CRISPR/Cas9-mediated knockout of a prolyl-4-hydroxylase subfamily in *Nicotiana benthamiana* using DsRed2 for plant selection

Pia Uetz | Stanislav Melnik | Clemens Grünwald-Gruber | Richard Strasser | Eva Stoger

1 Institute of Plant Biotechnology and Cell Biology, Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria
2 Core Facility Mass Spectrometry, University of Natural Resources and Life Sciences, Vienna, Austria

Correspondence
Eva Stoger, Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria.
Email: eva.stoger@boku.ac.at

Abstract
The properties of host plants used for molecular farming can be modified by CRISPR/Cas9 genome editing to improve the quality and yield of recombinant proteins. However, it is often necessary to target multiple genes simultaneously, particularly when using host plants with large and complex genomes. This is the case for *Nicotiana benthamiana*, an allotetraploid relative of tobacco frequently used for transient protein expression. A multiplex genome editing system incorporating the DsRed2 fluorescent marker for the identification and selection of transgenic plants was established. As proof of principle, *NbP4H4* was targeted encoding a prolyl-4-hydroxylase involved in protein O-linked glycosylation. Using preselected gRNAs with efficiencies confirmed by transient expression, transgenic plant lines with knockout mutations in all four *NbP4H4* genes were obtained. Leaf fluorescence was then used to screen for the absence of the SpCas9 transgene in T1 plants, and transgene-free lines with homozygous or biallelic mutations were identified. The analysis of plant-produced recombinant IgA1 as a reporter protein revealed changes in the number of peptides containing hydroxyproline residues and pentoses in the knockout plants. The selection of efficient gRNAs combined with the DsRed2 marker reduces the effort needed to generate *N. benthamiana* mutants and simplifies the screening processes to obtain transgene-free progeny.

KEYWORDS
CRISPR-based gene targeting, host plant engineering, molecular farming, *Nicotiana benthamiana*, plant-made proteins

1 INTRODUCTION

The CRISPR/Cas9 genome-editing system has been used to introduce targeted mutations in a wide range of plant species for both experimental purposes and the commercial development of new traits.\[1\] The system works by generating DNA double-strand breaks (DSBs) at predefined sites in the genome specified by a guide RNA (gRNA) that associates with the Cas9 endonuclease. The DSBs are typically repaired by the endogenous nonhomologous end-joining (NHEJ) pathway, which is prone to errors and usually results in the insertion or...
deletion of a few nucleotides at the target site. If the DSB is introduced near the beginning of the coding sequence, the resulting indel often causes a frameshift mutation that leads to premature termination during protein synthesis, which is a simple way to generate knockout mutants. More sophisticated outcomes can be achieved by tweaking the system components. For example, the provision of a repair template can be used to generate clean knockout and knock-in events by favoring the homology-dependent repair pathway. Furthermore, the expression of multiple gRNAs targeting different genes and/or a single gRNA targeting a conserved sequence in multiple genes can facilitate multiplex genome editing.

The CRISPR/Cas9 system has been applied in all major food/feed crops and has been used primarily to improve traits such as biotic and abiotic stress tolerance, yield, and nutritional quality. However, it can also be used to enhance the industrial performance of plants, including the optimization of recombinant protein manufacturing by molecular farming. Examples include the improvement of protein levels and structural properties by interfering with the expression of host genes encoding endogenous proteases and glycosyltransferases as well as targeting the RNA silencing machinery. Many such examples have been discussed in recent reviews.

One of the most widely used hosts for molecular farming is *N. benthamiana*—a close relative of common tobacco. This species has been used extensively as a model for the functional analysis of plant genes and interactions with pathogens because it has a short lifecycle and carries a mutation that makes it hyper susceptible to many plant genes and interactions with pathogens because it has a short life cycle and has been used extensively as a model for the functional analysis of plants, including the optimization of recombinant protein manufacturing by molecular farming. Examples include the improvement of protein levels and structural properties by interfering with the expression of host genes encoding endogenous proteases and glycosyltransferases as well as targeting the RNA silencing machinery. Many such examples have been discussed in recent reviews.

One of the most widely used hosts for molecular farming is *Nicotiana benthamiana*—a close relative of common tobacco. This species has been used extensively as a model for the functional analysis of plant genes and interactions with pathogens because it has a short lifecycle and carries a mutation that makes it hyper susceptible to many viruses. This prompted its development as an expression host for viral vectors, and more recently it has emerged as the key industrial platform for transient expression, particularly for the production of antibodies and vaccine candidates. For example, *N. benthamiana* was used to manufacture ZMapp, which is a cocktail of three IgG antibodies for the treatment of Ebola virus infections as well as clinical vaccine candidates against hepatitis B virus and influenza virus and, most recently, SARS-CoV-2.

