Genome Editing With CRISPR/Cas9 in *Pinus Radiata* (D. Don)

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Abstract

Background

To meet increasing demand for forest-based products and protect natural forests from further deforestation requires increased productivity from planted forests. Genetic improvement of conifers by traditional breeding is time consuming due to the long juvenile phase and genome complexity. Genetic modification (GM) offers the opportunity to make transformational changes in shorter time frames but is challenged by current genetically modified organism (GMO) regulations. Genome editing, which can be used to generate site-specific mutations, offers the opportunity to rapidly implement targeted improvements and is globally regulated in a less restrictive way than GM technologies.

Results

We evaluated U6 snRNA promoters from three different species that were able to drive expression of guide RNA (gRNA) for CRISPR/Cas9 genome editing in *P. radiata*. Using a single-copy cell wall gene GUX1 as a target, we have demonstrated genome editing using CRISPR/Cas9 in somatic embryogenic tissue and plantlets derived from the edited tissue. We generated biallelic INDELs with an efficiency of 15% using a single gRNA. Twelve percent of the transgenic embryogenic tissue was edited when two gRNAs were used and deletions of up to 1.3 kb were identified. However, the regenerated plants did not contain large deletions but had single nucleotide insertions at one of the target sites. We also assessed the use of CRISPR/Cas9 ribonucleoproteins (RNPs) for their ability to accomplish DNA-free genome editing in *P. radiata*. We chose a hybrid approach, with RNPs co-delivered with a plasmid-based selectable marker. A two-gRNA strategy was used which produced an editing efficiency of 33%, and generated INDELs, including large deletions. Using the RNP approach, deletions found in embryogenic tissue were also present in the plantlets. But, all plants produced using the RNP strategy were monoallelic.

Conclusion

We have demonstrated the generation of biallelic and monoallelic INDELs in the coniferous tree *P. radiata* with the CRISPR/Cas9 system using DNA and RNPs respectively. This opens the opportunity to apply genome editing in conifers to rapidly modify key traits of interest.

Background

Population growth, coupled with the need to transition from a petrochemical-based economy towards a more sustainable bio-based one, is predicted to increase the demand for wood and other forest-based products three-fold by 2050 [1]. This increased demand, together with the challenges associated with climate change and the need to increase agricultural production, will put further pressure on the area and quality of natural forests. It has been estimated that planted forests, which comprise only 7% of the global forest area, have the potential to supply two-thirds of global roundwood demand [2] and offer a

route to sustainably increase the production of forest products and reduce pressure on natural forests. To meet the increased demand, it will be necessary to further increase the productivity of planted forests.

There is a long history of productivity improvements in commercially planted conifer species through traditional breeding and silviculture [3, 4]. The use of genomic-based breeding technologies, particularly the implementation of genomic selection are also showing promise for implementation into breeding programs [5–7]. Long breeding cycles, large and complex genomes, variable juvenile-mature correlations, emerging pests and diseases, climate, and market changes provide challenges to breeding approaches that have to date led to moderate gains in conifers [6, 8].

Direct manipulation of conifer genomes offers a potentially more rapid route to trait improvement and allows the introduction of novel traits as well as improvement of existing ones. Demonstrated trait modifications in conifers include; insect resistance [9, 10], herbicide tolerance [11, 12], wood pulping efficiency [13, 14], stress tolerance [15] and sterility [16]. These technologies also enable production of rationally designed trees that produce biochemicals and biomass for specific purposes [17], yet, no modified conifers have been commercialized. These modification technologies require the introduction of new genes either via *Agrobacterium* or biolistic based methods [18]. However, the transformation of conifers is challenging, relying on complex somatic embryogenesis protocols, with many species or genotypes proving recalcitrant to somatic embryogenesis protocols and/or transformation [18]. The lack of efficient transformation systems for elite germplasm intended for large-scale production remains a major challenge for genetically modified varietal forestry [19].

