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DNA-based genetic markers for rapid cycling *Brassica rapa* (Fast Plants type) designed for the teaching laboratory

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We have developed DNA-based genetic markers for rapid cycling *Brassica rapa* (RCBr), also known as Fast Plants. Although markers for *B. rapa* already exist, ours were intentionally designed for use in a teaching laboratory environment. The qualities we selected for were robust amplification in PCR, polymorphism in RCBr strains, and alleles that can be easily resolved in simple agarose slab gels. We have developed two single nucleotide polymorphism (SNP) based markers and 14 variable number tandem repeat (VNTR)-type markers spread over four chromosomes. The DNA sequences of these markers represent variation in a wide range of genomic features. Among the VNTR-type markers, there are examples of variation in a non-genic region, variation within an intron, and variation in the coding sequence of a gene. Among the SNP-based markers there are examples of polymorphism in intronic DNA and synonymous substitution in a coding sequence. Thus these markers can serve laboratory exercises in both transmission genetics and molecular biology.

Keywords: Fast Plants, rapid cycling *Brassica rapa*, marker, SNP, education, DNA fingerprinting, genetic mapping

INTRODUCTION

Rapid cycling *Brassica rapa* (RCBr), also known as Fast Plants, are a widely used model organism in biology education. They were developed by selection of *B. rapa* for the traits of short time to flowering, rapid seed maturation, lack of seed dormancy, petite growth habit, and high female fertility (Williams and Hill, 1986). The result is a plant with a 7-week generation time that can be cultivated inexpensively by novices. Their fast growth occurs at room temperature under continuous illumination by household fluorescent lights with simple and inexpensive growing materials (Williams, 1997). In addition to the plant strains, the Wisconsin Fast Plants Program[1] has developed a large assortment of educational activities and support materials. Topics covered by RCBr activities include the effect of environment on plant growth, plant-herbivore interactions, hormones and growth, and genetics (Musgrave, 2000). Seed stocks and instructional kits are available from Carolina Biological Supply (Burlington, NC, USA). Seeds are also available from the Crucifer Genetics Cooperative (Williams, 1985).

Rapid cycling *B. rapa* is an excellent organism for teaching genetics. Cross-pollination is easy for students at all levels because like other *Brassica*, and unlike *Arabidopsis*, they are self-incompatible for pollination. Lessons in Mendelian inheritance are performed using RCBr stocks that vary in easily scored phenotypes such as stem color (purple versus non-purple) and leaf color (green versus yellow-green; Williams, 1985). There are also traits with complex inheritance such as trichome density which shows additive polygenic inheritance (Lauffer and Fall, 2000) and intensity of anthocyanin pigmentation which is both polygenic and affected by environment (Goldman, 1999). Some molecular genetic markers exist, but have been slower to develop (Wendell and Pickard, 2007).

Although there is a large set of DNA markers for *B. rapa* in the form of microsatellites and single nucleotide polymorphisms (SNP), they do not lend themselves well to the teaching laboratory where simple agarose slab gels are most common, time and budgets are limited, and the students using them are novices. Microsatellites are highly desirable genetic markers because they tend to have multiple alleles and thus be highly informative (Litt and Luty, 1989; Weber and May, 1989). An extensive list of microsatellite markers for *Brassica* developed by several groups can be found at the Microsatellite Information Exchange[2], and microsatellite markers that have been developed for *Brassica* crop species are usable and polymorphic in RCBr (Burdzinski and Wendell, 2007; Iniguez-Luy et al., 2009). Instructional use is difficult because the size difference between alleles is usually in the range of 2–20 base pairs which is best resolved in polyacrylamide gels. We have previously reported a set of selected microsatellites and protocols to make them work in a teaching laboratory environment using polyacrylamide mini gels (Wendell and Pickard, 2007). However, the need for polyacrylamide gels still creates a barrier to their use by instructors of undergraduate or advanced high school laboratories who either may not have the needed equipment in a teaching laboratory, or do not wish to work with polyacrylamide. Another type of DNA marker available for *B. rapa* are SNP (Park et al., 2009). SNPs have grown in significance as genetic markers because they are present at a high density in genomes and SNP genotype data can be collected using automated high throughput methods such as microarrays. Although a single SNP is not as informative as a single polymorphic microsatellite, due to SNPs generally having

[1] www.fastplants.org

[2] http://www.brassica.info/resource/markers/ssr-exchange.php

only two alleles, a string of SNPs can be just as informative as a single microsatellite with multiple alleles (Kruglyak, 1997). However, the methods used to routinely analyze SNPs such as microarrays or automated DNA sequencing cannot be expected to be readily available in an instructional laboratory. Even simpler methods such as TaqMan assays still require more sophisticated equipment (for real time PCR) than most instructional laboratories have on hand.

