Selected AFLP primer pairs for fine mapping in pea

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Introduction

Amplified fragment length polymorphism (AFLP) markers have proven useful in fine mapping of genomic regions containing genes of interest in cultivated plants (9). AFLPs have been used in pea to map *er2*, a gene that confers resistance to powdery mildew (8) and to identify quantitative trait loci (QTL) controlling seed weight (7). We used a commercially available AFLP kit (1) to test primer pair combinations on the parents of recombinant inbred line (RIL) populations and in bulk segregant analysis (BSA). Our objective was to identify the most informative primers based on clarity and repeatability of amplified fragments and to maximize the number of scoreable polymorphic bands for use in genetic linkage analysis and QTL studies.

Materials and Methods

Four parental pairs were surveyed to collect profiles of AFLP markers for mapping disease resistance traits. The first two parental pairs [Puget (PI608010)/90-2079 (PI557500) and Dark Skin Perfection (PI269772)/90-2131 (PI557501)] are being used to identify QTL associated with Aphanomyces root rot resistance. Parental polymorphisms were tested using all 64 combinations of eight *Eco*RI (selective nucleotides AAC, AAG, ACA, ACC, ACG, ACT, AGC, AGG) with eight *Mse*I (selective nucleotides CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT) primer pairs. Primer combinations that appeared useful were further tested on 127 F_{10}-derived RILs from the cross Puget × 90-2070.

The third parental combination, being used to identify markers for *Fw*, was Green Arrow (PI 614141) and PI 179449. The fourth parental combination was DP and JI 296 (PI 680204). All 64 primer pair combinations were surveyed on both the third and fourth parental combinations.

DNA was extracted from 1 g of leaf tissue using the method of Murray and Thompson (6).

The AFLP procedure described in the AFLP analysis system I manual (1) was used in all four experiments with minor modifications (1). The *Eco*RI and *Mse*I double digest products were ligated with the double-stranded DNA adapters provided by the manufacturer. In the first PCR (preamplification) of the restricted fragments, we used with the *Eco*RI plus one selective nucleotide (A) primer and the *Mse*I plus one selective nucleotide (C) primer were used in the amplification reaction and the thermocycler profile recommended (1). For the first three parental combinations, the second (selective) amplification was performed using *Mse*I plus three selective nucleotide primer mix from the kit (1) and *Eco*RI plus three selective nucleotide primers labeled with infrared fluorescent dyes (LI-COR, Lincoln, Nebraska). The PCR reaction was run in a total mixture volume of 22 µl and used the thermocycler profile recommended (1).

For the fourth parental combination, the second PCR was carried out in a total reaction volume of 15 µl, using *Mse*I and *Eco*RI plus three selective nucleotide primers synthesized by Operon Technologies (Alameda, CA). The cycle profile recommended (1) was run using 30 cycles in which the annealing temperature was lowered by 0.7°C.

The amplified fragments were separated by size on a 7% denaturing polyacrylamide gel. For the first three parental combinations the fragments were detected using a LI-COR automated sequencer, which employs infrared fluorescence to detect the amplified fragments (LI-COR, Lincoln, Nebraska). Data collection software created image files that were analyzed with Gene Profiler gel analysis software (Scanalytics, Fairfax, VA). The fourth parental combination employed a silver staining technique based mainly on the procedure described in Budowle et al. (2), to visualize the amplified fragments the bands were then scored manually.

Results

Out of the 64 primer pairs used in the first experiment, 43 of these combinations resulted in fingerprint profiles in which polymorphic fragments were scored present and absent between the two RIL parents, 90-2079 and Puget, using the LI-COR genotyping system (Table 1). The 29 primer pairs which generated the highest number of polymorphic bands between the two parents were selected and then used on the (Puget × 90-2079) mapping population. These 29 primer pairs highlighted by shading in Table 2, amplified 209 polymorphic markers we could clearly score. The primer pairs with poor fingerprint patterns (frequent missing lanes and poor amplification) in the first experiment also produced poor amplification and fingerprints in the third experiment. However, silver staining the amplified fragments produced scoreable fragments in 61 out of 64 primer combinations (Table 3). Two of the three AFLP primers that failed to produce data with silver staining also failed with the infrared fluorescent dyes in the first three experiments (Tables 1, 2, and 3).

Table 1. Number of polymorphic fragments by primer pair between the four RIL parents used in the first experiment. The first number is the minimum and the second was the maximum number of polymorphic bands among the four parental lines.