Over the last decade, genome editing, particularly the CRISPR/Cas9 system, has been widely used in plants, both for fundamental research and precision breeding [20–22], with the first genome-edited food introduced into the market in 2019 [23]. Novel traits or traits difficult to achieve by breeding, such as biotic- and abiotic-stress resistance [24–26], and sterility [27] can be generated by knockout-mediated trait improvement. Desirable traits can be fine-tuned by generating a range of alleles through either genome editing or base editing [28–31]. Successful demonstrations of editing have included trees like poplar and eucalyptus [32–35]. As far as we are aware, genome editing is yet to be demonstrated in coniferous trees.

Globally, organisms that have had foreign DNA introduced into their genome are considered to be GMOs and are subject to various levels of regulation. However, genome edited plants where the transgene has been removed by crossing and segregation, are not regulated as GMOs in many countries, including Australia, Argentina, Canada, Japan, and the USA [36, 37]. Yet, the European Union and New Zealand still considers such mutated plants to be GMOs and regulates them accordingly [38].

In conifers, removing transgenes by segregation is challenging due to their long breeding timescales. Genome editing mediated by direct delivery of CRISPR/Cas9 ribonucleoproteins (RNPs), circumvents the introduction of new DNA into the plant genome, and as above would not be regulated as GMOs in many countries [39]. The ability to produce edited plants without the requirement to undergo time consuming

breeding to remove transgenes makes the use of RNP-mediated editing particularly attractive for slow-breeding conifers.

Pinus radiata D. Don., a conifer species native to California, is the world's most extensively planted exotic softwood [40] due to its high productivity and suitability for the construction timber, furniture, pulp and paper industries [41]. It is predominantly planted in Australia, Chile, and Spain and is the dominant species in New Zealand planted forests, where it comprises 90% of the planted production forest area and contributes 1.6% to GDP [42]. We have investigated the use of CRISPR/Cas9 to edit the *P. radiata* glucuronic acid substitution of the xylan 1 (GUX1) gene [43, 44] and demonstrated genome editing using DNA and RNPs.

Results

Evaluation of RNA polymerase III promoters

To identify and evaluate the efficacy of promoters to express guide RNAs in *P. radiata*, we tested previously characterized U6 promoters from *Arabidopsis* and wheat and a novel promoter identified in the Douglas-fir genome sequence. A 549 bp promoter sequence upstream of the start of Douglas fir U6 snRNA gene was selected based on the presence of the putative conserved Upstream Sequence Element (USE) element (-63 bp to -55 bp) and TATA box (-29 bp to -22 bp) regulatory elements. A DNA sequence from the GUS gene was cloned downstream of each of the U6 promoters (Fig. 1A) to test its functionality in *P. radiata* using a fluorometric β -Galactosidase assay [45]. The U6 promoters from the three species did not show any significant difference (Fig. 1B). In subsequent work, the Douglas fir U6 promoter was used for single gRNA editing, while both the Douglas fir and wheat U6 promoter were used for two gRNA editing.

Genome editing with single guide RNA (gRNA)

We used the cell wall gene GUX1 (NCBI Accession No: MT628352), which previous work in our lab (unpublished) has shown to be present as a single copy, to investigate genome editing. Using a single gRNA at site1 (Fig. 2A), we generated 100 independent transgenic lines by co-transformation with two separate plasmids, one encoding CRISPR/Cas9 and the other, a gRNA and a selectable marker. The control lines were transformed with the plasmid containing the gRNA but without the plasmid encoding the CRISPR/Cas9. Transgenic embryogenic tissue growing on selective media was screened by Sanger sequencing of PCR products generated using primer1 and primer3 (Fig. 2A) flanking the targeted region to identify edited material. Initial testing identified 15 lines harbouring edits out of the 100 transgenic lines generated (additional file 2). All the edited lines had a single base pair insertion or deletion at the target cut site (Fig. 2B). Embryogenic tissue containing edits was matured to produce somatic embryos that were germinated to generate *in vitro* plantlets. Of the 15 edited lines, only 2 produced somatic embryos that were able to germinate. A total of 6 plantlets were produced, 5 plantlets germinating from the 24. DNA was extracted from needle material collected from these plantlets. The