In order to allow the use of DNA makers with RCBr under the conditions where these plants are most commonly used, in an undergraduate or advanced high school teaching lab, we have developed genetic markers specifically suited to use under simple conditions. The markers we report here have been selected for robust and reliable amplification by PCR, polymorphism in RCBr populations, and alleles that can be readily resolved in small conventional agarose slab gels. For repetitive DNA-based markers we have sought those with longer repeated element like variable number tandem repeats (VNTR) markers, rather than microsatellites, so that the size difference between alleles would be larger. For detection of SNPs, we have identified those that are reliably detected by the technique of PCR-RFLP (Konieczny and Ausubel, 1993).

MATERIALS AND METHODS

PLANT STRAINS

A variety of RCBr strains were used which vary in both Mendelian traits and DNA markers. One of the Mendelian loci is *anthocyanin-less* which has the recessive *anl* allele for lack of anthocyanin pigment (non-purple stem) and the dominant wild type *ANL* allele that allows anthocyanin production resulting in purple stems (Williams, 2007). The other Mendelian locus used is *yellow-green* which has the recessive *ygr* allele for yellow-green color and the dominant wild type *YGR* allele for normal green color (Williams, 2007). The Wisconsin Fast Plants strains Standard *B. rapa*; Purple Stem, Hairy; Non-Purple Stem, Hairless; and Non-Purple Stem, Yellow-Green Leaf were obtained from Carolina Biological Supply Company (Burlington, NC, USA). Strain DWRCBr70 is derived from a purple-stemmed RCBr population by selection for high intensity of purple color. DWRCBr52 was derived from a non-purple stem population by selection for low trichome density. DWRCBr60 was derived from a populations of plants with purple stems and yellow-green leaves.

DNA PURIFICATION

DNA was purified from leaf tissue using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following manufacturer's instructions with the exception that the tissue was disrupted using a ground glass homogenizer.

PCR

PCR was performed in a 10-μl reaction volume with 50 ng of template DNA and 10 pmol of each primer using either Accuprime Taq DNA polymerase and supplied Buffer I (Invitrogen, Carlsbad, CA, USA) or Syzygy Taq polymerase (Syzygy Biotech, Grand Rapids, MI, USA). The PCR program was an initial incubation at 94˚C for 2 min, followed by 25 cycles of 94˚C for 30 s, 61˚C for 60 s, and 72˚C for one 60 s, and a final incubation at 72˚C for 4 min.

DNA SEQUENCING

PCR amplicons were purified for DNA sequencing using a MinElute PCR Cleanup Kit (Qiagen Inc., Valencia, CA, USA) and their purity verified by analytical electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing reactions were performed using ABI BigDye Terminator v3.1 Cycle Sequencing Kit and analyzed using the Applied Biosystems ABI Prism 3730 DNA Analyzer at the Wayne State University Applied Genomics Technology Center[3].

ELECTROPHORESIS

PCR products were separated by electrophoresis in 1.2% agarose (Genetic Analysis Grade from Fisher Scientific, Waltham, MA, USA) in 7 cm long slab gels at 150 V for 30–50 min. Bands were detected by ethidium bromide staining.

IDENTIFICATION OF CANDIDATE VNTR

In order to identify sequences with potential VNTR polymorphism, bacterial artificial chromosome (BAC) sequences obtained from the *B. rapa* Genome Project[4] were searched on chromosomes 1, 2, 3, and 9. (This work was performed prior to the release of the complete *B. rapa* genome sequence.) The entire DNA sequence of each BAC was analyzed using the Emboss program eTandem[5]. eTandem generates a score based on the nature of the putative repeat; for a perfect repeat, the score is equal to the length of the entire repetitive sequence minus the first repeat. From the search results, only those potential VNTR's with a score greater than 20 and sequences with three or more repeats of 6–100 base pairs were selected, with preference to the longest repeats that were available and/or the highest number of repetitions. The rationale for this choice was that a larger repeat element would produce a larger size difference between alleles and more repeats would increase the probability of polymorphism. Finally only those results with a percent consensus among repeats of 80% or greater were chosen for further analysis.

PCR primers were designed to prime from the sequences flanking the candidate VNTR using Primer-BLAST[6]. Only primer pairs that were expected to amplify a PCR product size ranging from 200 to approximately 1000 bp were accepted.

