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A PCR Assay for the Anthocyaninless Mutation in Fast Plants and a Bridge Between Classical Genetics and Genomics

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A PCR assay to detect the mutation responsible for the non-purple trait in Rapid Cycling *Brassica rapa* (RCBr), a.k.a. Wisconsin Fast Plants and ways to incorporate it in the biology lab was developed. Purple vs. non-purple variation in RCBr is an easy-to-score Mendelian trait with non-purple being recessive to purple. My group has recently identified an insertion mutation in the gene for dihydroflavonol 4-reductase (DFR) as being responsible for the non-purple trait. A robust PCR assay detects the mutant and wild type alleles of this gene in any Fast Plant strain. In this mini workshop you will see the PCR assay and use the nucleotide sequence of the mutant and wild type alleles as a bridge between classical genetics, molecular genetics, and bioinformatics.
Keywords: Fast Plants, Mendelian genetics, molecular genetics, mutation, PCR

Introduction

Variation in stem color in Wisconsin Fast Plants is a simple Mendelian trait that is ideal for genetics education. Wild type Fast Plants have purple coloration that is most concentrated on the stems due to anthocyanin pigments. Nonpurple is a complete lack of anthocyanin and is recessive to purple. This is a single gene trait and the gene responsible was named *anthocyaninless*. For more information see https://fastplants.org/lessons/.

The nonpurple trait is due to an insertion mutation in the gene for dihydroflavonol 4-reductase (DFR). DFR is an essential enzyme in the anthocyanin biosynthesis pathway, and loss of function mutations in its gene are responsible for an anthocyaninless phenotype in many plant species. We genetically mapped the *anthocyaninless* locus to the chromosomal neighborhood of the *DFR* gene and found that nonpurple strains have and insertion of 384 bases in the fourth exon of DFR that is in complete linkage with *anthocyaninless*. All nonpurple strains of Fast Plants have this insertion which is predicted to alter the reading frame and introduce a premature stop codon (Wendell et al, 2016). Knowing the gene and mutation responsible for the nonpurple trait means that it can be used for both classical genetics and molecular genetics and give students a bridge between these two realms.

Lab Exercises

The mutant and wild type alleles can be detected by PCR and agarose gel electrophoresis (Figure 1). One application of this assay that I have used in my Genetics Laboratory course is for students to test the genotypes of purple plants to identify which are homozygous wild type alleles and which are heterozygous carriers of the mutant allele. Students are presented with pairs of purple plants to be cross pollinated and use the DNA test to determine their risk of having affected offspring. They then grow seedlings of this mating and compare the distribution of phenotypes to their prediction.

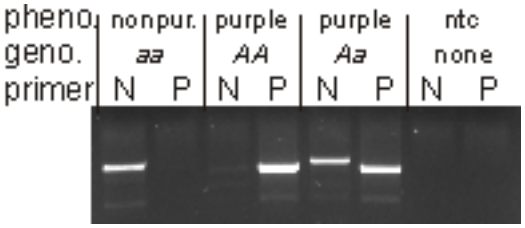

Figure 1. Detection of mutant and wild type alleles by PCR and gel electrophoresis.

The fact that the actual mutation is known enables lessons in gene structure and function and some elementary bioinformatics. The sequences of the *DFR* gene from Fast Plants strains Purple Hairy (*ANL/ANL*) and Nonpurple

Stem, Yellow Green Leaf (*anl/anl*) are deposited in Genbank (accession numbers KX185527 and KX379243, respectively). Predicted mRNA sequences for these two alleles are attached to this article as separate files. I teach a Genetics Laboratory course and have one session where the students analyze the mutant and wild type sequences. I start by having them align the wild type and mutant sequences to identify sequence variation. I use Clustal Omega but there are other alignment programs out there (http://www.ebi.ac.uk/Tools/msa/clustalo/). Students find that there are many positions in the gene where the sequence of mutant and wild type alleles differ. This raises the question as to which ones affect gene function. To evaluate where each mutation sits in the functional structure of the gene, we use NCBI Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form) with the genomic and predicted mRNA sequences. NCBI Splign will predict the exon structure and give a predicted polypeptide sequence. The students will need a lot of time for this, and I think its best just to let them explore for an extended time rather than directing them to the solution quickly. Given enough time, they will find that insertion mutation occurs in the fourth exon of the gene which alters the reading frame and introduces a premature stop codon (Wendell et al 2016).

Materials

Parental, F1, and F2 Fast Plant seeds can be obtained from Carolina Biological Supply (Burlington, NC). Complete information on Fast Plant cultivation can be found at the Wisconsin Fast Plants web site (fastplants.org). For reliable methods to obtain Fast Plants DNA for PCR, see our paper in an earlier volume of these proceedings (Wendell and Pickard, 2013).