PCR products spanning the region of interest were sequenced to confirm edits. All the plantlets as expected contained a single base insertion or deletion (Fig. 2C). No edits were found in the lines transformed with gRNA only constructs. To further characterize the type of insertions, we cloned the PCR products from the generated plantlets into an *E. coli* vector and sequenced 10 colonies derived from each plantlet. All insertions were either an adenine or cytosine base (Fig. 2C). No guanine or thymine insertion was detected in the edited plantlets. All the sequenced plantlets contained biallelic mutations, with one line being homozygous for the insertion (Fig. 2C). Different types of edits in the four edited plants (line 1 to line 5, Fig. 2C) originating from the same embryogenic tissue line was not expected. Line 3 died before analysis could be done. Based on the *in silico* translated peptide sequence, the single base pair insertion/deletion is predicted to produce a non-functional truncated protein due to premature termination by an in-frame stop codon.

Genome editing with two gRNAs

A strategy using two gRNAs targeting sites 1.2 kb apart within the GUX1 gene (Fig. 3A) was tested for its ability to produce deletions that could be easily screened by PCR. One hundred transgenic lines were generated of which 12 lines (12%) were successfully edited (additional file 2). The control lines were transformed with the plasmid encoding Cas9 without the gRNA. Two embryogenic lines with deletions greater than 1 kb were identified. Line 3 had a 1.2 kb deletion at the target cut sites, while line 2 had a 1.37 kb deletion 209 nucleotides downstream of target site2 (Fig. 3A). Other edited lines had single nucleotide insertions at one of the target sites (Fig. 3A). No edits were found in the 2 lines transformed with Cas9 only construct. When the edited embryogenic tissue was matured to produce embryos, line 2 produced a single plantlet while 4 plantlets were produced from line 3 (Fig. 3B). PCR products, amplified from DNA extracted from needles, were cloned and sequenced from these plantlets. The plantlets did not harbour the 1.37 kb or the 1.2 kb deletions identified in the embryogenic tissue, instead the plantlets had a single insertion at target site4 and were biallelic (Fig. 3B). Other successfully edited embryogenic lines did not produce plantlets. Like the earlier edits, these plantlets are predicted to introduce a premature stop codon producing a truncated protein.

Genome editing with ribonucleoprotein (RNP) complexes

To investigate the use of DNA-free genome editing in *P. radiata*, we tested the ability of directly delivered RNPs, comprising a Cas9 protein in complex with targeting gRNAs, to carry out editing. Because of challenges associated with direct selection of edited plantlets we used a hybrid approach to biolistically co-deliver RNPs with a plasmid-borne selectable marker to select for antibiotic-resistant transformed/edited cells [46]. Two different commercial SpCas9 proteins; Alt-R SpCas9 3NLS and Alt-R SpCas9 V3 were tested for their ability to edit the GUX1 gene. Guide RNAs with target site 142 bp apart (Fig. 4) were used with the intention of generating deletions. Twenty-two antibiotic-resistant transgenic embryogenic lines were generated for Alt-R SpCas9 3NLS and 12 lines for Alt-R SpCas9 V3 (additional file 2). Alt-R SpCas9 3NLS produced 5 edited embryogenic lines (22%) and Alt-R SpCas9 V3 produced 4

Processing math: 100%

lines (33%). Embryogenic tissue showed a variety of editing patterns (Fig. 4). Six

lines had a single nucleotide insertion at either one of the target sites. The single nucleotides inserted were either adenine, cytosine or thymine. Guanine insertions were not found in our analysis. Alt-R SpCas9 3NLS line 10 had a 142-nucleotide deletion between the target sites. Alt-R SpCas9 3NLS line 20 had a 1130 bp insertion at target site1 (Fig. 4) while Alt-R SpCas9 V3 line 3 had a 10 bp deletion at target site3 (Fig. 4). The edited embryogenic lines were then matured to produce embryos and subsequently plantlets. Germinated plantlets were then analysed by PCR amplification, cloning and Sanger sequencing. All the resulting plantlets maintained the INDELs seen in their respective embryogenic tissue, including the 142-nucleotide deletion in Alt-R SpCas9 3NLS line 10. Alt-R SpCas9 3NLS line 20 did not produce plantlets (Fig. 4). All embryos/plantlets produced using RNPs were monoallelic.

