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In Search of the t-DNA Insert: Genotyping Arabidopsis Thaliana Transgenic Plant Lines

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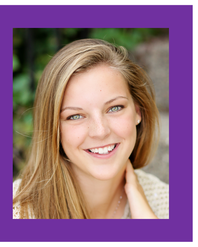

Abstract

Overall this project set out to isolate a homozygous plant line for a mutation in the gene that codes for 3-hydroxyisobutyrate dehydrogenase (HIBDH), an enzyme that catalyzes one of the reactions in valine catabolism. Using a reverse genetics approach, seeds heterozygous for the mutation were planted and DNA was subsequently isolated from leaves. The DNA was amplified using PCR with primers specific to the gene of interest and the t-DNA insert (the method for how the mutation was generated). Interesting PCR results led us to conclude that either the t-DNA insert was folded on itself or inserted in duplicate. Either way it meant that we weren't going to be able to properly identify homozygous mutant plant lines.

Concurrently, we worked to build a construct of HIBDH to create an eventual complement plant line. The HIBDH gene was cloned from *A. thaliana* and ligated into a binary vector then transformed into *E. coli*. These will then be used for another transformation into plants when a reliable transgenic plant line is available.

Background

- Increasing global population, changing climate, and insufficient farm land means research in plant science is important
- Humans cannot produce branched chain amino acids (BCAAs) like valine, leucine, and isoleucine though they are required for a functioning body, so we get them from plants
- Multiple enzymes in the valine catabolic pathway have shown to be vital for plant development and growth (Fig. 1)
- Lack of CHY4 is embryo lethal, lack of MMSD leads to significantly decreased germination rates[1]
- HIBDH might also be significant in the development of plants
- Goal was to characterize HIBDH through reverse genetics, taking away the function/production of the enzyme to see what effect that has on the plant
- 5k bp t-DNA sequence is inserted into the 1.4k bp HIBDH gene resulting in a HIBDH transgenic plant line[4]
- A complement plant line, one with the functioning gene inserted back into mutant plant line, is then developed to confirm phenotype changes are due to the lack of HIBDH
- Isolating a homozygous plant line is the starting point to any further research

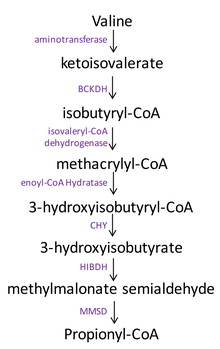

Figure 1. Valine degradation pathway. Reactions catalyzed by 3-hydroxyisobutyryl CoA hydrolase (CHY) and methylmalonate semialdehyde dehydrogenase (MMSD) flank HIBDH.

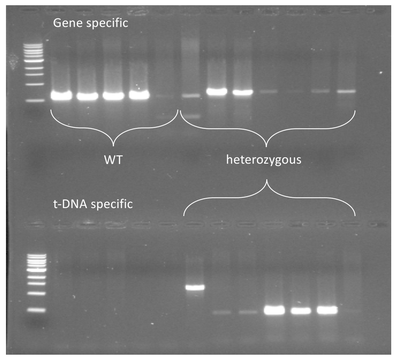

Figure 2. Sample genotyping PCR. Gene specific primers were used in in row 1 and t-DNA specific in row 2. Bands only in row 1, like in columns 1-5, indicate a homozygous wild type (WT) plant. Bands only in row 2 show a homozygous mutant plant line. Both rows having bands, as in columns 6-12, indicate that the DNA is heterozygous.

Genotyping – Results

DNA was isolated from plants and genotyped using t-DNA and gene specific primers. Heterozygous plants give bands for both reactions, homozygous mutants give PCR products with only t-DNA specific primers, and homozygous wild type (WT) give PCR products with only gene specific primers.

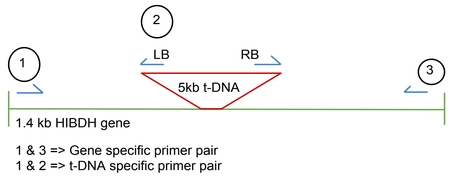

Figure 3. HIBDH gene showing the proposed site of the t-DNA insert as well as possible primer pairings for genotyping. Wild type plants will show PCR products when using primers 1&3 only. Transgenic plants will produce PCR products when using primers 1&2.

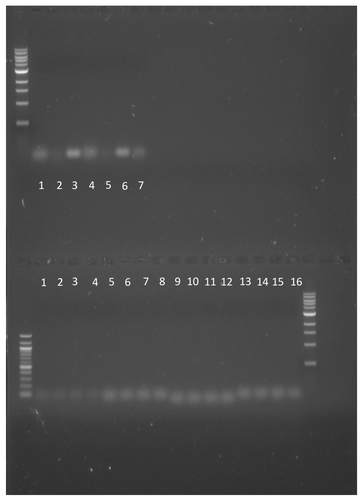

Figure 4. PCR various primer parings. Row 1 is a primers only control. Row 2, lanes 1-12 are RB primers. Row 2, lanes 13-16 are 4 different templates with a RB primer and a new gene specific primer.