Primers designed to amplify potential VNTR markers were tested for suitability by a series of criteria. First they were tested for the ability to robustly and reproducibly amplify a product, i.e., one could always detect a "bright" band on an ethidium bromide stained agarose gel. Those that passed the first test were used to screen for potential polymorphism in a sample of 12 random plants of the strain Standard *B. rapa* as well as the strains DWRCBr52, DWRCBr60, and DWRCBr70. When more than one band size was detected, the products were tested for evidence that they segregated as alleles of the same locus by genotyping individuals from an F_2 generation previously produced by crossing DWRCBr52 and DWRCBr70 strains (Burdzinski and Wendell, 2007). Any primer pair that amplified a product from more than one locus was discarded.

[3] agtc.wayne.edu
[4] www.brassica-rapa.org
[5] http://emboss.bioinformatics.nl
[6] http://www.ncbi.nlm.nih.gov/tools/primer-blast/

IDENTIFICATION OF SNPs IN RCBr

To identify SNPs in RCBr, we resequenced sequence-tagged sites (STS) chosen from those reported by Park et al. (2009). For each STS tested, PCR primers were designed using the program Primer-BLAST (see text footnote 6). PCR was performed on three individuals of each strain tested and the amplicons were pooled to provide template for sequencing. Such pools were generated for each of the strains DWRBr52, DWRBr60, and DWRBr70. The resulting sequence data was then aligned using ClustalW2[7] to identify SNPs between strains.

DEVELOPMENT OF PCR-RFLP MARKERS FROM RCBr SNPs

Single nucleotide polymorphisms were used to develop PCR-RFLP markers using a hierarchical approach. First, the nucleotide sequence surrounding each SNP was screened using NEBcutter V2.0[8] to identify those SNPs that resided within restriction endonuclease recognition sequences. Next, PCR primers were designed so that the position of the SNP, if cut by the enzyme, would produce restriction fragment lengths on a gel that could be easily resolved from each other and from the uncut band if present.

GENOMIC SEQUENCE DATA

Information on gene sequences and *Arabidopsis* homologs connected to the markers developed was obtained through the *Brassica* database BRAD (Cheng et al., 2011).

GENETIC MAPPING

Markers expected to be on chromosome A09 were genetically mapped relative to the *anthocyaninless* (*ANL*) locus in 81 test-cross progeny generated by crossing DWRCBr70 (*ANL*/*ANL*) with DWRCBr52 (*anl*/*anl*) and backcrossing to DWRCBr52. The order of all DNA markers was determined by their position in the *B. rapa* genome sequence available from BRAD (Cheng et al., 2011) and map distances in Kosambi centimorgans were calculated using MAPMANAGER (Manly et al., 2001). The position of the *anthocyaninless* locus was determined as that which gave map distances with the highest LOD scores.

RESULTS

VNTR-TYPE GENETIC MARKERS FOR RCBr

We have developed a total of 14 genetic markers that are based on a VNTR-type repetitive DNA and meet the criteria of robust and reproducible amplification, polymorphism in RCBr strains, and alleles that can be resolved on conventional agarose slab gels (**Table 1**). Markers are available on chromosomes A01, A02, A03, and A09. With the one exception of *D9BrapaS4* which has three alleles, all markers have only two alleles in RCBr populations surveyed. From these 14 VNTR-type markers we chose three to recommend most for use in an educational setting (**Table 2**) because they are most reliable in producing "bright" bands of alleles that are most readily resolved in small agarose slab gels (**Figure 1**). We subjected these three markers to further analysis including DNA sequence of their repetitive element to determine the nature of allelic variation.

[7]http://www.ebi.ac.uk/Tools/msa/clustalw2/
[8]http://tools.neb.com/NEBcutter2/

Table 1 | Variable number tandem repeat-type markers for rapid cycling *Brassica rapa*.

Name	Genome position[1]	Primer sequence
D1BrapaS1	A01:2129419..	GGAGGAGCAAGCAGGACCAGGA
	2130033	ACGCTGTGATTTGTTGCTTCCGA
D1BrapaS2	A01:1997477..	GCGATGCGTATTGGTGGCCG
	1998260	CCGTCGCCGGTTCACAAACCA
D1BrapaS3	A01:2092628..	AAGCAAAGCCAGCGGCGGAT
	2093030	GGCTGGTCACCCACAGGCAC
D1BrapaS4	A01:2125066..	TGGGCGTTGTTCTCATGTTCGGT
	2125999	ACCCGCCATTCTCCCCACCT
D1BrapaS5	A01:1975693..	TCCAAAGTTCTGCTCGAGGGTCC
	1976021	CCAGGAATGGCAGCATTAGACCGA
D1BrapaS6	A01:2129419..	GGAGGAGCAAGCAGGACCAGGA
	2130034	ACGCTGTGATTTGTTGCTTCCGA
D1BrapaS7	A01:4323134..	TGGTCCCTGGATGCGCGGAA
	4323402	GTGGCTGGTCACCGGTGTTGT
D2BrapaS1	A02:2005018..	CCGAACCGTCTCTAACCGAATCGC
	2005276	GGAGCTAGCATCGCTCGCGG
D2BrapaS2	A02:21600350..	ACTTTGTGAGCTTTGGCTGTTGGT
	21601504	AGCCCAGAAAGCGGTTCAGGA
D3BrapaS1	A03:25334204..	ATGTGGCGCGCTGCCATTGA
	25334706	CGCTAAGCATCCTTAACATTTTCGTGC
D9BrapaS1	A09:7345386..	CCAGCCAAATCGTCACTCATGCGA
	7345818	TGCATGCCTAAGAGTTTGGAGTAACAC
D9BrapaS3	A09:1547528..	TCGTGGGACGGTCCTCTTGCT
	1548201	ACCAAACTCTCCACCTCGGACA
D9BrapaS4	A09:28258896..	AGCGATGTAGCACCCGAGTCCA
	28259404	TCGAGCTGAGAGGGAAGCTGTGA
D9BrapaS5	A09:34072636..	CCTTGGCTGCATCAGGCGCA
	34072917	TCCAAAAGTGAGGCTGCCTTAGTGA

