Desiree	2	LBA4404	1	Medium	No	8/10	Short deletions and insertio	**0.22**	Yes	3.66
Desiree	2	LBA4404	6	Fast	No	NA		**1.42**	No	Under LOD
Desiree	2	LBA4404	4	Very fast	No	0/11		**0.58**	No	Under LOQ
Desiree	NT				No			**1.00**		Under LOD
Rywal	1	C58pMP90	9	Medium	No	0/9		**0.87**	No	Under LOD
Rywal	1	LBA4404	3	Very fast	No	0/10		**3.23**	Yes	Under LOD
Rywal	1	LBA4404	4	Very fast	No	NA		**3.19**	Yes	Under LOQ
Rywal	1	LBA4404	5	Very fast	No	NA		**1.62**	No	0.01
Rywal	1	LBA4404	8	Medium	Yes	1/8	Short deletions	**0.10**	Yes	0.85
Rywal	1	LBA4404	9	Fast	No	0/7		**1.34**	No	Under LOD
Rywal	1	LBA4404	11	Medium	No	NA		**2.25**	NA	Under LOQ
Rywal	1	LBA4404	18	Very slow	No	0/7			NA	
Rywal	1	LBA4404	19		No	0/7			No	Under LOD
Rywal	1	LBA4404	20		No	0/8			NA	
Rywal	NT				No			**1.00**		Under LOD

miRNA abundance correlates with the number and type of mutations

miRNA abundance: Relative miRNA abundance (relative to the endogenous control) determined by qPCR in the selected transgenic lines was normalised to the averaged expression in non-transgenics (NT), which was set to one. miRNA abundance is lower in the lines with higher number of mutated alleles (No. of mutated amplicons).

miRNA-editing efficiency with selected dual sgRNA constructs is high

Insert in the genomic DNA: To discriminate transgenic from NT plants, we amplified three regions of the T-DNA by PCR using genomic DNA as a template. Among transgenic plants, 10 contain complete T-DNA in the genome. We confirmed the presence of all T-DNA regions in the genome of transgenics with mutations. The majority of plants with no mutations do not contain any region of T-DNA in the genome.

Cas9 expression: Relative expression of Cas9 (relative to the endogenous control) was determined by qPCR. In all transgenic lines with Cas9 expression, we detected mutations, suggesting high efficiency of Cas9-editing. In addition, expression correlates with abundance of mutations. LOD/LOQ: limit of detection/quantification

We established fast and efficient pipeline for CRISPR/Cas9-mediated microRNA fine-tuning of miRNA expression in polyploid species and validated it on three miRNAs from potato. It consists of:

Design and assembly of CRISPR/Cas9 constructs → **Transient transfection of protoplast with constructs** → **HRM to select functional sgRNAs** → **Stable transformation of potato using functional sgRNAs** → **Selection of transgenic lines with desired mutations according to mutation types and miRNA abundance**