Original Paper Plant Biotechnology, **18** (3), 191-196 (2001)

Conversion of AFLP Markers Linked to the S^h Allele at the *S* Locus in Buckwheat to a Simple PCR Based Marker Form

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Received 6 November 2000; accepted 24 November 2000

Abstract

Using the technique of amplified restriction fragment length polymorphism (AFLP) analysis approximately 500 polymorphic loci were screened on the bulked segregant pools from F_2 progeny of the cross between *Fagopyrum esculentum* (pin) and *F. homotropicum*. The objective was to find those markers with tight linkage to the buckwheat homostylar locus, concerned with self-compatibility. Analysis of 123 F_2 plants identified nine markers that show no recombination in 36 recessive homozygous plants. In the nine markers, two (N2 and N7) were confirmed to derive from a single region. The N2 sequence was present only in *F. homotropicum* and was absent in common buckwheat, *F. esculentum*. Nucleotide sequence information from each flanking region of the two single locus markers was used to design region-specific primers for PCR amplification. N2 region-specific primer amplified a single fragment in *F. homotropicum* #1 but not in common buckwheat, *F. esculentum* #284 (pin). Whereas N7 AFLP marker was converted into a co-dominant marker for both parents. However, N7 marker showed size polymorphism between the parent lines. These markers can be utilized for fine mapping of the S^h allele in buckwheat, and for positional cloning of the gene.

Introduction

Polymerase chain reaction (PCR)-based markers are important to plant breeding programs and genome analysis because they are simple, fast and easy to handle. Many fingerprinting techniques based on PCR, such as simple sequence repeats (SSRs) or microsatellites (Tautz, 1989), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and amplified fragment length polymorphic DNA (AFLP) (Vos *et al.*, 1995) have been developed over the past several years. Sequence characterized amplified region (SCAR) (Paran and Michelmore, 1993) and sequence tagged sites (STS) (Williamson *et al.*, 1994) are also types of PCR markers but differ from former methods by having single or two bands.

Common buckwheat, *F. esculentum*, is a heteromorphic self-incompatible species. The self-incompatibility of this species is controlled by a single locus and the genotype for the short-stylar morph is considered to be heterozygous thrum as "*Ss*" and the long-stylar morph being imparted by the recessive homozygous pin as "*ss*" (Sharma and Boyes, 1960). They also postulated that buckwheat may contain an *S* super-gene (gene complex), similar to *Primula* (Dowrick, 1956). Campbell (1995) reported the production of a self-compatible interspecific hybrid between common buckwheat and a wild species, *F. homotropicum*, which was found in Yunnan province in China (Ohnishi, 1993). *F. homotropicum* shows middle-stylar (homostylar) morph, and is self-compatible and often cleistogamous. Genetic analysis using F_2 progenies derived from cross between common buckwheat and *F. homotropicum* indicates that the homomorphic flower type and the self-compatible trait are controlled by either a single dominant gene or two closely linked genes and exist as multiple alleles in *S* locus. Woo *et al.* (1997) suggested the genotype of *F. homotropicum* as S^hS^h and represented the relationship of dominance as $S>S^h>s$.

S^h allele-linked molecular markers in buckwheat

are important not only for breeding but also for understanding the mechanism of self-incompatibility. For these purposes, molecular marker techniques have been employed to find S^h allele-linked markers by using an F_2 population derived from the cross between common buckwheat, *F. esculentum*, and *F. homotropicum* (Aii *et al.*, 1998). Three RAPD markers that linked to the S^h allele have also been converted into SCAR markers (Aii *et al.*, 1999). One of these markers revealed co-dominance between *F. esculentum* and *F. homotropicum*. However, the RAPD and SCAR markers were not enough to saturate the markers around the S^h allele.