The optimization of *N. benthamiana* and other tobacco host species is challenging due to the large and complex allotetraploid genome, which means that multiple copies of each target gene are usually present and must be mutated simultaneously. Many *N. benthamiana* products have been manufactured in a transgenic line in which the N-linked glycosylation pathway is suppressed by RNA interference (RNAi), but silencing is incomplete leading to the production of some plant-type complex N-linked glycans. More recently, multiplex genome editing has been used for the same purpose. Several xylosyltransferases (XyIT) and fucosyltransferases (FucT) were knocked out in *N. benthamiana* using TALENS and the complete set of XyIT and FucT genes was subsequently knocked out using the CRISPR/Cas9 system, leading to products with no detectable plant-type complex N-linked glycans at all. Similar work has been reported in tobacco BY-2 cells. In the context of O-linked glycosylation, the knockout of a single prolyl-4-hydroxylase (P4H) gene via homologous recombination in the moss *Physcomitrella patens* showed promise for the production of recombinant human proteins devoid of undesir able plant-specific hydroxyproline residues. Most studies in higher plants, however, have focused on the synthesis of human mucin-type O-linked glycans rather than the suppression of plant-type O-linked glycans. We, therefore, targeted the *N. benthamiana* P4H4 gene family, which encodes some of the key metabolic enzymes responsible for the first step of plant-type O-linked glycosylation.

Gene-edited plant lines are usually produced by the in vitro regeneration of modified cells under selection, but this is a laborious and time-consuming process that involves the handling and genotyping of large numbers of plants. Typically, the CRISPR/Cas9 cassette is left in the genome, and further work is required to segregate the transgene from the mutations and thus generate transgene-free lines. The workload becomes more difficult to manage for the introduction of multiple mutations, and ideally, it would be possible to introduce all mutations and remove the transgene cassette in the T0 and T1 generations, respectively. Fluorescent proteins can be used for the noninvasive confirmation of transgene removal, and this has been applied to CRISPR/Cas9 editing in many species. Although green fluorescent protein (GFP) is widely used as a marker, its spectral properties overlap with several plant pigments and a preferable alternative is DsRed2, a mutant of the DsRed protein from the coral *Discosoma* sp., which is easily distinguished from plant cell autofluorescence. When the DsRed gene is linked to a transgene encoding the recombinant protein of interest, it can be used not only as a transgene marker but also in many cases as a semiquantitative indicator for the expression of the linked transgene.

Here we extend CRISPR/Cas9 genome editing in *N. benthamiana* by adding a DsRed2 reporter to simplify transgene tracking in the mutated population at any vegetative stage across the T0 and T1 generations. The vector, which produces multiple gRNAs released from a concatemer by Csy4 ribonuclease, was optimized by adding a scaffold attachment region (SAR) and a plastid-targeted DsRed2 sequence to maximize the local concentration of DsRed2 and thus reduce the limit of detection. As proof of concept, we targeted P4H group 4 (Nbp4H4) genes, which are strongly expressed in *N. benthamiana* leaves. Our system achieved strong DsRed2 fluorescence in *N. benthamiana* leaf tissues during transient expression and stable transformation experiments. This not only simplified the selection of T0 transformants likely to contain the desired mutations but also allowed the early identification of homozygous and biallelic quadruple knockout T1 seedlings lacking the SpCas9 transgene. Mass spectrometry analyses of a reporter protein harboring multiple proline and O-glycosylation sites showed some changes in the hydroxyproline content when produced in edited plants, thus confirming the effectiveness of the editing tool in a functional context.

## 2 EXPERIMENTAL SECTION

### 2.1 Plant material and growth conditions

*N. benthamiana* seeds were germinated in bulk in a small greenhouse box containing soil, which was placed in a growth chamber under
long-day (16-h photoperiod) conditions. We transferred 1-week-old seedlings into separate pots for maturation. Plants used for transient expression were kept at room temperature after agroinfiltration and were maintained in the greenhouse with a long-day photoperiod. Transgenic plants were generated by the Agrobacterium-mediated transformation of cotyledons. Sterile seedlings and tissue cultures were grown in an incubator at 25°C with a long-day photoperiod. After rooting, T0 primary transformants were transferred to soil and grown in a growth chamber under long-day conditions at 24°C. All subsequent generations were propagated directly in the growth chamber starting from seeds as described above.

2.2 | Phylogenetic analysis

We targeted the *N. benthamiana* P4H4 genes by locating all putative P4H sequences in the *N. benthamiana* genome databases using BLAST, starting with the protein and DNA sequences of the known homolog Nbv6.1trP32386.[43] We screened the *N. benthamiana* Sequencing Consortium (NbSC) database (https://www.nbenth.com/), the Queensland University of Technology database (https://benthgenome.qut.edu.au/) and the Sol Genomics Network (https://solgenomics.net/). The genomic DNA, gene and protein sequences were aligned using the MAFFT Algorithm L-INS-i.[44]

2.3 | Design of gRNAs

We used CCTop software (https://cctop.cos.uni-heidelberg.de:8043/) to identify putative target sites[45] with Tobacco *Nicotiana benthamiana* Niben101” set as the reference genome. The parameters were set to 20 nt maximum length and 12 nt seed sequence length allowing up to four mismatches for the identification of off-targets and up to two mismatches in the core region. We selected gRNAs with no predicted off-targets within other genes and that targeted the N-terminal region of the coding sequence of at least two *NbP4H4* homologs simultaneously (Supplementary Table S1).

