PROJECT PROFILE

Title**: Development of a genome editing platform for functional characterization of genes**

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Objectives:

- To analyze sequence variations in the *EcHKT1:1* gene in salt-tolerant and susceptible Eucalyptus clones.
- To develop a genome engineering platform in Eucalyptus and Casuarina for rapid functional analysis of genes involved in salt tolerance.

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SUMMARY

Use of guide RNAs that direct efficient gene editing without any off-target effects is important in the application of CRISPR/Cas technology. Methods for quick screening of efficient guide RNAs are important in trees like Eucalyptus and Casuarina, where in A. tumefaciens-mediated generation of gene-edited plantlets for assay of the edits can take more than a year. A rapid protocol for evaluating CRISPR Cas9 gRNA constructs and promoters for generating desired gene edits in Eucalyptus using a composite transgenic plant strategy was developed (Shamili et al., 2022; 2025). Similarly, to rapidly evaluate gene editing constructs in *C. junghuhniana*, a method for generation of composite transgenic plants through *A. rhizogene*-mediated transformation was also developed (Sangeetha, 2024).

The present study evaluated a synthetic promoter, MsPRP2, reported earlier to drive root-preferential and salt-inducible gene expression in Medicago sativa, Arabidopsis thaliana, and Glycine max, for generating the desired deletion in *EcHKT1;1* gene in *E. camaldulensis*. Two sgRNA sequences targeting the *EcHKT1;1* promoter and exon1 region separated by 1411 bp

were used to synthesize a polycistronic gRNA-tRNA cassette driven by the MsPRP2 promoter.

For rapid in-planta evaluation of this construct, it was cloned into CaMV-driven Cas9_1 and GFP-based transformation vectors for A. rhizogenes-mediated transformation. The pooled GFP-tagged roots generated 36 days after co-cultivation were used to evaluate the efficiency of the construct by PCR analysis and amplicon sequencing. While the control roots generated a 2375 bp amplicon, the GFP-tagged roots yielded a smaller 964 bp amplicon indicating the generation of the expected 1411 bp deletion in the *EcHKT1;1* gene. The RT-qPCR analysis showed a 3.22-fold (69 %) downregulation when compared to the wild A4RS roots.

To reconfirm these results obtained from GFP-tagged hairy roots, the *EcHKT1;1* gene editing constructs were also evaluated in whole plant transgenic events of Eucalyptus generated using A. tumefaciens. These transgenic events showed an expected 1411 bp deletion, in a heterozygous state. RT-qPCR analysis showed 2.08 fold (52 %) *EcHKT1;1* gene downregulation when compared to the control. The analysis was possible only 16 months after co-cultivation when sufficient leaves could be harvested. In contrast, the higher transformation efficiency of *A. rhizogenes*-mediated transformation, at around 20 % when compared to 0.59 % for *A. tumefaciens*, enabled pooling of GFP-tagged roots generated within 36 days from a larger number of composite transgenic plants.

This study thus demonstrates the feasibility of using rapidly generated GFP-tagged roots for quick evaluation of the efficiency of the MsPRP2 promoter and the selected gRNAs to generate desired gene deletions. Reports of the use of tissue preferential or conditional promoters to express gRNA constructs are few. MsPRP2 promoter could be used for targeting CRISPR/Cas9-mediated epigenetic modification like methylation or demethylation.

A. rhizogenes-mediated transformation was used to generate gene-edited composite transgenics. These were subjected to incremental salt treatment. At the end of 150 mM, it was observed that all 3 control A4RS pHKN29 Cas9_1 plants dried, while 1 out of 3 gene edited plantlets survived. Further, the amount of sodium concentration in these plants did not show any significance. The results indicate that generation of homozygous edits is required for generation of complete knockouts and phenotypic analysis.