[1]*Genome positions were determined by a BLAST search of the Brassica rapa genome sequence version 1.1 using the Brassica Database (BRAD).*

Table 2 | Best RCBr VNTR-type markers for classroom use.

Marker	Allele sizes[1]	Repeat motif[2]
D9BrapaS1	452/497	$(aataagctagtgaagaag)_{22}$
D9BrapaS4	318/462/515	$(gaaaaaacttcactttagctctaaagctaaaaaaga)_3$ $(aaagcttcaatttaagctct)_4$
D1BrapaS1	543/617	$(agttgctgtgtctcctgatgaaat)_{16}$

[1]*The allele sizes are those produced with primers given in* ***Table 1****.*
[2]*The number of repeats listed is in the largest allele.*

D9BrapaS1 resides in a segment of DNA on chromosome A09 that does not contain any annotated genes or other genomic features. Nucleotide sequencing confirms that it contains a VNTR-sized repetitive DNA element (**Table 2**). However, the two alleles present in RCBr do not differ in the repetitive DNA sequences, but instead vary in a 53-bp insertion/deletion in the single-copy DNA flanking the repetitive element (**Figure 2**). Alignment of these alleles with the *B. rapa* genome sequence indicates almost 100% identity except for the 53-bp segment.

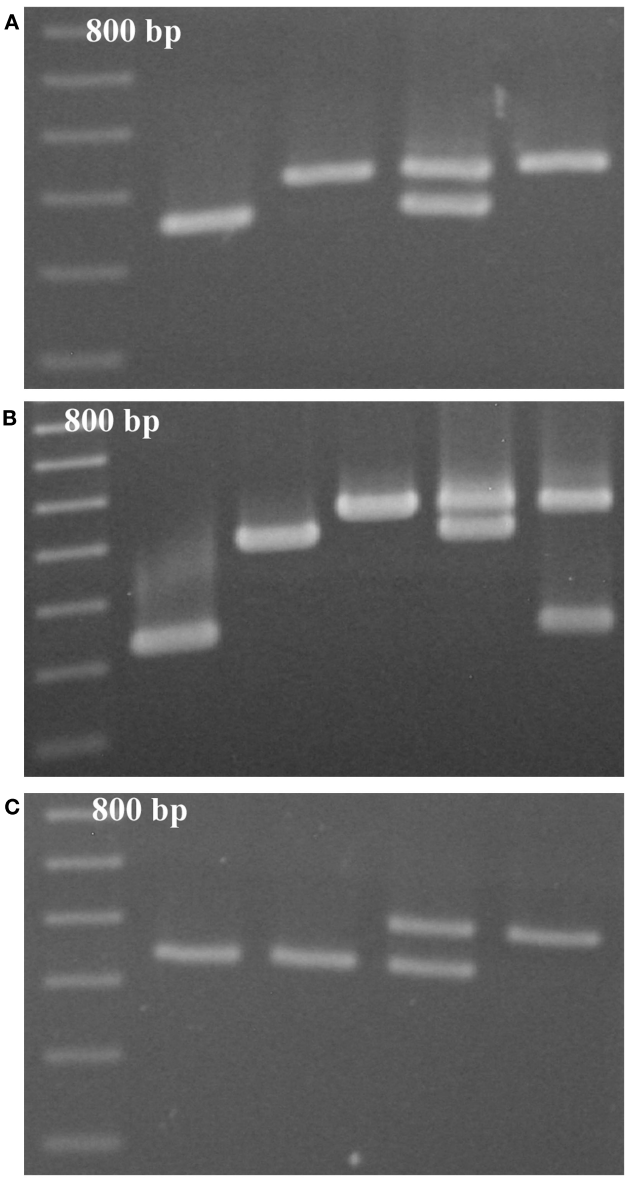

FIGURE 1 | Resolution of alleles of VNTR-type markers (A) *D9BrapaS1*, (B) *D9BrapaS4*, and (C) *D1BrapaS1* on 1.2% agarose gels. The first lane in each gel is a 100-bp DNA ladder.

