Brief Communication

Nicotine-free, nontransgenic tobacco (Nicotiana tabacum L.) edited by CRISPR-Cas9

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Worldwide, approximately 1.1 billion people are smokers and more than 7 million people die from the negative effects of smoking every year (WHO report, 2017). One of the main natural ingredients causing dependence on tobacco is nicotine. Tobacco with a lowered nicotine content could help people to overcome their nicotine addiction. Nicotine-free (or nicotine reduced) cigarettes may contribute to reduce the number of smokers and nicotine consumption, thus reducing the risk of death from tobacco use. Most genes involved in the nicotine biosynthesis in tobacco are known and well characterized (Dewey and Xie, 2013). This opens the possibility to employ genetic engineering approaches to alter the alkaloid content of the plant, and in particular to reduce the nicotine content. Nicotine itself is composed of a pyrrolidine and a pyridine ring, which are synthesized in independent pathways (Figure 1a). Recent approaches dealt with the silencing of upper pathway genes encoding the putrescine N-methyltransferase (PMT) or A622, a phosphatidylinositol-4-phosphate (PIP)-family member of NADPH reductases. The applied RNA silencing methods either resulted in the increased biosynthesis of other alkaloids like anatabine (i.e. Wang et al., 2009) or were only successful in hairy root cultures and BY-2 cells, but not in whole plants (Kajikawa et al., 2009). The final oxidation step in the biosynthesis of nicotine, as well as anatabine and anabasine, is proposed to be catalysed by flavoproteins of the berberine bridge enzyme-like (BBL) family (Kajikawa et al., 2011). The knockdown of the three most highly expressed BBL genes (BBLa–BBLc) by RNAi or the knockout with EMS-induced mutations resulted in a reduction of the nicotine content without increasing the content of other alkaloids (Kajikawa et al., 2011; Lewis et al., 2015). Recently, the BBL gene family in tobacco was expanded by the identification of BBLd.2 and BBLe, leading to six known isoforms (Kajikawa et al., 2017). Thus, the simultaneous knockdown of these BBL genes is a promising approach to generate a nicotine-free tobacco plant.

We aimed for a simple CRISPR Cas9-based knockout strategy and searched for an identical target sequence present in all six published coding sequences (BBLa, BBLc, BBLd.2 originated from Nicotiana sylvestris; BBLb, BBLd.1, BBLe from Nicotiana tamen-tosiformis; Kajikawa et al., 2011, 2017) to enable the use of a single-guide RNA. Except for the PAM sequence, the chosen target sequence is identical in all six BBL sequences (Figure 1b). Mismatches to other sequences of the N. tabacum genome were excluded by BLAST search. We cloned the 20 base pair target sequence between the ubiquitin 6-26 promoter from Arabidopsis thaliana and the chimeric sgRNA. The gene cassette was subsequently transferred into the transformation vector pCas9-TPC carrying a bar-gene as selection marker (Fauser et al., 2014).

After transformation in Nicotiana tabacum L. plants ‘Virginia Smoking Tobacco’ (Strictly Medicinal Seeds LLC, United States), we regenerated ten plants, denoted them as T0 generation and analysed the plants with regard to their nicotine content. Extraction of alkaloids was done from grounded leaves, and nicotine levels were analysed with GC-FID. The change in nicotine levels ranged from unchanged (T0 5) over a medium reduction of 65% (T0 3) to a reduction of around 95% (T0 1 and T0 4) compared to the wild type, indicating that not all BBL loci were knocked out. Sequencing the fragments of genomic DNA from T0 1 and T0 4 plants showed no editing of BBLe, whereas all other BBL genes were mutated. The T0 plants 1, 3 and 4 were chosen for further characterizations in following generations. Rooted plantlets were cultivated in a plant chamber for self-pollination in order to produce T1 seeds. To enable further gene editing with CRISPR Cas9, we selected transgenic T1 plants with phosphinothricin (PPT). Transgenic T1 plants were cultivated until flowering and T2 plants were grown from collected seeds. This growing cycle was continued to obtain T3 plants. While the nicotine content in plant T1 1.2 did not decrease further, a decrease in nicotine content by 95% was observed for plant T1 3.1. The nicotine level of plant T1 4.11 was as low as in the T0 generation.