PCR primer sequences and working stock solution concentrations are given in Table 1. To detect the wild type allele of DFR, PCR is performed with primer CF that primes upstream of the insertion site and primer PR that primes downstream of the insertion site. PCR with primer CF and primer NR will detect the mutant allele (Figure 2). For PCR, we combine the following: 12 ul Mean Green 2X Master Mix (Empirical Biosciences, Grand Rapids, MI), 5 ul primer CF, 5 ul of either primer NR or PR and 2 ul of DNA sample. It is important to perform PCR with the primer stocks at the concentrations given in Table 1 because nonspecific products tend to be produced when primers are in excess.

Table 1. PCR Primers

Primer	Sequence	Stock
CF	CACGAAGATTTCCACCGAAGC	960 nM
NR	TTGACGGCGTGCTAAATTGG	960 nM
PR	TGGACCGATCACCAATGTCG	480 nM

Normally, to detect an insertion mutation, you can use a pair of PCR primers that flank the insertion site and observe the insertion by a band of increased size. However, the insertion in the Fast Plant DFR gene has a palindromic structure that makes PCR across the entire insert very difficult (Wendell et al, 2016). Therefore I detect the mutant allele with one primer anchored in the insertion that primes "outward" from the insertion and another primer anchored in the gene, thus avoiding the palindrome (Figure 2).

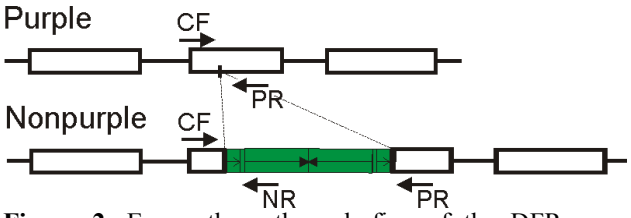

Figure 2. Exons three through five of the DFR gene showing the position of the insertion in the recessive nonpurple allele. A combination of PCR primers CF and PR detects the purple allele, while a combination of CF and NR detects the nonpurple allele.

PCR is performed with the following protocol: 94° C for 2 minutes to denature genomic DNA; 30 cycles of 94° C for 30 seconds, 61° C for 60 seconds, 72° C for 60 seconds; 72° C for 4 minutes, and a hold at 12° C. PCR products can be resolved in 1.2 % agarose gels run at 150 V for 30 minutes. I use SYBR Safe (Life Technologies) to detect bands which can be visualized on either an ultraviolet or blue light transilluminator.

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Acknowledgments

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About the Author

Doug Wendell is a faculty member at Oakland University and teaches genetics and genetics laboratory. His does research in plant genomics and has recently gotten into environmental DNA research. He also spends a considerable amount of time herding cats.

Appendix A
Predicted mRNA Sequences

>Purple_DFR_predicted_mRNA
AAATGTTTTCTTATAGCACGAAGATTTCCACCGAAGCTACGTAGTAAACTCTTTCTCAAAGCATAATCCATCTTTCACA
CACAAAGATGGTAGCTCACAAAGAGACCGTGTGCGTAACCGGCGCATCAGGATTCATTGGTTCATGGCTCGTGATGCGG
CTACTGGAACGTGGTTATTTTGTCCGTGCCACTGTTCGCGATCCTGGAAATTTGAAGAAAGTGCAACATCTTCTTGATT
TGCCAAACGCGAAGACGCAACTCACTTTATGGAAAGCCGATTTATCTGACGAAGGAAGCTACGATGACGCCATAAACGG
ATGCGGCGGCGTTTTCCACATAGCAACTCCCATGGATTTTGAATCTAAGGACCCCGAGAACGAAGTGATAAAACCGACA
GTGAATGGAGTGTTGGGGATAATGAAAGCATGTGATAAGGCAAAAACCGTACGAAGAATTGTGTTTACTTCGTCTGCTG
GAACGGTTAATGTTGAGGAACACCAGAAAAATGTCTATGATGAAAACGATTGGAGTGATCTTGACTTTATCATGTCCAA
GAAGATGACAGGATGGATGTATTTCGTGTCGAAAACGTTAGCCGAGAAAGCAGCTTGGGATTACGCGAAGGAAAAAGGA
ATAGATTTCATTAGTATTTTCCCGACATTGGTGATCGGTCCATTTATAACAACATCTATGCCGCCTAGCCTTATTACCG
CGCCCTCTCCTATCACTCGTAACGAGGCACATTACTCCATCATAAGACAAGGACAGTATGTCCACTTGGACGATTTATG
CAATGCCCATATATTCTTGTACGAACAAGTTGCTGCCAAGGGACGTTATGTTTGTTCCTCTCACGATGCAACGATTCTT
ACTATCTCCGAGTTTCTCAGGCAAAAATATCCAGAATATAACGTGCCTTCAACGTTTGAAGGAGTGGATGAGAATCTAA
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GGCAGTGATGATAATAAGGAGATTAAAAACGGATCTGCAGGTTTAACTGATGGTATGGTAGCTTGTAAGAAGACCGAAC
CAGGGATGGCCGGCGAGAAAGCCGATAGTCACATGTCGGCACAGCAGATCTGTGCTTAGAAATTGAACCCGTATCTAAT
ATGGATCTATTGTCTTATGAATGTGTGATTTGTTTTTTTTTCTTTTTAAGTTTGTATGAAAGTGTTGTGGACTTATTTA
TGGTATATGATATCATGTGTGAGTGTGAAAAATTATG

