Establishment of NAC Protein Knockout Mutants Using CRISPR-cas9

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Introduction

The vascular system in plants is responsible for the effective transport of water, nutrients, and photosynthates. This network supports the growth of large plant species, and elucidating the processes of vascular tissue development is crucial for horticultural purposes. The process of both xylem and phloem cell differentiation is tightly controlled by various regulatory proteins. Microarray analysis revealed that several members of the NAC family proteins are expressed during phloem development. We are interested in elucidating the functions of these NAC genes in phloem cell differentiation.

• Figure 1. Figure representing co-expression network of genes during phloem development. Late, middle and early stage genes are indicated. (Kondo et al. 2016)

It has been established that NAC028 and NAC057 are late stage markers of phloem differentiation and that they play a role in enucleation of phloem cells. Observation of the coexpression network of phloem genes indicated that NAC020 is
expressed early on during phloem development while NAC028 and NAC057 are expressed in the middle stages. Furthermore, it was observed in the lab that overexpression of NAC020 results in the disruption of APL expression. APL is a known master regulator of phloem cell differentiation, and therefore we hypothesize that NAC020 is an early stage inhibitor of phloem differentiation. However, the precise function of NAC020 remains unknown, as do the functions of NAC028, and NAC057.

In this project, we worked to establish mutants of these three NAC proteins in order to observe the effect of a knockout mutation on vascular development.

**Methods**

**Plant Materials**

*Arabidopsis thaliana* accession Columbia was used as the wild type plant species. These plants were used for the establishment of *nac020* mutants and will be used for the establishment of *nac020/nac028* and *nac020/nac057* double mutants.

**Designing sgRNA Construct Oligos**

The DNA sequences for NAC028 and NAC057 were found on the Arabidopsis tair website and sgRNA construct options were generated using the CRISPR direct website. Highly specific sequences were analyzed and the most appropriate oligos were ordered to induce a mutation 3 base pairs upstream of the PAM sequence.

**Oligo Annealing and Ligation**

Sense and antisense sgRNA strands were annealed in TE buffer at 95°C. The double-stranded sgRNA constructs were then ligated into linearized pEn_Chimera vector
using T4 Ligase. The reaction was allowed to proceed for 2 hours at room temperature.

**Bacteria Transformation**

DH5α competent cells were used for transformation. Cells were thawed on ice and 50 μL were used for each plasmid. 5 μL of respective vector was added to competent cell aliquots, tubes were kept on ice for 15 minutes, and then heat shocked at 42°C for 60 seconds. Tubes were returned for ice for 5 minutes, 1 mL of SOC media was added to each tube, and cell cultures were then incubated for 1 hour at 37°C. Tubes were spun down for 3 minutes at 6000 rpm, 900uL of supernatant was disposed of, and the pellet was resuspended in the remaining supernatant. Suspended cells were then pipetted onto agar plates with ampicillin, spread evenly, and allowed to incubate at 37°C overnight.

**Colony PCR**

Transformed colonies were checked for insertion of sgRNA construct via colony PCR. ExTaq His Polymerase was used with ExTaq buffer. The corresponding forward sgRNA oligo was used as the forward primer, and a primer present downstream of the insertion site inside the vector was used as the reverse primer. The DNA region containing the sgRNA construct was amplified for 30 cycles. The PCR product was imaged on a 1% agarose gel stained with Ethidium Bromide and captured with UV light.

**Gateway Reaction**
The sgRNA constructs were transferred from entry vector, pEn_Chimera, to
destination vector, pde_cas9, via gateway reaction. LR Clonase II enzyme was used
with TE buffer to perform the reaction.

**Results and Discussion**

**Sequencing and Genotyping of T3 nac020 Plants**

![Sequencing data](image1)
![Agarose gel](image2)

**Figure 2.** Left: Sequencing data featuring the PAM sequence and the region shortly upstream for seedling nac020 T3 #7. Right: Agarose gel showing the result of cas9 genotyping PCR.

The T3 generation of nac020 mutants was previously established in the lab. I
sequenced the NAC20 gene to check for homozygous mutants. We determined that
there was both an insertion and deletion mutation three base pairs upstream of the
PAM sequence. We performed genotyping to determine which T3 mutants had the
cas9 gene. We identified four seedlings that did not have the cas9 gene.

**Insertion of sgRNA Constructs Into Entry and Destination Vectors**

![Images of inserts](image3)
Figure 3. Left: Colony PCR of DH5α cells transformed with entry vector containing double-stranded sgRNA construct. Right: Colony PCR of DH5α cells transformed with destination vector containing double-stranded sgRNA construct.

The sgRNA construct oligos were ligated into the entry vector. The plasmids were then grown in competent E. coli cells and ligation was verified by colony PCR. The constructs were then successfully transferred to destination vector via gateway reaction, and the destination plasmid was transformed into competent E. coli cells. Successful insertion into destination vector was verified by colony PCR. Destination vectors were then isolated and sequence was confirmed by sequencing.

Conclusions

We can conclude that sgRNA constructs were successfully annealed and ligated into the entry vector, and we can conclude that the entry vector was successfully transformed into DH5α competent cells. This was confirmed by colony PCR and subsequent imaging of the PCR product on an agarose gel. Furthermore, we can conclude that the gateway reaction was successful, and that destination vector with the sgRNA construct inserted was transformed into DH5α competent cells. This was also confirmed by colony PCR.

We also concluded that homozygous mutations were established in the T3 generation of nac020 mutant plants. This was determined by analyzing the sequencing results obtained from isolated DNA of nac020 T3 seedlings. Furthermore, genotyping of all T3 plants indicated that four seedlings did not retain the cas9 gene. Therefore, these four seedlings will be used for characterization of nac020 mutant phenotype, as well as for the establishment of nac020/nac028 and nac020/nac057 double mutants.
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Citations