Discussion

The CRISPR/Cas9 system is a powerful tool for targeted mutagenesis. It has been widely applied in model species and increasingly in plants of agronomic or horticultural importance [47, 48] and trees [35, 49, 50]. Here, we demonstrate CRISPR/Cas9-mediated genome editing in the commercially important coniferous tree *P. radiata* using both transgenic-mediated production of the editing complex and through the direct delivery of RNPs.

Heterologous RNA polymerase III U6 promoters have been widely used to transcribe guide RNAs in plants [51]. To select promoters suitable for the efficient production of gRNAs in *P. radiata* we tested two of these, previously characterized from *Arabidopsis* [52] and wheat [53]. We also tested a novel U6 promoter isolated from Douglas fir. The lack of significant differences in the transcriptional efficiencies among the 3 U6 promoters demonstrates similar functionality in their respective species.

CRISPR/Cas is much simpler to implement than the other reported systems such as ZFN and TALEN [54, 55]. High-efficiency editing can be achieved even in large and complex plant genomes at each of the multiple targeted genomic loci [56, 57]. In this study we demonstrate that this system can efficiently, and site-specifically, edit an endogenous gene in *P. radiata* to produce mono- and bi-allelic mutations.

When editing with a single gRNA using a transgenic approach all edited plantlets had both alleles edited. Targeting the GUX1 gene with a single gRNA produced single nucleotide INDELS. We did not see large deletions using a single gRNA as are frequently seen in other plant species [58–60]. In single gRNA edited plantlets all the insertions were either a cytosine or an adenine. The pattern of insertion or deletion is determined by nucleotide – 4 from the protospacer associated motif (PAM) site [61]. The – 4 nucleotide in the gRNA site1 (Fig. 2A) is an 'A', which is predicted to mainly show double-strand break repair via insertion. A repeat of the PAM-distal nucleotide adjacent to the cut site is favoured over other nucleotides resulting, in this case, to be adenine [62]. When editing with a single guide RNA all plants were biallelic (carried edits in both alleles). However, only 20% of the edited plants that were regenerated carried the same edit on both alleles. Such biallelic edits are desirable for implementation as producing biallelic edits through crossing and segregation of trees carrying monoallelic ones would be time-consuming.

Screening of embryogenic tissue edited with two gRNAs identified large deletions in embryogenic tissue. However, none of the plantlets generated from this material contained these large deletions, instead the plantlets that were regenerated had only a single nucleotide insertion at one of the two target sites. This may be the result of the embryogenic tissue being chimeric because of delayed Cas9 cleavage occurring in the proliferating embryogenic tissue. This could result in plantlets being generated from differently edited sectors [63, 64]. Similar results have been observed in rice, where genomic deletions were present in transformed callus lines and plants with genomic deletions were rarely found [65]. Furthermore, the rate of genomic deletions with multiple gRNAs is considerably lower than single-site mutagenesis [66], which could have reduced the frequency of plants with large deletions.

It has been suggested that delayed Cas9 cleavage, after the first cell division, in proliferating cells can result in the production of chimeric editing [63, 64]. This may be occurring in the proliferating embryogenic material with plants being generated from embryogenic material derived from different edited cells within the cell line to those identified in the initial screening. This may also explain why embryos carrying different edits were generated from the same embryogenic mass in experiments using a single gRNA.