2.4 | Construct design and cloning

We developed two CRISPR/Cas9 binary vectors (pBV114 and pBV113) containing the DsRed2 gene. The vectors were identical except that the former contained a SAR between SpCas9 and DsRed2 transcriptional units (Figure 1). The vectors were generated by combining conventional restriction and ligation techniques with Golden Gate assembly, using primers synthesized by Sigma-Aldrich (Germany) and cloning reagents from Thermo Fisher Scientific (Austria).

The concatenated gRNA cassettes were assembled as previously described.[42] Briefly, we added SapI and AarI restriction sites to vector pJET1.2 (Thermo Fisher Scientific) to produce the modified vector pUV1. This allowed the Golden Gate assembly of gRNA cassettes at the SapI site, followed by transfer to the destination vector pBVM5/pBVM5.2 using the AarI site. Vectors pYLCV1 and pOGS41, containing the Centrum yellow leaf curling virus (CmYLCV) promoter[46] and an optimized sgRNA scaffold[47] were used as templates for the amplification of the corresponding cassette elements. Partially overlapping protospacer halves together with Esp3I restriction sites were incorporated into the primers such that unique complementary ends were formed by restriction, ensuring ordered ligation of the elements and the reconstruction of the protospacers in the resulting cassette. Likewise, SapI sites flanking the outermost elements of the cassette allowed its incorporation into pUV1 for amplification in *Escherichia coli*.

After sequencing, the intermediate plasmids containing the assembled gRNA cassette to confirm the lack of mutations, the gRNA module controlled by the CmYLCV promoter was transferred to binary vectors pBVM5 and pBVM5.2 to create the final vectors pBV113 and pBV114, respectively. The binary vector pBVM5 was constructed using parts originating from vectors pDIRECT_21C[42] pTRAKt_HC,[48] B357p9ioR-35sCasWT (DNA Cloning Service, Germany) and pDsRed2 (Clontech/Takara, USA). The DsRed2 gene was fused to a plastid transit peptide sequence from the barley starch synthase I gene[41] and was also equipped with a C-terminal 3xFLAG tag sequence. Vector pBVM5.2 is a derivative of pBVM5 with the SAR removed.

2.5 | Transient expression in *N. benthamiana*

The binary vectors were introduced into chemically competent *Agrobacterium tumefaciens* strain GV3101(pMP90) using the freeze-thaw method. Overnight cultures containing pBV113 or pBV114 were prepared from a single colony in a YEB medium supplemented with 25 mg L⁻¹ gentamycin and 100 mg L⁻¹ spectinomycin. The next day, the cultures were diluted to OD₆₀₀ = 0.4, and a syringe was infiltrated into the youngest fully expanded leaves of 5-week-old wild-type plants by agroinfiltration.[11] The tissues were collected 5 days post infiltration (dpi), snap-frozen in liquid nitrogen, and stored at –80°C for further analysis.

2.6 | Stable transformation of *N. benthamiana*

*NbP4H4* knockout lines were established by germinating sterile *N. benthamiana* seeds on plates containing half-strength Murashige and Skoog (MS) medium (Duchefa, Netherlands) at pH 5.7, supplemented with 2.4 g L⁻¹ phytagel and 3% w/v sucrose. The plates were nonhermetically sealed with Parafilm and grown in a growth chamber at 25°C with a 16-h photoperiod. On the sixth day, the seedlings were inoculated with *Escherichia coli* carrying pBV113. The original method was modified by centrifuging the *A. tumefaciens* culture and resuspending the resulting pellet in a 1 ml induction medium (MS medium plus 50 g L⁻¹ sucrose and 1.8 g L⁻¹ glucose, pH 5.6) supplemented with 200 μM acetylseringone and incubating...
the cultures at 28°C in the dark for 1 h with gentle shaking. The suspension was then diluted with MS medium to OD₆₀₀ = 0.4. The cotyledons were separated from the roots and transferred to a Petri dish, where they were pricked with a sterile toothpick dipped in the A. tumefaciens suspension. After inoculation, the cotyledons were placed adaxial side up on a cocultivation medium (CCM), which is MS medium containing 3% w/v sucrose, 1× Gamborg vitamins, 10 mM MES-hydrate, 2 mg L⁻¹ BAP, 100 μM acetylsyringone and 2.4 g L⁻¹ phytagel (pH = 5.7), and incubated for 3 days in the dark at 25°C. Subsequently, the cotyledons were transferred to a selection medium (TSM1), which is MS medium containing 3% w/v sucrose, 1× Gamborg vitamins, 2 mg L⁻¹ BAP, 100 mg L⁻¹ kanamycin and 100 mg L⁻¹ timentin solidified with 0.8% w/v agar (pH = 5.7), for the selection of transgenic explants and suppression of A. tumefaciens overgrowth. The plates were sealed with Parafilm and placed in a growth chamber with a 16-h photoperiod for 2 weeks. The transformed cotyledons were then cut away from the stem with a sterilized scalpel and placed onto fresh TSM1. Subculturing was carried out every 2 weeks, and healthy-looking shoots were cut from the calluses and transferred into rooting medium (TRM), which is MS medium supplemented with 2.4 g L⁻¹ phytagel, 100 mg L⁻¹ kanamycin, and 100 mg L⁻¹ timentin, for root induction. Putative transgenic explants with nascent roots were transferred to sterile 0.125-L plastic cups containing TRM. Finally, well-rooted plants were transferred to soil in pots (height = 8 cm). Seeds from Cas9-positive T0 plants were germinated directly in the soil and the seedlings were screened to identify transgene-free T1 plants.

