Phenotypic characteristics of tobacco plants harboring mutations in nicotine biosynthesis genes from *PMT* and *QPT* gene families

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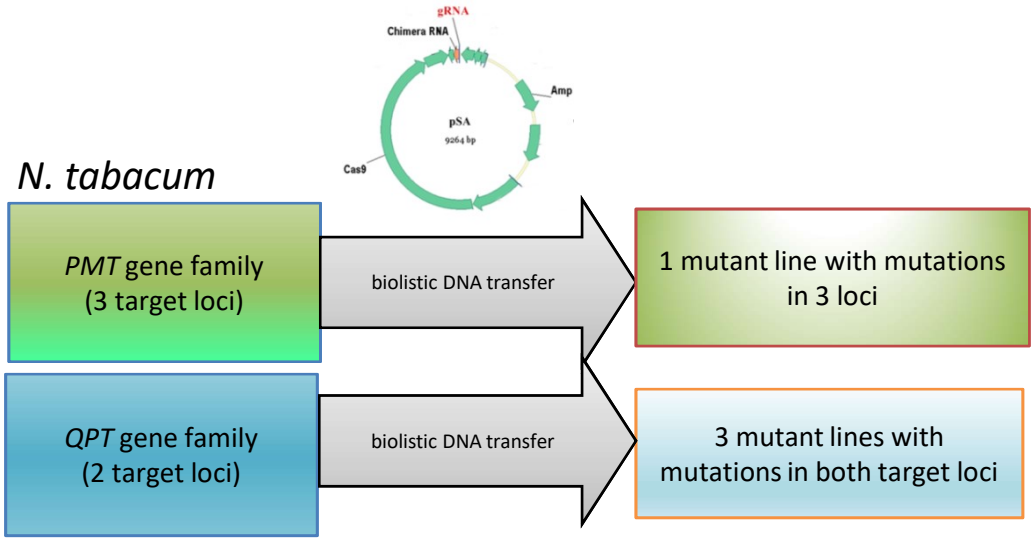
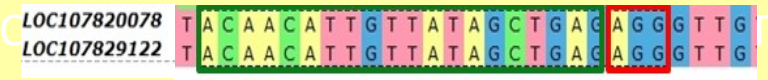
Nicotine accumulation is often a disadvantageous feature when using tobacco in biotechnological approaches. The decrease in nicotine levels can be achieved by knocking out respective key biosynthesis genes.

In the present study, Cas9/gRNA technology was used for targeted mutagenesis of the nicotine biosynthesis genes *PUTRESCINE N-METHYLTRANSFERASE* (*PMT*) and *QUINOLINATE PHOSPHORIBOSYLTRANSFERASE 2* (*QPT2*).

Two genetic constructs, a vector carrying cas9 and gRNA expression units without an antibiotic resistance gene, and a pBI121-based plasmid containing a kanamycin resistance gene and a GUS reporter gene, were delivered to tobacco leaf explants using biolistic DNA transfer. The design of the cas9/gRNA vector involved gRNAs simultaneously guiding the Cas9 nuclease to target motifs conserved across all three genes of the *PMT* gene family (LOC107799426, LOC107799425, LOC107770255) and the *QPT2* gene family (LOC107820078 and LOC107829122).



Conservativeness of the target site QPT_exon 5 (underlined by PAM)



Conservativeness of the target site PMT_exon 2 (underlined by PAM)

The aim of the study is to characterize obtained lines of mutant plants and identify mutations.

Methods and results

Specific pairs of PCR primers were designed to each target site using Perl primer Software. We use selected primer pairs for PCR and Sanger sequencing reaction.

Three clonally maintained T0 lines were obtained in which both *QPT2* genes exon 5 proved mutated.

locus /clone population	QPT1 mut	QPT2 mut	QPT3 mut
LOC107820078	Biallelic mut, -9/?	Biallelic mut, -7/-5	Biallelic mut, +1/?
LOC107829122	Biallelic mut, -10/+1	Homozygote, +46/+46	Homozygote, -4/-4

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QPT2_ LOC107829122_allele1 +1bp  ACAACATTGTTATAGCTGGAGAGG
QPT_ LOC107829122_allele2  -10bp ACAACATTGTTA----------GGGTT-TTCTCAATTTT
QPT2_LOC107829122_ex 5     WT    ACAACATTGTTATAGCTG-AGAGGGTTGTTCTCAATTTT
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In the T0 and T1 generations, plants carrying mutations in individual genes of the PMT family were identified.

clone population \locus	LOC107770255	LOC107799425	LOC107799426
PMT(1) T0	mut –3 nt	mut –1 nt	mut –1 nt