In this study, we identified nine AFLP markers linked to the S^h allele in buckwheat. Using those S^h allele-linked AFLP markers, we demonstrate a procedure to convert dominant AFLP fragments to simple, co-dominant PCR-based markers. Our procedure relies upon TAIL-PCR to characterize genomic regions adjacent to the AFLP fragment and upon the development of PCR strategies to capitalize on the genomic differences. For large-scale and locus-specific applications, such conversion techniques are necessary and will become increasingly important because of the popularity of the AFLP procedure, its immediate applicability to a very broad range of plant species, and its ability to rapidly identify linkages to genes of interest. Our converted markers are well adapted to large-scale, region-specific applications as they are reliable, rapid and simple to generate.

Materials and Methods

Plant materials and DNA extraction

In order to identify AFLP markers linked to homostylar locus, an F_2 population of 123 plants was constructed by self-pollination of F_1 plants between common buckwheat, *F. esculentum* (#284), and a wild species, *F. homotropicum* (#1) (Campbell, 1995). The female parent #284 was a heteromorphic self-incompatible pin-style (*ss*). The male parent #1 was homomorphic, self-compatible, and later identified as S^hS^h. Genomic DNA extraction was followed after the method of Aii *et al.* (1998).

AFLP analysis

AFLP analysis was performed according to the manufacturer's (Gibco-BRL) instructions except that all PCR reactions were performed without [^{32}P]dCTP. PCR products were analyzed on 5% denaturing polyacrylamide gels. The gel was prepared with 5% acrylamide solution, 7M urea and 1xTBE buffer (89 mM Tris-borate plus 2.5 mM EDTA, pH 8.3). The gel was pre-run at a constant 80 W until the temperature reached 50 ℃ before loading. Gels were run at a constant 55 W for 2.5 h. Silver staining procedure was performed according to the manufacturer's (Promega) instructions.

Cloning and sequencing of AFLP products

Specific AFLP fragments were excised directly from the denatured polyacrylamide gels on the glass plate using a razor blade. The fragments were used directly as template in a total PCR reaction volume of 50 μl. The PCR profile and reaction components were the same as those described above. The specific PCR products were cloned into pGEM-T vector (Promega) and sequenced using Dye Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) with an automated DNA sequencer (ABI PRISM^{TM} 310).

Southern hybridization

DNA samples from #284 (*ss*) and #1 (S^hS^h) were digested with endonuclease, *Eco*RI. Digested DNAs (5 μg/lane) were electrophoresed in 0.9% agarose gels and transformed to nylon membranes using 0.4 N NaOH. Probe labeling and signal detection were performed according to the manufacturer's instructions using AlkPhos DIRECT labeling and detection Kit (Amersham Pharmacia Biotech).

TAIL-PCR

The specific primers (N2F1, N2F2, N2F3, N2R1, N2R2, N2R3, N7F1, N7F2, N7F3, N7R1, N7R2, N7R3) were designed from the sequence of AFLP fragments obtained (**Table 1**). In addition, three arbitrary degenerate (AD) primers AD1, AD2 and AD3 were designed and commercially synthesized

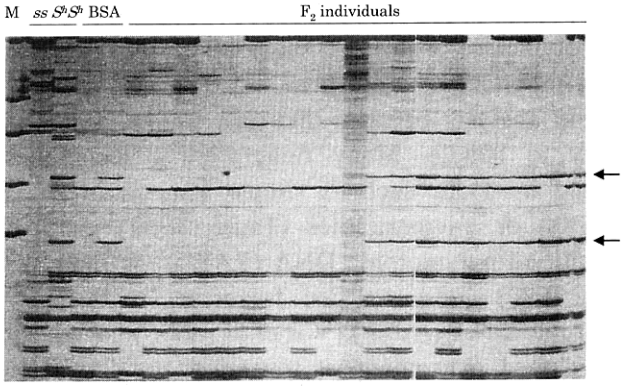

Fig. 1. AFLP fingerprints generated with primer pair E-ACG × M-CAA for DNAs from donor plants, *F. esculentum* #284 pin (*ss*) and *F. homotropicum* #1 (S^hS^h), bulked samples of F_2 pin (BP, *ss*) and homostyly (BH, S^hS^h) and F_2 individuals. Notice the AFLP fragment linked to the S^h allele (*arrow*). This band (N4) is present in the S^h bulk, absent in the *s* bulk, and segregating in the F_2 population. Molecular marker indicated as M.

