Optimization of PCR conditions for DNA Amplification of Common Buckwheat Using EST Primers

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ABSTRACT

Under optimal conditions the PCR reaction is very efficient; microgram quantities may be synthesized from a single molecule of substrate DNA. DNA of four lines of common buckwheat (Kyusu, Canada, Miyazaki and Botansoba) was used to optimize PCR reaction and cycling program of 26 primers for DNA amplification of common buckwheat. Annealing temperature (Ta), PCR cycle number and $MgCl_2$ concentration were considered optimum if the single clear band was observed. Of the 26 primers Ta of only 10 primers could be optimized. Three primer pairs performed best at Ta of 54^oC. The optimum concentration of $MgCl_2$ was found to be 1.5mM for all primer pairs. Similarly the number of PCR cycles was found to be 40 for all 10 primer pairs except for primer pair 57. Optimized PCR conditions were used for subsequent studies such as transferability of EST primers to other *Fagopyrum* species and construction of linkage map.

Key words: Annealing temperature, buckwheat, EST markers, optimization of PCR conditions

INTRODUCTION

The polymerase chain reaction (PCR) has become an indispensable tool of molecular biology. In the PCR technique, DNA is amplified in vitro by a series of polymerization cycles consisting of three temperature-dependent steps: DNA denaturation, primer-template annealing and DNA synthesis by a thermostable DNA polymerase. The PCR sample may be single- or double-stranded DNA or RNA (Rychlik et al 1990).

Under optimal conditions the PCR reaction is very efficient; microgram quantities may be synthesized from a single molecule of substrate DNA. Ordinarily, several experiments are required to achieve optimal conditions for PCR, even if good primers are chosen, ie those that do not form dimers by annealing of their 3'-termini and that are specific to one sequence in the substrate DNA (Rychlik et al 1990). Optimization of PCR involves testing a number of variables. Condition should produce high yields of specific DNA target sequences. Since no single set of conditions can be applied to all PCR amplifications, individual reaction component concentrations, and time and temperature parameters must be adjusted within suggested ranges for efficient amplification of specific targets (Applied Biosystem 2006).

With some combinations of primers and genomic DNA templates, a non discrete size range of amplification products, appearing as a smear as visualized on a gel could be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA (Williams et al 1990). Inclusion of formamide in the PCR eliminates most of the nonspecific products and increases the efficiency of the amplification (Sarkar et al 1990). Formamide is found to be a simple and inexpensive method of increasing the specificity of PCR.

There is obviously no single set of conditions that can be applied to all PCR amplifications. For example, depending on the length and sequence of primer used, the annealing temperatures of the PCR can differ from each other. Finding the optimal or the most stringent annealing temperature is a prerequisite for reliable results and efficient amplification of a specific target (Ishii and Fukui 2001). The requirement of an optimal PCR reaction is to amplify a specific locus without any unspecific by-products. Therefore, annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches the reaction (Henegariu 2006). For any given primer pair, the PCR program can be selected based on the composition (GC content) of the primers and the length of the expected PCR product. In the majority of the cases, products expected to be amplified are relatively small from 0.1 to 2-3 kb (Henegariu 2006).

EST-based markers are associated with the coding regions of the genome therefore, they enhance molecular germplasm evaluation by capturing variation across transcribed regions and in genes of known function (Buhariwalla et al 2005). In this article we experimentally determine the optimal annealing temperature (TaOPT) values for several expressed sequence tag (EST) primer pairs which were designed from the cDNA sequences from bud and style of common buckwheat (*Fagopyrum esculentum*, 2n = 2x = 16) (Jyotaro Aii 2006, personal communication). Optimal $MgCl_2$ concentration and number of PCR cycles were also determined.

MATERIALS AND METHODS

Plant materials and DNA extraction

DNA samples of four lines of common buckwheat (Kyusu, Canada, Miyazaki and Botansoba) was used to optimize the annealing temperature (Ta), number of PCR cycles and $MgCl_2$ concentration for 26 primer pairs (Table 1). Leaves from 22 day old seedlings were collected in plastic bag and kept at -20°C. DNA was extracted from the frozen dried leaf using the DNeasy Plant Mini Kit (Qiagen, Japan). The extracted DNA was stored at -20°C and used directly without diluting for further study.

PCR reaction

Except three variables (Ta, PCR cycle and $MgCl_2$ concentration) all other PCR protocols were same. Amplification reactions were performed in 30 µl reaction volume consisting of 14.7 µl sterile distilled water, 6 µl 5 x buffer (50 mM Tris-HCl pH 8.3, 250 mM KCl, 500 µM dNTP, 0.1% Triton X-100, 0.05% Gelatin, 7.5 mM $MgCl_2$), 3 µl of each primer of 2 µM concentration, 0.3 µl Taq DNA polymerase and 3 µl template DNA. Two concentrations of $MgCl_2$ (1.5mM and 2.0mM) were tested in final PCR reaction. DNA amplifications were performed in thermal cyclers (GeneAmp PCR system 9700 and 2700, and TAKARA) using the following cycling condition: one cycle of 94°C for 3 min; different cycles of 94°C for 0.30 min, different annealing temperatures for 0.30 min, 72°C for 0.30 min; one cycle of 72°C for 7 min followed by soaking in 4°C.

