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Summary:
Arabidopsis thaliana plants are ideal for genetic and molecular studies and are commonly used as a model organism. Its hermaphroditic flowers allow for self-fertilization, promoting inbreeding and homozygosity. Arabidopsis thaliana's small size and its short generation time allows for maintenance of large multigenerational populations in an accelerated timeframe with respect to plants. Within the A. thaliana genome, membrane receptor and ligand proteins encoded by the RLK (receptor-like kinase) and the SCRL (S-Locus cysteine-rich like) gene families, respectively, are suspected of initiating signal transduction pathways involved in plant development, plant defense, and other aspects of plant reproduction. Arabidopsis thaliana plants are susceptible to T-DNA insertion of transgenes via Agrobacterium infection. This allows for reverse-genetic manipulation, especially via the CRISPR/Cas9 technology. The plant CRISPR system in pHEE401 uses an egg cell-specific promoter coupled with a stabilizing rbcS-E9t terminator for early and sustained expression of the Cas9 gene. In this project, we developed a CRISPR construct, designated pDP4g03230CR2, which targets the Arabidopsis thaliana RLK At4g03230 gene for functional analysis via CRISPR-induced silencing in future experiments.

In order to accomplish this, two target sequences from the coding region within exon one of the At4g03230gene that had a restriction enzyme site coincident with the Cas9 site and few homologous off-target sites were chosen using the CRISPR-P program. These two target sequences were incorporated into primers CRAt4g03230FP and CRAt4g03230RP facilitating PCR amplification of portions of two sgRNA genes within template DNA pCBC-DT1T2. The PCR product was inserted into CRISPR Ti plasmid pHEE401 via a cut-ligation reaction. Heat shock transformed bacteria that was kanamycin resistant/spectinomycin sensitive was used for isolation of putative pDP4g03230CR2 DNA. Spectinomycin sensitivity, PstI digestion and nucleotide sequence analysis of pDP4g03230CR2-C indicated successful cloning of the designed PCR fragment with the two selected sgRNA guidance sequences into the modified Ti plasmid, pHEE401.

RNA-Seq data revealed expression of the At4g03230gene in dry seeds and anthers containing pollen before and at opening of the flower. At4g03230 transcripts were also moderately expressed in the root without the apex. GUS reporter gene analysis of the At4g03230gene revealed expression in immature anthers from all early stages to before flower opening as well as in the flower nectaries. In the future, A. thaliana gametes and seeds can be transformed via Agrobacterium containing pDP4g03230CR2-C using the floral dip method. The seed can be grown on GM/hygromycin agar plates and the selected transgenic plants screened for phenotypic changes in dry seeds, anthers containing pollen before and at opening of the flower,

the root without the apex and the nectaries. The CRISPR-induced mutation of putative knockout plants with a consistent phenotypic aberration will be characterized using the restriction site loss assay.