Table 1. The amplification primers for TAIL-PCR and region-specific PCR.

Primer name	Sequence
N2F1(T)[a]	5′-GTGTTTGTAGTCGTAAGTTTCTTC-3′
N2F2(T)	5′-GTTTCTCCCCCCCTACTC-3′
N2F3(T)	5′-GTTCGTTCTAACAACGTCGAATGA-3′
N2R1(T)	5′-CTCTTAATGGTTGAAGTAAGCTGC-3′
N2R2(T)	5′-GCTGCAACAATCTTGCTTGTTTTGG-3′
N2R3(T, 1)	5′-CGCCGCTGTTACCTCTCAT-3′
N7F1(T)	5′-GAAATCACCCATGGAGTAAGTG-3′
N7F2(T)	5′-CACCCATGGAGTAAGTGTTTCC-3′
N7F3(T)	5′-GGAGACCATGCGCTCTACAA-3′
N7R1(T)	5′-GCCAAACATCTCGCGTACCAG-3′
N7R2(T)	5′-TCGCGTACCAGAGGGTGTGC-3′
N7R3(T, 1)	5′-CCTTTGTGAATGAGGTACCCAC-3′
AD1(T)	5′-NGTCGASWGANAWGAA-3′
AD2(T)	5′-GTNCGASWCANAWGTT-3′
AD3(T)	5′-WGTGNAGWANCANAGA-3′
N2-Mse+213(1)	5′-CTCCAAACACCCGTTTAGATAGG-3′
N7-Eco+710(1)	5′-CACGCAACCAGGTGAACCTACC-3′

[a]T, TAIL-PCR primers; 1, region-specific PCR primers

(Gibco-BRL) (**Table 1**). TAIL-PCR was performed according to the method of Liu and Whitter (1995). The TAIL-PCR products were cloned and sequenced by the same procedure as described above.

Region-specific PCR

The region-specific PCR primers were designed from the flanking and internal region of each AFLP fragment (**Table 1**). PCR was carried out under the following conditions: 25 cycles at 95 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 30 sec and finally at 4 ℃. Amplified PCR products were resolved by electrophoresis in a 1.6% agarose gel.

Results

Screening of AFLP markers linked to the S^h allele in buckwheat

In the 123 F_2 progenies, 87 had homomorphic flowers and 36 heteromorphic flowers (**Table 2**), which followed 3 : 1 segregation ratio by the chi-square test. These results suggest that homomorphism was controlled by a single dominant locus, *S* locus, as designated previously by Woo *et al.* (1997). Consequently, AFLP and bulked segregant analyses were employed to identify molecular markers linked to S^h allele in the present study. Of 64 primer combinations screened, 36 amplified 62 polymorphic products in one bulk but were absent in the other. Nine of these 62 fragments showed no recombination in each flower phenotype of 10 individual F_2 plants. Furthermore, these nine AFLP fragments showed no recombination in 36 F_2 recessive homozygotes as pin (*ss*), indicating that they were tightly linked to the S^h allele and designated as N1, N2, N3, N4, N5, N6, N7 N8 and N9, respectively (**Fig. 1, Table 3**).

Table 2. Segregation of the flower types in F_2 progeny.

No. of homomorphic flower plants	No. of heteromorphic flower plants	$X^2(3:1)$	P
87	36	1.20	0.3-0.1

Characterization of AFLP fragments

The nine AFLP fragments linked to S^h allele were cloned. The identities of the cloned fragments to the corresponding AFLP markers were verified by Southern hybridization. The hybridization patterns were found to be identical in each case, indicating the authenticity of the cloned fragments (data not shown). At least five different individual clones with respect to a single AFLP clone were sequenced. All clones had *Eco*RI and *Mse*I adapters, which were used for AFLP reaction on both ends of PCR product, respectively. Sequencing results indicate that each of the nine AFLP markers derived from different region because there is no significant

Table 3. AFLP fragments linked to the buckwheat S^h allele.