>nonpurple_DFR_predicted_mRNA
AAATGTTTTCTTATAGCACGAAGATATCCACCGAAGCTACATAGTAAACTCTTTCTCAAAGCATAATCCATCTTTCTAA
CACACAAAAATGGTAGCTCACAAAGAGACCGTGTGCGTAACCGGCGCATCAGGATTCATCGGTTCATGGCTCGTGATGC
GGCTACTGGAACGTGGTTACTTTGTCCGTGCCACTGTTCGCGATCCTGGAAATTTGAAGAAAGTGCAACATCTTCTTGA
TTTGCCAAACGCGAAGACGCAACTCACTTTATGGAAAGCCGATTTATCTGACGAAGGAAGCTACGATGACGCCATAAAC
GGATGCGACGGCGTTTTCCACATAGCTACTCCCATGGATTTTGAATCTAAGGATCCCGAGAACGAAGTGATAAAACCAA
CAGTGAATGGAGTGTTGGGGATAATGAAAGCATGTGATAAGGCAAAGACCGTACGAAGAATTGTGTTTACTTCGTCTGC
TGGAACGGTTAATGTTGAGGAACACCAGAAAAATGTCTATGATGAAAACGATTGGAGTGATCTTGACTTTATCATGTCC
AAGAAGATGACAGGATGGATGTATTTCATGTCGAAAACGTTAGCCGAGAAAGCAGCTTGGGATTACGCGAAGGAAAAAG
GAATGAGAATTCGGCCAAAAAAAACCTCAACTTTACACGAATTGCCAAAACAAACATGAACTTTGGGGCTGGCCAAAAA
AACACCAAACTTTCATTGACTTTAGAATTAATTAACAATGTTTTCGCTGACTTGCCAATTTAGCACGCCGTCAACAAAT
TTAACAGAAATATTTGACGTCAAATATTTCTGTTAAATTTGTTGACGGCGTGCTAAATTGGCAAGTCAGCGAAAACATT
GTTAATTAATTCTAAAGTCAATGAAAGTTTGGTGTTTTTTTGGCCAGCCCCAAAGTTCATGTTTGTTTTGGCAATTCGT
GTAAAGTTGAGGTTTTTTTTGGCCGAATTCCCAAAAAGGAATAGATTTCATTAGTATTATCCCGACATTGGTGATCGGT
CCATTTATAACAACATCTATGCCGCCTAGCCTCATTACCGCGCTCTCTCCTATCACTCGTAACGAGGCACATTACTCCA
TCATAAGACAAGGACAGTATGTGCACTTGGACGACTTATGCAATGCTCATATATTCTTGTACGAACAAGCTGGTGCCAA
GGGACGTTATGTTTGTTCCTCTCACGATGCGACGATTCTTACTATCTCCGAGTTTCTCAGGCAAAAATATCCAGAATAT
AACGTGCCTTCAACGTTTGAAGGAGTGGATGAGAATCTAAAGAGCATTATGTTCAGTTCCAAGAAGCTGATTGATATGG
GATTTAACTTCAAGTATAGTCTCGAGGATATGCTGGTGGAATCGATTGAGACATGTCGTCAAAAGGGTTTTCTCCCTTC
ACTTTACCGGAACATTTGAAATCTGAGGACAAAGTTCCGGGCAGTGATGACAATAAGGAGATTAAAAACGGATCTGCAG
GTTTAACTGATGGTATGGTAGCTTGTAAGAAGACCGAACCAGGGATGGCCGGCGAGAAAGCCGATAGTCACATGTCGGC
ACAGCAGATCTGTGCTTAGAAATTAAACCCGTATCTAATATGGATCTATTGTCTTATGAATGTGTGATTTGTTTTTTTT
TTGTTCTTTTTAAGTTTGTATGAAAGTGTTGTGGACTTATTTATGGTATCTGATAGTATCATGCGTGAGTGTGTAACAT
TATG

Note to user: these are predicted cDNA sequences of the DFR gene from Fast Plants in FASTA format. They are based on the predicted mRNA sequence of the Chinese cabbage DFR gene. The predicted nonpurple sequence also assumes that this allele can be sucessfully transcribed and processed, but that has not been verified experimentally.

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