2.7 Mutant screening

Genomic DNA was isolated from agroinfiltrated N. benthamiana leaves using the NucleoSpin Plant II kit (Macherey-Nagel, Germany). To assess gRNA efficiency, each targeted exon was amplified using DreamTaq polymerase (FisherSci, Austria) and gene-specific primers based on the N. benthamiana draft genome sequence (Supplementary Table S2). The amplicons were characterized by agarose gel electrophoresis and Sanger sequencing (Microsynth, Switzerland). Transient expression data were compared with the sequencing chromatograms to determine mutation frequencies, and editing events were identified using TIDE (http://shinyapps.datacurators.nl/tide/) to track indels by decomposition. Knockout scores for gRNAs were calculated as the sum of all significant indels (p < 0.001) not including ±3 and multiples thereof (no frameshift expected).

Genomic DNA from stably transformed T0 plants was isolated from leaf tissues. A Retsch mill was used as suggested, and snap-frozen leaf tissue (50 mg) from 4-week-old N. benthamiana plants was ground with magnetic beads. The extraction buffer contained 1% DTT instead of β-mercaptoethanol, and after incubation with isopropanol, the solution was directly centrifuged at 13,000 × g for 20 min at 4°C. The pellet was then washed with 70% ethanol. T0 plants were first screened by PCR using primers 35S_F and DM1Cas9-304_R (specific for SpCas9) to confirm the presence of the transgene (Supplementary Table S2 and Figure S1B). Cas9-positive plants were then screened to determine the gRNA efficiencies and to identify homozygous lines for the next generation. T1 plants were screened for the absence of the SpCas9
transgene, the presence of induced mutations, and to confirm the results of fluorescence analysis.

2.8 | Fluorescence analysis

A white light source with a green excitation filter was used to visualize macroscopic DsRed2 fluorescence, which was observed through a red filter as previously described.[40] Infiltrated leaf tissues were observed with a Leica DM5500B fluorescence microscope equipped with a DFC 300 FX camera and DsRed filter set, and with a Leica SP5 confocal microscope. Samples were mounted in tap water on a glass slide. DsRed2 was excited at 561 nm and fluorescence emission was monitored at 573–642 nm. Images were captured using Leica Application Suite v4.10.0.

2.9 | Production and peptide analysis of recombinant IgA1

Three individual biological replicates for the edited and two for the wild-type plants were produced by infiltrating the construct into the respective 5-week-old plants to obtain 13 g of leaf material for each sample. Transient expression and purification of IgA1 from N. benthamiana wild-type or T2 plants of the NbP4H4 KO-line pBV113-4N1 (not showing DsRed fluorescence nor containing the Cas9 editing transgene, the presence of induced mutations, and to confirm the results of fluorescence analysis. peaks were summed, using the quantification software Skyline. Values were averaged over three biological replicates (n = 3) for the mutant and two biological replicates for the wild type (n = 2) and the standard deviation was calculated.

3 | RESULTS

3.1 | Design of gRNAs targeting N. benthamiana P4H4 genes

We selected NbP4H4 as our target because transcriptome analysis has shown high corresponding transcript abundance in N. benthamiana leaves (Gene Expression Atlas v6, https://benthgenome.qut.edu.au/). We used the published NbP4H4 sequence data[43] to screen all available N. benthamiana sequence resources with BLAST. N. benthamiana has a 3.1-Gbp allotetraploid genome spanning 19 chromosomes, and two independently assembled draft genome sequences[25,50] are publicly available via the SOL Genomics Network (https://solgenomics.net/) and the Queensland University of Technology website (https://benthgenome.qut.edu.au/). Furthermore, both PacBio and hybrid assemblies of the LAB strain genome[51] have been made available under “reserved analyses” status via Apollo (https://apollo.nbenth.com/).