AFLP primer pair	Fragment size (bp)	Marker name	No. of copy
E-ACT × M-CAT	160	N1	low
E-ACC × M-CAT	175	N1	single
E-ACC × M-CAG	227	N3	high
E-AAC × M-CAG	183	N4	high
E-ACA × M-CAT	113	N5	high
E-ACA × M-CTA	158	N6	high
E-ACA × M-CTC	130	N7	single
E-ACA × M-CTC	274	N8	high
E-AGT × M-CTT	142	N9	high

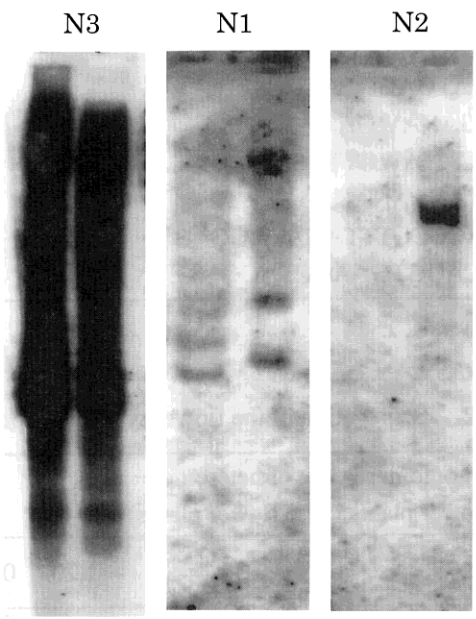

Fig. 2 Southern hybridization with DNA fragments cloned from the AFLP markers N3(A), N1(B), N2(C), to DNA from *F. esculentum* #284 pin (*ss*) (left lane) and *F. homotropicum* #1 (S^hS^h) (right lane).

homology among those clones.

To determine whether each AFLP marker was derived either from one of the multi-copy sequences or from the single-copy one, DNA samples prepared from common buckwheat, *F. esculentum* #284 pin (*ss*) and *F. homotropicum* #1 (S^hS^h) were digested with *Eco*RI, electrophoresed in agarose gels, and transferred to membrane filters. Six of nine clones, N3, N4, N5, N6, N8 and N9, strongly hybridized to a large number of different DNA fragments, suggesting that they derived from highly repetitive DNA sequences (**Fig. 2A**, **Table 3**). One of nine clones, N1, was also shown to hybridize to repetitive DNA fragments but the number of the hybridized bands were less than those with N3, N4, N5, N6, N8 and N9 (**Fig. 2B**, **Table 3**). In contrast to these clones, two AFLP clones, N2 and N7, were hybridized to a single DNA fragment, suggesting

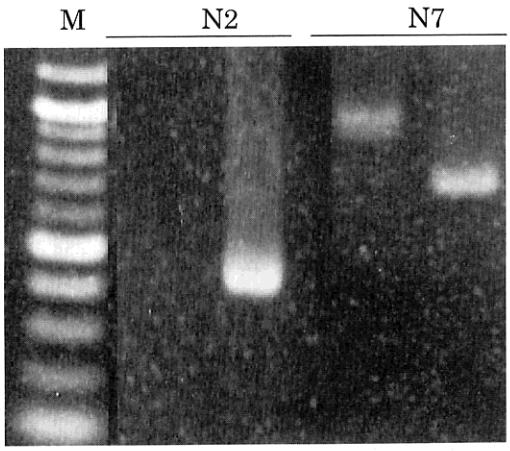

Fig. 3 Polymorphisms detected with region-specific markers, N2R and N7R, between *F. esculentum* #284 pin (*ss*) (left lane) and *F. homotropicum* #1 (S^hS^h) (right lane). 100bp ladder is indicated as M.

that they derived from a single region in buckwheat genome (**Fig. 2C**, **Table 3**). Especially, N2 was hybridized to only *F. homotropicum* #1 (S^hS^h), indicating that this sequence is specific for *F. homotropicum* genome.