Primers	M-CAA[1]	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC[2]	–[3]	–	–	–	–	–	–	–
E-AAG	–	6–9	12–15	7–8	–	12–14	4–5	5–8
E-ACA	–	6–9	10–13	7–10	2–4	13–16	3–4	9–12
E-ACC	1–5	12–17	4–7	13–20	–	4–5	4–6	2–5
E-ACG	–	6–7	7–10	7–10	–	8–9	4–5	5–6
E-ACT	–	14–21	6–10	5–7	–	8–10	5–7	4–5
E-AGC	3–5	5–6	12–13	4–7	–	6–10	2–4	4–6
E-AGG	–	9–12	10–13	6–8	–	7–10	–	–
Max.	10	81	81	70	4	74	31	42
Min.	4	58	61	52	2	58	22	29

[1] M indicates *Mse*I primer 5'GAT GAG TCC TGA GTA A+NNN 3'
[2] E indicates *Eco*RI primer 5' GAC TGC GTA CCA ATT C+NNN 3'
[3] - indicates poor amplification reaction with the primer pair

Table 2. Number of polymorphic markers generated per primer pair combination in the ('Puget' × 90-2079) RIL mapping population and amplified from 29 selected primer combinations. Primer combinations were selected from the first experiment based on clarity, repeatability, and the number of polymorphic markers generated.

Primers	M-CAA[1]	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC[2]	–[3]	–	–	–	–	–	–	–
E-AAG	–	3	6	3	–	7	–	5
E-ACA	–	–	3	2	–	8	8	5
E-ACC	–	9	–	4	–	–	–	–
E-ACG	–	8	5	11	–	6	–	7
E-ACT	–	12	5	11	–	11	–	–
E-AGC	–	5	10	–	–	4	–	8
E-AGG	–	9	11	14	–	9	–	–
Total	–	46	40	45	–	45	8	25

[1] M indicates *Mse*I primer 5'GAT GAG TCC TGA GTA A+NNN 3'
[2] E indicates *Eco*RI primer 5' GAC TGC GTA CCA ATT C+NNN 3'
[3] Blank cell indicates not done.

Table 3. Number of polymorphic bands by primer combination pair between 'DP' and JI 296 visualized by silver staining.

Primers	M-CAA[1]	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC[2]	–[3]	15	10	6	12	5	3	2
E-AAG	7	24	7	4	11	17	9	3
E-ACA	6	9	17	7	17	13	8	7
E-ACC	4	8	11	3	16	7	–	5
E-ACG	–	10	7	3	11	15	7	6
E-ACT	8	11	15	3	5	21	15	9
E-AGC	8	9	7	5	9	13	3	14
E-AGG	15	14	18	4	13	15	12	15
Total	48	100	92	35	94	106	57	64

[1] M indicates *Mse*I primer 5'GAT GAG TCC TGA GTA A+NNN 3'
[2] E indicates *Eco*RI primer 5' GAC TGC GTA CCA ATT C+NNN 3'
[3] – indicates poor amplification reaction with the primer pair

Discussion

In Table 4, we summarize our conclusions on the optimal primer pairs (41) of these 64 for use on pea using the LI-COR system. Pea had more good AFLP primer pairs than either rape seed or potato, but fewer than the other crops tested by Gibco/BRL including barley, lettuce, maize, pepper, sunflower, and tomato (1). In our study, the primer pairs highlighted in Table 4 has served as a template for identifying useful AFLP markers for fine mapping in RILs using the standard protocols reported here in infrared fluorescent dye-labeled primer experiments (1, 5).

Table 4. Guidelines for primer pair selection for creating a linkage map in pea using LI-COR.

Primers	M-CAA[1]	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E–AAC[2]	–[3]	–	–	–	–	–	–	–
E–AAG	–	Y	Y	Y	–	Y	Y	Y
E–ACA	–	–	Y	Y	Y	Y	Y	Y
E–ACC	Y	Y	Y	Y	–	Y	Y	Y
E–ACG	–	Y	Y	Y	–	Y	Y	Y
E–ACT	–	Y	Y	Y	–	Y	Y	–
E–AGC	Y	Y	Y	Y	–	Y	Y	Y
E–AGG	–	Y	Y	Y	–	Y	–	–

[1] M indicates *Mse*I primer 5'GAT GAG TCC TGA GTA A+NNN 3'
[2] E indicates *Eco*RI primer 5' GAC TGC GTA CCA ATT C+NNN 3'
[3] - indicates poor amplification reaction with the primer pair with infrared

Silver staining allowed us to visualized more scoreable polymorphisms than with the infrared fluorescent dye-labeled primer experiments using the procedures and pea genotypes presented. The protocol used with the LI-COR IR[2] could be modified in order to produce the same results as was found analyzing inter-simple sequences repeats used in conjunction with the same LI-COR instrument in this research (3). AFLP experiments should also be run on a larger set of pea genotypes in order to enhance our results obtained in this study.

Other AFLP primer pairs are interesting for use on pea linkage studies using the AFLP technique. For mapping *er*2, a powdery mildew resistance gene in pea, Tiwari et al (8) reported on the use of an additional 64 AFLP primer combinations by adding eight more *Mse*I plus NNN. Other AFLPs of interest for use on pea are those reported by Timmerman-Vaughan et al (7) based on *Pst*I/*Mse*I double digests. The amplification

profiles of the 64 primer pairs we tested on pea using LI-COR sequencer are posted on the cool season food legume genome database at http://coolgenes.cahe.wsu.edu/.

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