Genome editing with RNPs has been successfully applied in several species using protoplasts [39, 67–69] and immature embryos [70, 71]. In our study we have successfully edited *P. radiata* somatic embryogenic cells and produced plantlets using RNPs. All RNP edited plantlets were monoallelic, whereas plasmid-mediated CRISPR/Cas9 genome-edited plants were all biallelic. RNPs have a limited time frame for cas9 protein to cleave DNA to mediate editing before they are degraded by endogenous proteases, resulting in a higher likelihood of monoallelic mutation [72]. All culture and transformation experiments were carried out at 23 °C which is below the optimum for the Cas9 protein activity (37 °C) [73]. This sub-optimum temperature, combined with the short half-life of the RNP complex, may have contributed to the lack of biallelic edits. In contrast to editing using the transgenic delivery of the editing complex, deletions identified in embryogenic tissue were also present in the needles of plantlets produced from this tissue. This is likely also a product of the limited window of activity for editing by RNPs. Edited tissue is likely to be homogenous, as editing would only occur in the cells prior to their first division.

Genome editing efficiency from methods using plasmid DNA and RNPs cannot be compared directly due to the different types of molecules and concentrations used for editing. The efficiency of genome editing using transgenic (12–15%) and RNP (22–33%) based delivery of the editing machinery are similar to those using comparable approaches in other plant species [48] and will support the development of efficient editing platforms.

Conclusions

In this work we have demonstrated genome editing in *P. radiata* using both transgenic and RNP approaches. As far as we are aware, this is the first published demonstration of genome editing in a conifer species. This opens the way to apply genome editing for both fundamental science and

economically and environmentally important trait improvements. Optimisation of RNP-based protocols, to achieve transgene free delivery, would facilitate rapid deployment where DNA-free editing is not regulated as a GMO.

Methods

Plant material

All experiments were carried out using previously cryopreserved somatic embryogenic cell lines of *P. radiata* that were initiated at Scion. These lines were established from immature embryos harvested from green cones and proliferated on Glitz2, a modified Litvay media [74] before being cryopreserved. Briefly, Somatic embryogenic tissue recovered from cryopreservation was transformed either with plasmid DNA or ribonucleoproteins using biolistics methodology. Transformed embryogenic tissue was then proliferated on selective media with antibiotics before transfer to maturation, embryo germination and multiplication/elongation media to produce plantlets. Full details of tissue culture and transformation procedures are provided in additional file 1.

Promoter identification and evaluation

To evaluate the efficacy of *Arabidopsis thaliana*, wheat (*Triticum aestivum*, *T. durum*) and *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir) U6 promoters and their functionality in conifers, nucleotide sequences of *A. thaliana* U6-26 snRNA gene (NCBI accession no: X52528) [75] and wheat U6 gene sequence (NCBI accession no: X63066) [53] were used as queries for BLASTN searches in a locally hosted *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir) genome database V. 1.0 [76]. To test U6 promoters in conifers, each U6 promoter was fused to a DNA fragment of the GUS gene from nucleotide 426 to 691 (NCBI accession no: AF502128) [52]. Six thymidine nucleotides were added to the 3' end of the GUS fragments → serve as a start site for transcription. A 27 bp sequence from the 5' end of *Arabidopsis* U6 RNA gene was added to the 5' end of the GUS gene fragments to facilitate U6-specific 5' capping and increase the accumulation of GUS transcripts. [77]. The three U6 promoters with GUS fragment were synthesized by GenScript, Inc. (Piscataway, NJ, USA).

Selection of target sequences, vector construction and plant transformation

All editing targeted the GUX1 gene (NCBI accession no: MT628352) in *P. radiata*. Prior to gDNA design the genomic sequence of GUX1 was recovered from the genotype to be edited. Primers, Primer2 (5' CGATCTC T GGC T T GAGG3) and Primer4 (5' TGC GTGTAGC T A T GCAG3 (Fig. 2A), were used to sequence both alleles. For editing, gDNAs site1: 5' GCAG ∇ GATCACATG ∇ CAGAGG3, site2: 5' A T TAGGGACTCGG ∇ AGGGGG3, site3: 5' GG T GTA T GCTATC ∇ CGGCGG3 and site4: 5' AGCTGCAGG T GG ∇ ACTGCGG3 were selected from regions without allelic differences (Fig. 2A). To increase the efficiency of genome editing gRNAs were designed according to the protocols of Dang et al. [77]. A U6-26 terminator was added to the 3' end of the gRNA structure. We used the

online tools E-CRISP [78] and CRISPOR [79] to identify target sites in the gene (Fig. 2A). The target sites were selected based on efficiency and activity scores [80–82]. All vectors were synthesized by GenScript (Piscataway, NJ, USA).