Region specific PCR

TAIL-PCR can amplify DNA sequences outside a characterized region (Liu and Whittier, 1995). In the present study, TAIL-PCR primers are designed based on the known sequence to point away from each other into the unknown DNA sequence. TAIL-PCR primers were derived from N2 and N7 amplified flanking region of both N2 and N7 fragments in *F. homotropicum* #1 (S^hS^h) and *F. esculentum* #284 (*ss*). TAIL-PCR products derived from the flanking region of N2 and N7 markers were cloned and sequenced. Based on the sequence information from each TAIL-PCR product, region-specific primers were designed and used for PCR amplification with parents, *F. homotropicum* #1 (S^hS^h) and *F. esculentum* #284 (*ss*). N2 region-specific primer amplified a single fragment in *F. homotropicum* #1 (S^hS^h)

but was absent in common buckwheat, *F. esculentum* #284 pin (*ss*) (**Fig. 3**). Region-specific primer derived from N7 AFLP marker in *F. homotropicum* #1 (S^hS^h) was approximately 700 bp in size, as compared to the 1,000 bp fragment amplified from common buckwheat, *F. esculentum* #284 pin (*ss*) (**Fig. 3**). This result indicates that a dominant N7 AFLP marker was converted into a co-dominant marker showing size polymorphism. These new markers were designated as N2R and N7R respectively.

Discussion

The AFLP is a powerful technique for marker-assisted plant breeding and the construction of high-density molecular linkage maps of plant genomes. Many more polymorphic DNA markers can be detected with this technique than with any other PCR-based marker detection systems (Vos *et al.*, 1995). In this study, a total of 64 AFLP primer combinations were screened, and then nine AFLP fragments were tightly linked to the S^h allele at the *S* locus in buckwheat. In the previous study, three RAPD markers linked to the S^h allele were identified using 372 RAPD primer sets (Aii *et al.*, 1998). This result indicates that AFLPs have a clear advantage over RAPD markers in terms of the number of sequences amplified per reaction and their reproducibility.

Attempts have been made to convert poorly adapted molecular markers to the forms useful for large-scale, region-specific applications. For example, RAPD markers have been successfully converted to sequence characterized amplified region (SCAR) markers (Paran and Michelmore, 1993; Aii *et al.*, 1999). These markers are generated by sequencing ends of RAPD bands shown to be linked to genes of interest. In some cases, co-dominant SCARs have been developed from dominant RAPD markers (Aii *et al.*, 1999). AFLP markers have also been converted to simple PCR based markers as SCARs (Cho *et al.*, 1996). However it is difficult to design specific primers because PCR products from RAPD is 500-1,500 bp in length, while the average size of AFLP fragments of interest identified in this study was less than 345 bp, including those contributable to the *Eco*RI and *Mse*I adapters. Also, we were not able to derive region-specific markers from the highly repetitive AFLP sequence. Bradeen and Simon (1999) demonstrated that the flanking region of AFLP fragments were amplified by inverse-PCR and converted AFLPs to allele specific markers using outside sequencing information of AFLP fragments. In this study, only AFLP fragments derived from single-copy sequence were converted to region-specific markers. Those from multi-copy AFLP fragments were unsuccessful because their sequences' origins were different. Thus, for the conversion of the AFLP to region-specific markers, we demonstrated that it is important to first determine the nature of the cloned AFLP markers, for instances copy number and distribution in the genome.

In this study, we developed two region-specific markers linked to the S^h allele at the *S* locus in buckwheat. One marker, N7R, revealed co-dominance in showing size differences and the other one, N2R, amplified a specific sequence in *F. homotropicum* #1. These markers will facilitate marker-assisted selection of the S^h allele in buckwheat breeding programs and will also be useful as genetic markers for high resolution mapping of the S^h allele. Our next objective will be the screening of flanking markers as AFLP markers to construct a high-density map for positional cloning of S^h allele in buckwheat. It will lead to the detailed understanding of the S^h allele in various buckwheat species. The use of flanking markers will also provide more effective selection for the trait by reducing errors due to recombinants.

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