We carried out a phylogenetic analysis of probable targets together with other members of the same gene family by constructing multiple alignments of the coding sequences as well as protein alignments of the putative catalytic domains, revealing a total of four NbP4H4 candidate genes (NbSC assembly v3.5 IDs: Nbl13g09210.1, Nbl09g08890.1, Nbl04g13800.1, and Nbl09g22440.1). These are described herein as NbP4H4_1–4. The correspondence between the NbSC and SOL Genomics Network gene models is straightforward for NbP4H4_1 (Niben101Scf09150g02004.1) and NbP4H4_2 (Niben101Scf02818g01017.1), whereas NbP4H4_3 is incorrectly annotated in the SOL Genomics Network and appears as three separate genes (Niben101Scf04674g00006.1, Niben101Scf04674g00009.1, and Niben101Scf04674g00010.1). Similarly, NbP4H4_4 is represented by two genes (Niben101Scf16019g00005.1 and Niben101Scf16019g00008.1). This is likely to reflect the misinterpretation of long introns within NbP4H4_3 and NbP4H4_4 as intergenic regions. The nomenclature of the NbP4H4 genes targeted in this study according to their ID in each of the databases is summarized in Supplementary Table S3.

Having established plausible gene models for the four NbP4H4 homologs, we attempted to identify common gRNA targets to allow the editing of all four genes using the minimal complement of gRNAs while also complying with standard requirements such as no off-targets or disruptive secondary structures. Given the sequence variability among the four genes, we only identified one pan-specific gRNA (G3). The other seven gRNAs target multiple exons in the first half of NbP4H4_1 and NbP4H4_2 (Figure 1). The gRNA sequences were then inserted into the binary vector, creating final vectors pBV113 and pBV114 for N. benthamiana transformation experiments. The vectors...
TABLE 1 (A) Six transgenic T0 plants (NbpBV113_1 to NbpBV113_6) showing clear DsRed2 fluorescence were analyzed for mutation frequencies at the six target sites

| Guide | Target(s) | NbpBV113_1 | NbpBV113_2 | NbpBV113_3 | NbpBV113_4 | NbpBV113_5 | NbpBV113_6 |
|-------|-----------|------------|------------|------------|------------|------------|------------|
|       |           | Mutation frequency (%) | KO score (%) |
| G3    | P4H4_1    | n/a        | 93.2       | 96.3       | 91.5       | 94.9       | n/a        |
|       | P4H4_2    | 33.5       | 31.0       | 35.6       | 10.8       | 8.3        | 38.2       |
|       | P4H4_3    | 36.6       | 30.6       | 94.0       | 91.6       | 89.1       | 98.9       |
|       | P4H4_4    | 70.7       | 65.3       | 99.3       | 92.8       | 91.6       | 99.3       |
| G7    | P4H4_1    | 43.9       | 33.3       | 94.0       | 96.0       | 91.7       | 98.0       |
|       | P4H4_2    | 42.5       | 32.6       | 60.3       | n/a        | 70.5       | 63.4       |

Note: Amplicons spanning these target sites were sequenced and the mutation frequencies were evaluated using TIDE. Mutation frequency is a percentage that refers to all indels (p < 0.001) after decomposition, whereas the knockout (KO) score is a percentage that refers only to indels leading to a frameshift.

(B) Mutations in T1 plants lacking the Cas9 transgene (confirmed both by PCR and the lack of DsRed2 fluorescence). The lengths and types of the indels are indicated (deletion –, insertion +, wild-type wt). The zygosity of the mutation was determined according to the presence or absence of wild-type sequences and the number of overlapping chromatogram traces. Heterozygous (HET) = one allele is wild-type, the other is mutated. Biallelic (BIAL) = both alleles are mutated, but the mutations are distinct. Homozygous (HOM) = both alleles carry the same mutation.

| Guide | Target(s) | INDELs/Zygosity/T - DNA present |
|-------|-----------|---------------------------------|
| G3    | P4H4_1    | +1/+1 HOM no                    |
|       | P4H4_2    | -1/-1 HET n/a                   |
|       | P4H4_3    | -5/-5 HOM no                    |
|       | P4H4_4    | +1/+2 BIAL no                   |
| G7    | P4H4_1    | +1/-1 BIAL no                   |
|       | P4H4_2    | n/a HET +2/-1 BIAL no           |

Note: n/a = not available because the sequence chromatogram comprised too many overlapping traces to be properly analyzed for indels (after including three technical replicates).