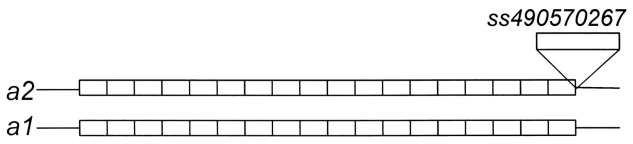

FIGURE 2 | Size variation between the alleles of *D9BrapaS1* in RCBr is due to a single insertion *ss490570267*.

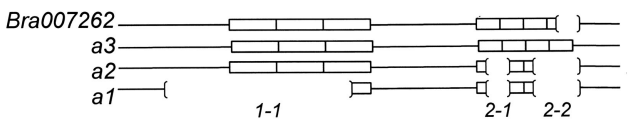

FIGURE 3 | Size variation between the alleles of *D9BrapaS4* in RCBr is due to a multiple indels designated by 1-1 (*ss490570298),* 2-1 (*ss490570286),* and 2-1 (*ss490570293).*

This indel has been deposited in to the dbSNP[9] database (ss490570267).

D9BrapaS4 contains a compound repetitive DNA element (**Table 2**) and the variation between the three alleles we have identified is in these repetitive sequences (**Figure 3**). A search of the *B. rapa* genome indicates that it resides within the first intron of the predicted gene *Bra007262*. Alignment of the comparable portion of the *Bra007262* sequence indicates that the largest allele of *D9BrapaS4* is nearly identical to the *Bra007262* sequence in the BRAD database except for a 20-bp deletion in Bra007262 in the repetitive DNA region of the marker. The indels responsible for the variation between the RCBr alleles have been deposited into the dbSNP database. ss490570286 and ss490570293 are responsible for the size difference between alleles 2 and 3, while the addition of ss490570298 produces allele 1.

D1BrapaS1 contains multiple tandem copies of a 16-bp repeat (**Table 2**), and the variation producing the fragment length difference between alleles is within the repetitive DNA, but the alleles do not vary from each other in numbers of whole repeats. Rather, each allele has several indels (relative to the other alleles) which are mostly smaller than 16 bp. Due to the repetitive DNA sequence, multiple sequence alignments are possible and we cannot presently identify the exact position of the indels.

A search of the *B. rapa* genome finds that the repetitive DNA element at the core of *D1BrapaS1* is in the second exon of the predicted gene *Bra011448* and within its predicted open reading frame. *Bra011448* is a predicted gene based on similarity to *Arabidopsis thaliana* gene *AT4G33500*. Both of these homologous genes are predicted to encode proteins with a protein phosphatase 2C (PP2C) domain near the C-terminus and a ribonuclease E (rne) domain in the second exon. Analysis of the predicted amino acid sequence of Bra011448 using NCBI Conserved Domain Search[10] finds that the *D1BrapaS1* repetitive DNA lies within the predicted ribonuclease E (rne) domain. Comparison of the nucleotide sequence of the two alleles of *D1BrapaS1* with *Bra011448,* as well as the sequence within AC189637.2 deposited in GenBank, shows that each has a different combination of indels, but all preserve the overall reading frame (**Figure 4**). They are all in-frame deletions except for one case in allele 2 of *D1BrapaS1* where two subsequent deletions preserve the reading frame. In contrast, there is very little variation in the section of the gene predicted to encode a PP2C domain. The nucleotide sequence of predicted exons 6, 7, 8, and 9 of *Bra011448* from the RCBr stocks homozygous for either allele of *D1BrapaS1* is 97% identical to the sequence in the BRAD database and there are no deletions.

[9]http://www.ncbi.nlm.nih.gov/projects/SNP/

[10]http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?

SNPs DETECTABLE BY PCR-RFLP

Among SNPs that we identified within RCBr populations, we found two that were readily assayable by PCR-RFLP under classroom conditions (**Table 3**). Both of these were detected with primers that amplified robustly in RCBr and when digested with the appropriate enzyme produced bands that were readily resolved within the same gel (**Figure 5**). The C/G polymorphism identified by *Park9-Hae*III lies within the third exon of predicted F-box protein gene *Bra026987* on chromosome A09. The substitution is a synonymous polymorphism in the third position of a serine codon. The T/C polymorphism identified by *Park14-Eco*RI lies within the seventh intron of *Bra013780*, a predicted transmembrane protein involved in defense or cell death.