Screening and analysis

For initial screening, DNA was extracted using CTAB [83] from 30–50 mg of post bombardment embryogenic tissue growing on selective media and screened for editing by PCR and sequencing. PCR was performed using 50 ng of genomic DNA and Q5 polymerase (NEB) following the manufacturer's instructions with annealing conditions of 60°C for 30 seconds and using primer1 (5' *CTG* ∇ ∇ *CCT* ∇ *CTGC* T *C3*) and primer3 (5' *CAGAG* T *G* ∇ *GAGTG* T *GCAT3*) (Fig. 2A). PCR products were either gel extracted or purified using exonuclease I and shrimp alkaline phosphatase (Exo-SAP) and sequenced by Sanger sequencing using primer2 and primer3 (Fig. 2A). Sequencing results were analysed by manual screening and the online tools ICE (<https://ice.synthego.com/>) and TIDE (<http://shinyapps.datacurators.nl/tide/>) [84]. Embryogenic tissue confirmed to harbour GUX1 edits were matured to produce somatic embryos and these were germinated and multiplied. Putatively edited plantlets were then characterized by cloning of PCR products generated from the plantlets into an *E. coli* vector and sequencing of 10 independent clones for each plantlet.

Abbreviations

CRISPR

Clustered regularly interspaced short palindromic repeats

ICE

Inference of CRISPR Edits

INDEL

Insertion or deletion

PCR

Polymerase chain reaction

snRNA

Small nuclear RNA

TALEN

Transcription activator-like effector nucleases

TIDE

Tracking of Indels by Decomposition

ZFN

Zinc finger nuclease

Declarations

Ethics approval and consent to participate

Processing math: 100%

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

CP performed transgenic genome editing with single and double gRNAs. LP performed genome editing with RNP. BG conducted all transformations and plant culture. CR provided all tissue culture material and conducted maturation media development. MS and GT conceived and directed the work. All authors read and approved the manuscript.

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Processing math: 100%

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Figures

Figure 1

Evaluation of RNA polymerase III U6 promoters. A. Graphical representation of the construct used. A 27 bp fragment from the 5' end of the *Arabidopsis* U6 RNA $\geq$ was added $\rightarrow$ 5' end of the GUS fragment to increase transcripts accumulation. B. Comparison of GUS expression driven by different U6 promoters by fluorometric β -Galactosidase assay. AtU6 – *Arabidopsis thaliana* U6-26 promoter, DfU6 – Douglas fir U6 promoter, TaU6 – *Triticum aestivum* U6 promoter. Data are mean $\pm$ SE (n=6).

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Figure 2

Editing of GUX1 with a single guide RNA. A. Graphical representation of the GUX1 gene sequence showing the gRNA target sites and primer binding sites. B. Pattern of edits identified in the 15 somatic embryogenic tissue lines. C. Allelic variations identified in different plantlets. Edited plantlet line numbers are denoted in parenthesis. CRISPR gRNA sites are underlined. Red text denotes the PAM site, blue text denotes insertions and "-" denotes deletions. 'Y' denotes a cytosine or thymine nucleotide. Scale bar 100 bp.

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Figure 3

Editing of GUX1 with two guide RNAs. A. Pattern of edits identified in the 12 somatic embryogenic tissue lines. B. Details of edits identified in needle tissue from germinated plantlets. CRISPR gRNA sites are underlined. Red font denotes the PAM site and blue font denotes insertions and "-" denotes deletions. 'R' denotes an adenine or guanine nucleotide, 'M' denotes an adenine or cytosine nucleotide.

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Details of editing in GUX1 using RNPs. Somatic embryogenic tissue and needles from germinated plantlets showed the same editing pattern. The red arrow shows the location of 1130 bp insertion. CRISPR gRNA sites are underlined and the PAM sequence is denoted by red nucleotides and "-" denotes deletions.

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