To evaluate DsRed2 as a visual selection marker for transgenic plants, we visualized the macroscopic and microscopic fluorescence of agroinfiltrated N. benthamiana leaves at 6 dpi. Macroscopic fluorescence was observed in all parts of the infiltrated leaf (Figure 3A) and was clearly distinguished from the autofluorescence of chlorophyll and other pigments in wild-type plants (Figure 3B). Epifluorescence and confocal microscopy showed, as expected, that DsRed2 was localized in the plastids of agroinfiltrated plants (Figure 3B) but not in wild-type plants (Figure 3D). Confocal microscopy confirmed that the autofluorescence detected at the excitation wavelength of chlorophyll was not detected at the lower wavelength used to excite DsRed2 (Figure 3D).

3.2 Determination of gRNA efficiencies and confirmation of DsRed2 fluorescence

We assessed the relative efficiencies of the eight gRNAs by transient expression. DNA was isolated from N. benthamiana leaves at 6 dpi and amplified by PCR using primers spanning each target site. The amplicons were then sequenced and the efficiency of each gRNA was determined using TIDE (Figure 2). The efficiency of the pan-specific gRNA (G3) differed at each target, ranging from 10.9% in NbP4H4_1 to 41.4% in NbP4H4_2, 42.3% in NbP4H4_3, and 46.5% in NbP4H4_4 (Figure 2A). All but one of the other gRNAs also induced mutations, with efficiencies ranging from 8.0% (G5, targeting NbP4H4_1) to 40.2% (G6, targeting NbP4H4_2). The transient expression experiments not only determined the efficiency of the individual gRNAs but also confirmed that the binary vector system as a whole was functional.

3.3 Generation, selection, and analysis of P4H4 quadruple knockouts

We combined gRNAs G3 and G7 and placed them in tandem in binary vector pBV113 for stable transformation experiments, thus targeting six potential mutation sites (two each in NbP4H4_1 and NbP4H4_2, and one each in NbP4H4_3 and NbP4H4_4). N. benthamiana
cotyledons were transformed with vector pBV113 and transgenic explants were selected based on their resistance to kanamycin and the presence of macroscopic DsRed2 fluorescence, which was visualized using a simple lamp and filter combination (Supplementary Figure S1A). We recovered 14 T0 plants and transferred them to the soil. Six continued to show strong DsRed expression (Supplementary Figure S1C) and were screened by PCR for the presence of the SpCas9 gene, which was confirmed in all cases (Supplementary Figure S1B). PCR with exonspecific primers for each NbP4H4 gene followed by Sanger sequencing revealed genome editing events at all six target loci (Table 1A). Most
FIGURE 3  Monitoring the transient expression of DsRed2 as a visual marker. (A) Wild-type Nicotiana benthamiana plant infiltrated with vector pBV113 containing a scaffold attachment region (blue dot, left of leaf vein) or pBV114 (blue dot, right of leaf vein) in daylight (left panel) and under a green light/red filter (right panel). (B) Wild-type N. benthamiana plant, no treatment, in daylight (left) and under green light/red filter (right). (C) Confocal microscopy of sections of N. benthamiana leaves infiltrated with vector pBV113 compared to (D) an untreated wild-type plant: DsRed2 fluorescence located in plastids (left), plastid autofluorescence (middle) and merged image (right). No DsRed2 signal was observed in the wild-type control tissue (D). All pictures were taken 6 days post infiltration. (E and F) Representative samples of two T1 transgenic lines germinated on half-strength MS medium, pictured in daylight (right panels) and displaying DsRed2 fluorescence (left panels). (E) Mutant line NbpBV113_3 and (F) NbpBV113_4. All pictures were taken with the same camera exposure times.

of the mutations were single-nucleotide insertions or short deletions, but we also recovered two inversion events. Remarkably, all six plants had mutations in all four target genes, and the vast majority of mutations resulted in a frameshift. Plants NbpBV113_3 and NbpBV113_4 showed an editing efficiency close to or above 90% in at least four target sites, indicating a high probability of heritability.[53] These plants were allowed to self-pollinate and produce seeds.

3.4  Identification of transgene-free T1 plants by DsRed2 screening

We germinated 20–50 seeds per line and visualized macroscopic DsRed2 fluorescence to distinguish between plants with transgene expression and potential negative segregants (Figures 3E,F). Individual plants lacking a DsRed2 signal were tested by PCR to confirm
the absence of the SpCas9 cassette, and the mutant genotype was verified by Sanger sequencing (Supplementary Figures S2A,B). The zygosity of the mutations was determined by checking for the presence or absence of wild-type sequences and the number of overlapping chromatogram traces. Accordingly, we were able to readily identify a transgene-free plant among the progeny of NbpBV113_4, which contained biallelic or homozygous KO mutations in all four NbP4H4 genes (Table 1B). No visual phenotype correlating with the quadruple knockout was detected. However, a phenotype segregating with the SpCas9 cassette was observed in line NbpBV113_3, where the fluorescent plants were smaller than wild-type plants of the same age (Figure 3E). This phenotype was probably caused by a genomic integration effect.