LINKAGE OF A09 MARKERS TO THE *ANTHOCYANINLESS* LOCUS

Among the markers we have found to be most suitable for teaching laboratory use, two of the VNTR-type and one of the SNPs were expected to be on chromosome A09, the chromosome which we previously reported to hold the *anthocyaninless* locus (Burdzinski and Wendell, 2007). We genotyped 81 progeny of a testcross between the purple (*ANL/ANL*) DWRCBR70 and non-purple (*anl/anl*) DWRCBr53 strains and found that the *anthocyaninless* locus most likely resides within the 6.3-Mb interval between *D9BrapaS4* and *Park9-Hae*III (**Figure 6**).

MARKER POLYMORPHISM IN FAST PLANTS STRAINS

To assist instructors who obtain RCBr as Fast Plants seeds from Carolina Biological Supply, we have surveyed the allele frequencies of the markers in four popular Fast Plants strains (**Table 4**). For each strain, we determined the genotype of 20 randomly chosen plants grown from seeds obtained directly from Carolina Biological Supply. Only the strain "Standard *B. rapa*" was polymorphic for all five markers tested. It was also the only strain in which we detected all three alleles of *D9BrapaS4*. For each marker, the strains tested usually had the same major allele. The only two exceptions to this pattern were *D9BrapaS1* in the Purple Stem, Hairy strain and *Park14-Hae*III in the Non-purple Stem, Hairless strain. The distribution of the genotypes in the plants tested did not deviate from Hardy-Weinberg expectations (not shown).

NEW RCBr STRAINS WITH DEFINED MARKER GENOTYPES

We have developed three strains of RCBr with genotypes optimized for use of these markers in an instructional lab. The strains vary in both the easy to score Mendelian loci *anthocyaninless* (purple or non-purple stem color) and *yellow-green* (green or yellow-green leaf color), and the DNA markers we have developed. For most markers, a strain is fixed for a particular allele so that crosses between strains will be fully informative (**Table 5**).

DISCUSSION

The DNA-based genetic markers that we have developed were intentionally designed for science education which is the main use of RCBr (also known as Fast Plants). They can be used as both markers for transmission genetics and provide the basis for extensions into molecular biology. The DNA polymorphisms responsible for the observed alleles of *D9BrapaS1*, *D9BrapaS4*, *Park9-Hae*III, and *Park14-Eco*RI have been deposited into the dbSNP database (see text footnote 9) so that when students identify alleles of these markers using basic agarose gels as shown in **Figure 1**, they can then obtain the sequence data underlying these polymorphisms. The sequence data obtained can then be the basis of further exploration of the *B. rapa* genome through the BRAD database[11]. The markers turn out to represent a wide variety of genomic features. Of the VNTR-type markers, one is

[11] http://brassicadb.org/brad/blastPage.php

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Bra011448    MKTFEAEENLVVEPTATVAL--SPDELVDVSPEENLVVEPTATVAVSTDELVVVSPEEDLVVEPTATVAVSTDELVVVSPEE
AC189637.2   MKTFEAEENLVVEPTATVAV--STDELVVVSPEENHVVEPTATVALSPDELVDVSPEEN----------------------
D1BrapaS1a1  MKTFEAEENLVVEPTATVELDVSPDEPVVVSPEENLVVE----LDVSPDELVVVSPEEKLVVEPTATVEL------------
D1BrapaS1a2  MKTFEAEENLVVEPTATVELDVSPDELVAVSPDENLVVEPTATVTASPDELVAMPPDELVDVASNEIVAVSPDELVAVSPDE

Bra011448    DLVVEPTATVAVSTDELVVVSPEEDLVVEPTATV--AVSTDELVVVSPEEDLVVEPTATVAVSPDELVVTSPDELISTSEAT
AC189637.2   -LVVEPTATVAVSTDELVVVSPEEDLVVEPTATV--AVTPDELVDVPPEENLVVEPTATVAVSPDELVVTSPDELISTSEAT
D1BrapaS1a1  ----------DLSPDELVVVPPEEKLVVEPTAIVELDVSPDELFVVSPEEKLVVEPTATVAVTPDELAAVSPDELVSTSEAT
D1BrapaS1a2  NLVVEST--VAASPDELVALPPDDLVDVAPNELV--AVSPDELVTVSPDENLVVEPTATVAVTPEEPVAVSPDELISTSEAT
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FIGURE 4 | Four different alleles of *D1BrapaS1* show four different combinations of in-frame deletions in *Bra011448*. The sequence shown is the portion of *Bra011448* that is predicted to encode an *rne* domain and lies within the marker *D1BrapaS1*. *AC189637.2* and *Bra011448* are Chinese cabbage sequences from public databases and *D1BrapaS1a1* and *D1BrapaS1a2* are from RCBr.