In order to identify nicotine-free tobacco plants carrying knockouts in all six BBL genes, progenies of plant T0 4 up to generation T3 were screened initially with regard to their nicotine content. The GC analysis of the nicotine content of the analysed T2 and T3 plants resulted in minimal peaks with retention times identical to the nicotine standard. To ensure the correct identification of the peak as nicotine, a GC-MS measurement was performed. A m/z of 162.23 identical to nicotine was detected with a signal-to-noise ratio intensity of nearly 1:1. Since the signal-to-noise ratio of the peaks was too low for an automated peak detection, a manual analysis of the peak area was performed for estimation of the residual nicotine content. It was calculated to 0.06 mg g per DW nicotine in the T2 4.11.1 and to 0.04 mg g per DW nicotine in the T3 4.11.1.2 plant (Figure 1c), which means a reduction of 99.6% and 99.7%, respectively, compared to the wild type. Based on these results, the plant T3 4.11.1.2 was considered as nicotine-free.

Finally, to check whether all 12 loci were knocked out by our approach, a PCR-based method was applied. The combination of a vector-based cloning strategy for the amplicons followed by Sanger sequencing with the direct sequencing of the PCR product.
renders whole genome sequencing unnecessary. The precondi-
tion for that approach was an insertion of a single nucleotide
three base pairs upstream of the PAM sequence resulting in a
frameshift, that is a knockout. In contrast, in Arabidopsis and rice,
insertions or deletions of several base pairs were observed after
nonhomologous end joining (NHEJ) events (Jiang et al.,
2013). However, for BBLa, sequencing results showed either the
insertion of the base guanine or thymine, whereas sequencing
results of the other BBL genes always showed the same base pair
insertion (Figure 1d). To confirm these results, fragments of the
BBL genes were amplified and sequenced directly. The previous
results were confirmed by analysis of the sequencing trace of
the samples (Figure 1e). Except from BBLa, the sequencing trace
of the fragments showed a distinct signal peak for the appropriate
base insertion. For BBLa, the sequencing trace showed a double
peak for thymine and guanine, which is consistent with the
results from the cloning experiments, in which both base pair
insertions were detected.

To prove that the nicotine-free plant is nontransgenic, leaf discs
were cut out and transferred, after surface sterilization, to MS
medium with PPT for selection. As control, a transgenic T 1
generation plant was used. After 2 weeks, leaf discs of the T1
plant were still green and even started to grow, whereas the leaf
discs of the nicotine-free plant died. This result was additionally
confirmed with PCR using primers that bind inside the transfor-
mation cassette thereby spanning the terminator of Cas9 and
the PPT gene (Figure 1f). As a positive control (PC), a T0 plant
was used, as a negative control the wild type (wt). (g) Phenotype of
a wild-type plant and the nicotine-free plant (T3 4.11.1.2).
nicotine-free plant a PCR product was obtained. Thus, the nicotine-free plant was declared as nontransgenic.

Analysis of the GC measurements showed that the alkaloids anatabine, nornicotine and anabasine were present in wild-type extracts, but the latter two at the detection limit. The chromatogram of the nicotine-free plant showed again traces of anabasine and nornicotine, as well as a reduced peak area for anatabine. Additionally, 1H-NMR measurements verified that no substantial changes in the primary metabolism occurred, showing that the complete knockout of the BBL gene family had no negative impact on other biosynthetic pathways using the tested growth conditions. Finally, no changes in the phenotype were observed (Figure 1g).

Conclusion

The generation of a nicotine-free and nontransgenic tobacco plant enables the introgression of this plant into other tobacco varieties and the reduction of overall nicotine and alkaloid content. Furthermore, the use of a single gRNA for the complete knockout of all BBL-relevant genes makes it easy to apply this method to other tobacco varieties and species, for example Nicotiana benthamiana that are commonly used as heterologous production platforms. This will expand the production spectrum of N. benthamiana to the biotechnological production of plant-made pharmaceuticals beyond antibodies.

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Author contributions

F.S. conceived the study. F.S and J.S designed the experiments and wrote the manuscript. J.S. performed the experiments. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing financial interests.

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