№ plant T1 /locus	PMT4 LOC107770255 mut	PMT3 LOC107799425 mut	PMT2 LOC107799426 mut
1	– 3 nt	– 1 nt	– 1 nt
2	– 3 nt	– 1 nt	– 1 nt
3	– 3 nt	– 1 nt	– 1 nt
6	– 3 nt	– 1 nt	large deletion
7	large deletion	– 1 nt	– 1 nt
8	– 3 nt	– 1 nt	– 1 nt
9	large deletion	– 1 nt	– 1 nt
11	large deletion	– 1 nt	– 1 nt
13	– 3 nt	– 1 nt	– 1 nt
14	– 3 nt	– 1 nt	large deletion
15	– 3 nt	– 1 nt	– 1 nt
16	large deletion	– 1 nt	– 1 nt
17	– 3 nt	– 1 nt	large deletion

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mut PMT_LOC426   AGTCTGATTACCAAGA-GTCATGCTCTTTGAGGTAATTAAT
mut PMT_LOC425   AGTCTGATTACCAAGA-GTCATGCTCTTTGAGGTAATTAAT
mut PMT_LOC255   AGTCTGATTACCAAGA---CATGCTCTTTGAGGTAAATAAT
SR1              AGTCTGACTACCAAGATGTCATGCTCTTTGAGGTAAATAAT
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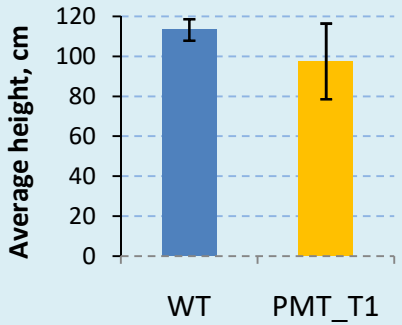
We estimated 10 morphological parameters of mutant plants and carried out biochemical analysis for nicotine content in the leaves.

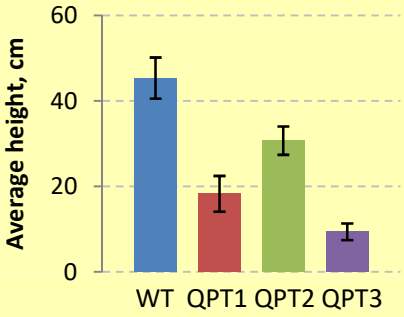
PMT mutant plants

The initial assessment of viability and morphology did not reveal any differences between T1 mutant plants and their wild-type counterparts.

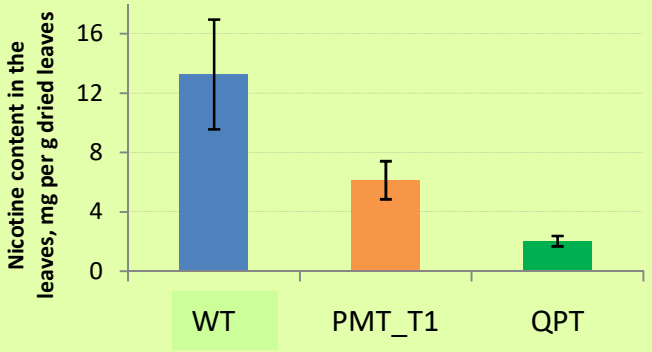


QPT mutant plants

QPT2 (for each line 1,2,3) loss-of-function plants exhibited severe phenotypic abnormalities such as inhibited growth, longostyly, a decrease in pollen fertility, and the absence of viable seeds.



The nicotine content in the leaves of *PMT* mutants (n=13 plants) was reduced to approximately 50% as compared to the controls. The nicotine content in the leaves of *QPT2* mutants (sum of lines 1,2,3) was 6.5 times lower than the that of wild-type leaves.

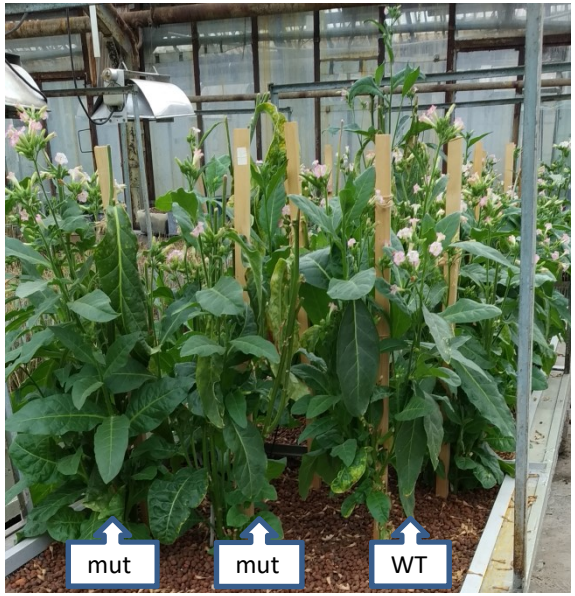


Conclusion:

A decrease of the nicotine content in *PMT* mutant plants reveals that knockout of this gene is can be used to reduce tobacco toxicity. The knockout of *QPT2* is inappropriate to use for obtaining low-nicotine tobacco varieties.

The severe abnormalities associated with the knockout of *QPT2* suggests that these genes' function is essential also for other processes than nicotine production in *N. tabacum*.

PMT mutant plants and WT plant



QPT mutant plants and WT plant

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