Table 3 | Single nucleotide polymorphism of rapid cycling *Brassica rapa* detected by PCR-RFLP.

Name	Genome position and primer sequences	SNP and ss#	Enzyme	PCR-RFLP, fragments
Park 9	A09:34639078 TCCTCAGCTGCTTTAGCCTC TTGCGACAAAGAAACACAGC	C/G ss490571632	*Hae*III	1022/310 + 712
Park 14	A01:7729329 TGTGCTGTAACTGCAAAGCA CGCAAATCACGAGTTCTTCA	T/C ss490571635	*Eco*RI	477 + 839/262 + 215 + 839

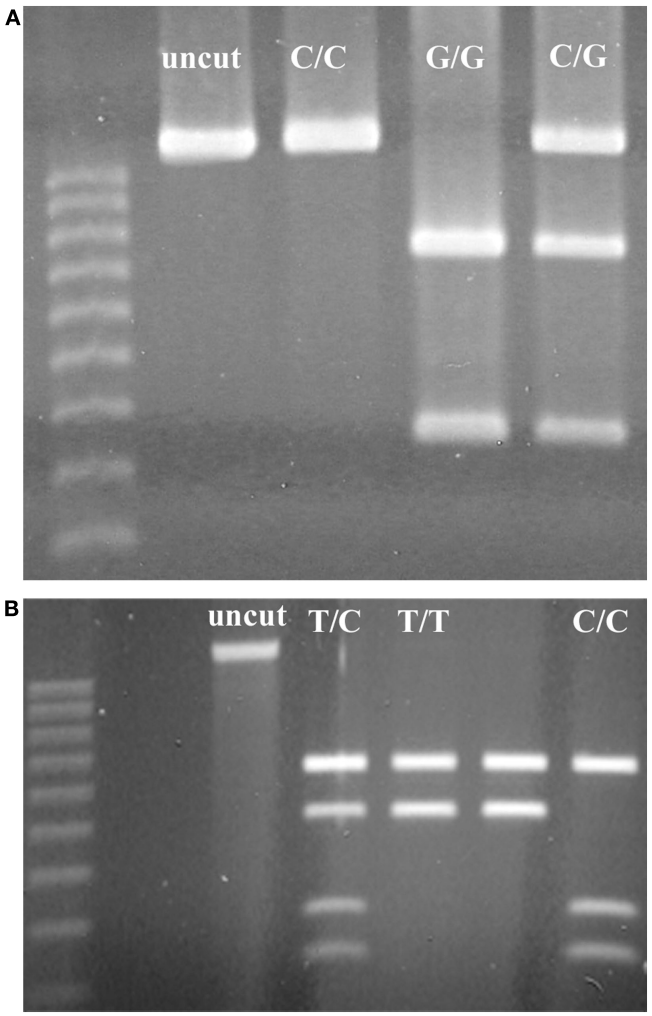

FIGURE 5 | Resolution of alleles of PCR-RFLP markers (A) *Park9-Hae*III (ss490571632) and (B) *Park14-Eco*RI (ss490571635) on 1.2% agarose gels.

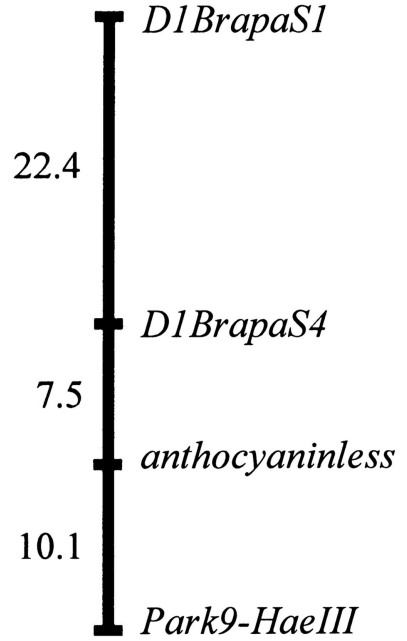

FIGURE 6 | Genetic map of the *anthocyaninless* locus relative to A09 marker reported in this paper. Map distances are Kosambi centimorgans.

in a non-genic region, one is within the intron of a gene, and one is within the open reading frame of a gene. Of the SNPs, one is in an intron and one is in the open reading from of a gene, although it is a synonymous substitution. Instructors can use the DNA sequence information we present here to develop lessons for students to study the possible impact on gene function of the sequence variation of the alleles.