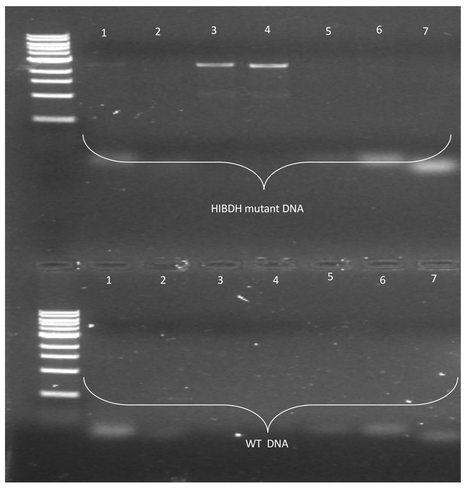

Figure 5. PCR results with only one primer. Lanes 1-7 correlates to different t-DNA specific primers. Only having one primer should not produce a PCR product.

Figure 6. *Arabidopsis thaliana* in the growth room at 2.5 weeks old.

Genotyping - Conclusion

None of the available primer pairs gave reliable or usable results. As in figure 1, left border (2) primers produced bands in WT plant PCRs when paired with the expected gene specific primer (1). Right border (RB) primers gave no bands anywhere when paired with its associated primer (3). When the pairings were switched (i.e. 2&3), the LB primers gave promising bands only for potential HIBDH knockout plants while RB continued to yield no bands. Products for this PCR were larger than expected so we sent the sample to be sequenced with poor results. To trouble shoot, PCR with only the LB primers was tried and bands were produced, indicating that the primers were binding in at least 2 places. This means that there are more t-DNA inserts in the *A. thaliana* gene than originally thought or that the insert was folded in on itself, exponentially increasing the difficulty of using this plant strain. We've requested a mutant line that was recently characterized for further analysis in this project.

References

[1]Gipson, Andrew B., Kyla J. Morton, Rachel J. Rhee, Szabolcs Simo, Jack A. Clayton, Morgan E. Perrett, Christiana G. Binkley, Erika L. Jensen, Dana L. Oakes, Matthew F. Rouhier, and Kerry A. Rouhier. "Disruptions in Valine Degradation Affect Seed Development and Germination in Arabidopsis." *The Plant Journal* 90.6 (2017): 1029-039. [2]Curtis, M. D. "A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta." *Plant Physiology* 133.2 (2003): 462-69. [3]Hyvonen, Marko. "Guide to Expression Construct Cloning." (2004): 1-16. [4]Krysan, P. J., Young, J. C., Sussman, M R. "T-DNA as an Insertional Mutagen in Arabidopsis." *The Plant Cell Online* 11.12 (1999): 2283-290. [5]Schertl, Peter, Lennart Danne, and Hans-Peter Braun. "3-Hydroxyisobutyrate Dehydrogenase Is Involved in Valine and Isoleucine Degradation in A. Thaliana." *Plant Physiology* (2017): n. pag.

Complement Line - Results

Developing a complement plant line starts with inserting the HIBDH gene into a binary vector which can be transformed into *E. coli* and plants.

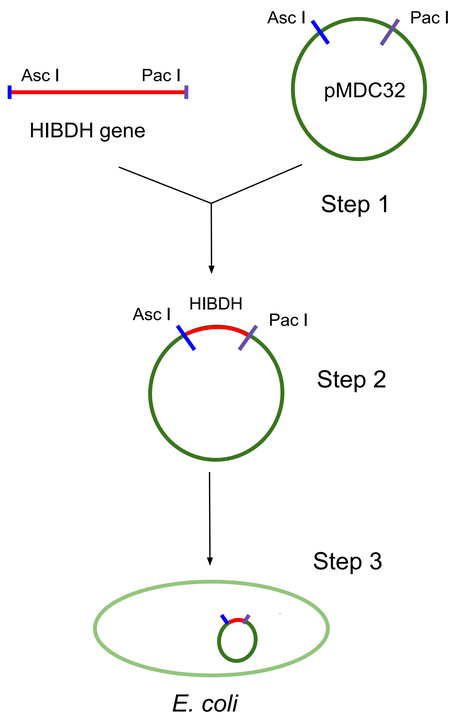

Figure 7. Scheme of the steps required for building a functional construct. Step 1 is isolating the AtHIBDH gene as well as choosing a vector. Step 2 is digesting then ligating the pieces together. Step 3 is transforming the new plasmid into competent *E. coli* cells

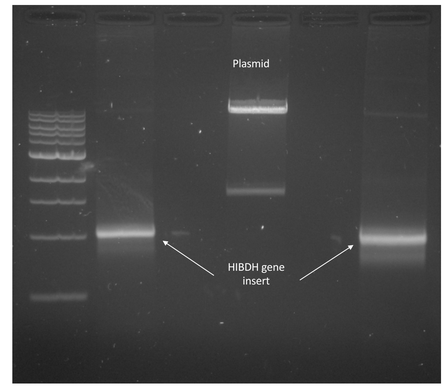

Figure 8. PCR of the restriction digest of both plasmid (pMDC32) and the insert. Bands were then purified and ligated together. Step one of figure 7.

Figure 9. Final restriction digest of completed plasmid in *E. coli*. 1-4 are samples, 5 is a control of the plasmid without the HIBDH insert. Confirmation of step 3 in figure 7.

Figure 10. PCR of *E. coli* colonies that shows the HIBDH insert has been successfully transformed. Primers specific to the ends of the insert were used. Step 3 of figure 7.

Complement Line - Conclusion

PCR, sequencing, and restriction digests suggest we have made the correct construct for a future complement line. The next step is transforming the vector into agrobacterium and then into a *A. thaliana*. The bacteria will insert their DNA into the plant cells where it will then be expressed by the plants and transferred to any offspring.

Acknowledgements

Professor K. Rouhier for guidance. This project was funded by the Kenyon Summer Science Scholars program.