3.5 | Prolyl 4-hydroxylation and O-glycosylation status of recombinant IgA1

It is well known that the members of the P4H enzyme family are required to initiate the O-glycosylation of arabinogalactan-proteins, extensins, and recombinant glycoproteins produced in plants.\textsuperscript{[34]} To assess the functional consequences of knocking out the P4H4 subset of these enzymes we used quadruple knockout plants for the recombinant production of IgA1 and subsequently analyzed its proline-rich hinge region. In LC-ESI-MS spectra of IgA1 peptide HYTNPSQDGVTVPCKVPSPPTPSPTPSPSCCHPR (Figure 4A) the conversion of up to five proline residues to hydroxyproline could be detected as well as the presence of additional pentoses, indicating arabinosylation. Relative quantification of glycoforms was performed to monitor changes due to genome editing. Since many of the glycosylated peptides were present only in low quantities, their total amount was summarized for each hydroxyproline-residue and compared to peptides with oxidized prolines lacking any pentoses (Figure 4B). A reduction in the relative amounts of peptides containing one to four hydroxyprolines could be observed for the reporter protein produced in the NbP4H4 knockout mutant compared with the wild type, whereas peptides containing five hydroxyproline residues were present in similar amounts in both groups. Surprisingly, however, the relative amount of the unmodified hinge region was slightly reduced and the overall amounts of glycopeptides with pentoses were increased for the mutant, possibly indicating a rebalancing effect due to the remaining P4H activities. This indicates that members of the P4H4 subfamily contribute to hydroxyproline formation in a recombinant protein, resulting in a shift in glycosylation pattern, but further studies are needed to elucidate the exact mechanisms.

4 | DISCUSSION

The functional analysis of plant genes by mutation is hampered by the presence of paralogs or homeologs with redundant or overlapping roles, particularly in polyploid species such as \textit{N. benthamiana}. The same issue arises when attempting to modify plants to remove undesirable properties, which is necessary during the domestication of crops, but also for the development of plants as a production platform for valuable molecules including recombinant proteins. We therefore established a rapid and straightforward procedure for multiplex genome editing in \textit{N. benthamiana} followed by the selection of transgene-free mutant lines.

As proof of concept, we targeted a subgroup of \textit{NbP4H} genes encoding prolyl-hydroxylases responsible for the first committed step of plant-type O-linked glycosylation.\textsuperscript{[34]} There are major differences between O-linked glycosylation in plants and mammals, which makes it advantageous to eliminate the endogenous pathway in plants. Blocking the first step (the conversion of proline to hydroxyproline in the sequence –Ser-X-X-Pro-) is a promising strategy to achieve this. However, the complete abolition of NbP4H activity is challenging because the allotetraploid \textit{N. benthamiana} genome contains a large number of \textit{P4H} genes falling into at least four homology groups (\textit{NbP4H1}, \textit{NbP4H4}, \textit{NbP4H9}, and \textit{NbP4H10}).\textsuperscript{[43]} \textit{NbP4H4}, which shows the highest transcriptional activity in leaves, appears to comprise four homologous candidate genes. It can be difficult to correctly identify and differentiate homologs in \textit{N. benthamiana} due to sequencing errors, incorrect annotations, and the diverse genotypes and accessions used in different experiments.\textsuperscript{[54]} We therefore cannot completely rule out the possibility that even more \textit{NbP4H4} genes remain to be discovered in this species. Indeed, two of the \textit{NbP4H4} genes targeted in this study had been incorrectly annotated in one genome assembly, probably due to the presence of long introns that were misinterpreted as intergenic regions.

Despite the extensive sequence divergence between the homeologous \textit{NbP4H4} loci, we found one target site (G3) that was conserved in all four genes. However, a single gRNA targeting multiple loci can be insufficient for the complete abolition of gene function because the mutation efficiency often varies between sites. We therefore utilized a multiplex gRNA system in which concatenated transcripts of up to eight gRNAs are processed by Csy4 ribonuclease.\textsuperscript{[42]} Testing was carried out by transient expression, which allowed the rapid screening and comparison of gRNAs. Although there is no guarantee that the results can be replicated in stably transformed lines, it nevertheless allows the exclusion of gRNAs with very low efficiency. Ultimately, we selected two gRNAs (G3 and G7, the former targeting all four genes and the latter targeting \textit{NbP4H4_1} and \textit{NbP4H4_2}) making a total of six target sites.