Three of the markers form a linkage group with the *anthocyaninless* locus allowing them to be used in laboratory projects in genetic linkage and mapping. They are also excellent tools for projects such as paternity testing. We have previously described a laboratory project using RCBr to perform paternity testing, but the previous design used microsatellite markers (Wendell and Pickard, 2007) which can pose difficulties for lab instructors due to the need for polyacrylamide gels to resolve them. However, the markers we report here can be detected and alleles resolved in the most simple agarose slab gels.

The data we provide on population allele frequencies in RCBr strains will be valuable to instructors using these markers for educational labs. For example, we previously described a simple lab project in which the students perform paternity testing by pollinating one plant ("Mother") with a mixture of pollen from two other plants ("Alleged Fathers"), but success in this project requires that the Alleged Fathers have different alleles for the markers used (Wendell and Pickard, 2007). An instructor who wishes to perform this project using Fast Plants obtained from Carolina Biological Supply would be best served using the markers *D9BrapaS1* and *Park14-Eco*RI since these are polymorphic in all strains tested (**Table 4**). To work with a great degree of polymorphism, an instructor should use the strains described in **Table 5** because they vary greatly in their genotypes for both our markers and simple Mendelian traits. Seeds for these strains are available by request to Douglas Wendell[12].

The VNTR-type markers that we developed are not as polymorphic as expected for repetitive DNA-based markers. Except for *D9BrapaS4*, we have only found two alleles for each of the markers despite testing numerous RCBr strains, whereas VNTR markers used in mapping and DNA fingerprinting typically have multiple alleles (Nakamura et al., 1987). This could result if the repetitive DNA elements that we have selected are not prone to polymorphism, but could also result if or the RCBr populations have a low rate of polymorphism. The latter explanation is consistent with previous work in which we tested microsatellite markers

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Table 4 | Estimated marker allele frequencies in fast plants strains from Carolina Biological Supply.

Marker	Allele	Strain and catalog number			
		Standard *Brassica rapa* 158804	Purple stem, hairy 158810	Non-purple stem, hairless 158812	Non-purple stem, yellow-green leaf 158842
D9BrapaS1	1	0.21	0.53	0.15	0.53
	2	0.79	0.47	0.85	0.47
D9BrapaS4	1	0.15	–	–	0.03
	2	0.06	–	0.13	–
	3	0.79	1.00	0.87	0.97
D1BrapaS1	1	0.97	1.00	1.00	0.84
	2	0.03	–	–	0.16
*Park9-Hae*III	C	0.09	0.03	–	0.23
	G	0.91	0.97	1.00	0.77
*Park14-Eco*RI	T	0.30	0.38	0.82	0.25
	C	0.70	0.63	0.18	0.75

Table 5 | New RCBr strains with defined marker genotypes.

Marker	Allele	DWRCBr53	DWRCBr76	DWRCBr91
D9BrapaS1	1	0.0	1.0	0.0
	2	1.0	0.0	1.0
D9BrapaS4	1	0.0	1.0	0.0
	2	1.0	0.0	0.0
	3	0.0	0.0	1.0
D1BrapaS1	1	1.0	0.0	1.0
	2	0.0	1.0	0.0
*Park9-Hae*III	C	0.0	0.8	0.0
	G	1.0	0.2	1.0
*Park14-Eco*RI	T	1.0	0.4	0.4
	C	0.0	0.6	0.6
Anthocyaninless	*ANL*	0.0	1.0	0.0
	anl	1.0	0.0	1.0
Yellow-green	*YGR*	1.0	1.0	0.0
	ygr	0.0	0.0	1.0

that had been developed for *Brassica* crop species for the usefulness in RCBr. Out of 37 primer pairs that amplified a product in RCBr DNA we only found 22 to be polymorphic and only 11 that had more than two alleles in RCBr (Burdzinski and Wendell, 2007), despite the fact that microsatellites usually have multiple alleles.

The reader may wonder why we only report two SNP markers given that SNPs are abundant in organisms, and other groups have reported huge lists of SNPs for *B. rapa* (Li et al., 2009; Park et al., 2009). We did find several other SNPs (not shown) that lie within restriction sites but we were unable to design a PCR-RFLP around them that gave legible bands. The main source of the problem was the difference in size between "cut" and "uncut" alleles when detected by PCR-RFLP in ethidium bromide stained gels. Because the intensity of staining of DNA in gels by dyes, whether fluorescent or visible, is proportional to the mass of DNA in a band, we encountered a problem of markers where the lower molecular weight bands of the cut allele were too faint for student to reliably detect. Another complication was that in some cases the restriction enzyme that recognized the SNP also had multiple recognition sites close to the SNP.

In addition to developing markers and plant strains, we have developed classroom-tested protocols for their use. We make these publically available at the web site humangeneticsmustard.blogspot.com and will be adding more instructor resources as we develop them.

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