The presence of a strong and constitutively expressed fluorescent marker cassette enabled us to visually identify and thus positively select for plants expressing the SpCas9 transgene during the transformation process, and negatively select for such plants in later generations when we were seeking to propagate transgene-free lines. Previously reported editing vectors with integrated visual markers showed fluorescence mostly at the seed stage\textsuperscript{[35,55]} or need special equipment and transformation methods to enrich for the desired mutants.\textsuperscript{[56]} Others have used fluorescent proteins in cotransformation experiments with double T-DNA binary vectors without an editing cassette\textsuperscript{[57]} or with multiple \textit{A. tumefaciens} strains, needing confocal microscopy to evaluate the results.\textsuperscript{[57]} In contrast, our system allows
the selection of fluorescent explants in any generation as soon as the leaf tissues develop. The presence of the SpCas9 cassette in the selected transgenic lines was confirmed by PCR and correlated with the visible fluorescence, indicating that DsRed2 is suitable as a marker to report the presence of adjacent transgenes, as already reported for antibody-producing transgenic maize plants. All six T0 plants displaying strong and evenly distributed fluorescence in the leaves also carried targeted mutations in the four NbP4H4 genes, and two of the plants showed editing efficiencies close to or above 90% at four of the target sites. It is tempting to speculate that selection for strong DsRed fluorescence may also favor the high expression of the Cas9 and gRNA cassettes located on the same T-DNA, increasing the mutation efficiency, in agreement with the finding that Cas9 availability is a limiting factor in multiplex genome editing. However, this hypothesis would need to be confirmed with a larger sample of plants.

The ultimate aim of genome editing in plants is the generation of offspring that carry the desired homozygous or biallelic knockout mutations in all target genes but lack the SpCas9 transgene because this avoids the possibility of subsequent off-target mutations and also allows the direct comparison of mutant and wild-type plants without potential pleiotropic effects caused by transgene expression or integration. Transgene elimination is also important for plant breeding because it ensures regulatory compliance and genetic stability. Finally, transgene elimination is required for the stacking of traits by the sequential combination of mutations. Traditional methods for the negative selection of transgenic plants include the use of markers that...
confer sensitivity to particular herbicides or antibiotics\cite{58} and the use of fluorescent markers that allow visual screening.\cite{59} Our vector system used DsRed2 to identify negative segregants in the T1 population derived from the two T0 plants with the highest editing efficiency. We analyzed the T1 seedlings devoid of fluorescence and confirmed both the absence of the SpCas9 transgene and the heritability of the mutations. Despite analyzing only three individual siblings per line, we were able to identify quadruple knockout plants with homozygous or biallelic mutations in all the target genes.

The loss of P4H activity in Arabidopsis thaliana was reported to cause root-hair phenotypes and problems with cell wall assembly.\cite{60} We did not observe any obvious phenotypes associated with the NbP4H4 quadruple knockout, perhaps because we selected only one particular subfamily of P4H enzymes. On a functional level, targeting this subset of enzymes in our study led to distinct changes in recombinant IgA1 peptides containing hydroxyproline residues and pentoses. Some of these changes, like slightly decreased amounts of the unmodified hing region or overall increased amounts of glycopeptides with pentoses, were unexpected for a P4H knockout but may be explained by isoform-specific substrate preferences of the remaining P4H enzymes and other unknown factors. Indeed, a total of four homology groups of putative P4H genes have been described in \textit{N. benthamiana}, each one comprising many paralogous and/or homoeologous copies, and the functional characterization of one representative gene from each group revealed very similar enzymatic activities but slightly different substrate preferences.\cite{43}

This highlights the difficulties faced when editing combinations of such genes to suppress the plant-specific oxidation of prolyl residues in recombinant proteins. However, improvements in multiplexing strategies and simple screening procedures, such as those demonstrated here, will facilitate the combined knockout of further members of this enzyme family to develop plant hosts in which the O-linked glycosylation pathway is disabled specifically in the tissues used for recombinant protein production.

5 | CONCLUSION

The identification of mutant genotypes or phenotypes following multiplex genome editing in plants often involves the expensive and labor-intensive screening and/or sequencing of large numbers of individuals. We have established a simple and rapid workflow for the generation and identification of homozygous or biallelic higher-order mutants in \textit{N. benthamiana} within one generation while keeping the number of T0 and T1 plants and the corresponding sequencing efforts to a minimum. The process was based on the adaptation of existing multiplexing elements to function efficiently in the model plant \textit{N. benthamiana} and combines transient expression to confirm the efficiency of different gRNAs and the use of DsRed as a reliable indicator for the presence of the editing cassette in the leaf tissues of T0 and T1 transgenic plants. We were able to recover transgene-free T1 progeny plants with biallelic or homozygous mutations in all four \textit{NbP4H4} target genes, showing changes in hydroxyproline formation on a glycoprotein reporter. These plants represent a valuable starting material for further functional comparisons and for additional rounds of editing to achieve the abolition of P4H activity and generate new host plants for molecular farming.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Richard Strasser \(\text{https://orcid.org/0000-0001-8764-6530} \)
Eva Stoger \(\text{https://orcid.org/0000-0002-7651-7992} \)

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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