Analysis of PCR products

PCR products were analyzed in 2% Agarose gels containing 0.0025% ethidium bromide in 1 x TAE buffer. Electrophoresis was run at 100 V for 45 min. Gels were visualized and documented using Kodak 1D software (Kodak EDAS290). Annealing temperature, PCR cycle number and $MgCl_2$ concentration were considered optimum if the single clear band was observed. If the band was not observed, annealing temperature was decreased and in the case of multiple bands, the temperature was increased. The number of PCR cycle was increased if there was single band with low intensity and cycle number was decreased in the case of multiple and unclear bands. In some cases of very faint single band (low intensity), concentration of $MgCl_2$ and number of cycle were increased. In the multiple bands with high and low intensity the number of cycle was decreased. The experiment was repeated until the single clear band of tested primers was achieved.

Table 1. Primers used for optimizing PCR conditions (Kindly provided by Jyotaro Aii, Japan 2006)

PN	Primer	Sequence	Product length, bp	Tm	GC content, %
3	Fe_cb_0006_primer_4	GAGGCTACGGATTTTCTGCC	320	61.091	55.000
		TCCTCGTCCTCTTCCTCCTC		60.870	60.000
5	Fe_cb_0012_primer_2	CAAGCCAACAAGCTGGAGAA	307	61.481	50.000
		AATGGGAGAATGCTTAGTTGCTTAC		61.137	40.000
7	Fe_cb_0014_primer_0	CAGCATACCAATGGCAGAGAA	536	61.172	47.619
		TCGATACGATAACCGGAAACA		60.329	42.857
8	Fe_cb_0016_primer_0	TCCAAATAGTCCCAACTTTACGC	50	61.506	43.478
		GTTGTTAATGCCGATTGCCG		63.507	50.000
19	Fe_cb_0043_primer_2	GTTTCATGGCCGGTTTCATC	604	62.543	50.000
		CACGAGAAAAGGAGCACAGTC		60.043	52.381
24	Fe_cb_0052_primer_0	CGAGCAAAACATCTCATTCAGG	343	62.022	45.455
		TTTCAGCCTCACGAGTTCCA		61.917	50.000
26	Fe_cb_0061_primer_0	AATGTGGAGACGTGGGTGAG	157	60.987	55.000
		GATTTATGAGCGCCAGAGCA		61.413	50.000
28	Fe_cb_0066_primer_3	CCGAAACAGAAAGCATTACGA	401	60.250	42.857
		AAACAATGGAAGGAGGCAGG		61.370	50.000
37	Fe_cb_0089_primer_0	TCCTCCTTGAAATATCCTTCCC	448	60.609	45.455
		TCCATACAGACGCGATTCTTG		61.172	47.619
43	Fe_cs_0035_primer_0	TATTCTTTGATCTGGCCGGG	497	62.192	50.000
		CTGGTTGTGGTGGTTGTGTG		60.949	55.000
45	Fe_cs_0063_primer_2	AATGATGTCCTCCGCCACC	490	63.242	57.895
		TTAGAACTGACCAGCGGCAC		61.375	55.000
46	Fe_cs_0064_primer_0	AGCACCACCACCACCTCTTC	487	62.518	60.000
		TTCCCATACTCTTCGGTGTCCT		62.104	50.000
47	Fe_cs_0067_primer_0	ACTGGTTCTTGGTGAAGGGG	288	61.306	55.000
		CCCATTTCCCATTGGCTTT		61.934	47.368
54	Fe_cs_0171_primer_0	AGTGTTTAGGATGGCGAGAGG	231	60.635	52.381
		TCTCATGGGTACAATCGGTCA		61.313	47.619
55	Fe_cs_0173_primer_2	CTTTTCCGCCTCCTCTTCTC	578	60.455	55.000
		CCACTCTCCCTAACACTTGACC		60.031	54.545
57	Fe_cs_0177_primer_2	GCAGCCAGCAGTCGAGAAAC	428	63.553	60.000
		GACAGCGCACAATCACACAA		61.944	50.000
63	Fe_cs_0207_primer_1	GCAGCTGATTACGGCGTTC	276	61.879	57.895
		TGTTTGGTTTACTTGGCCCTC		61.239	47.619
65	Fe_cs_0214_primer_0	TCCGACATTGCGAATAACCA	651	62.331	45.000
		AGGGAGGGAGAGAGGGAAAA		61.429	55.000
68	Fe_cs_0221_primer_3	TATGAGCAAGAGCGAATGCC	225	61.413	50.000
		TGAGGGTGGATGTTTTGACC		60.758	50.000
69	Fe_cs_0222_primer_0	TGGCTGATAGCGGTGAAAGA	377	61.848	50.000
		GGATTGTGGGCTGACAAAGA		61.046	50.000
70	Fe_cs_0224_primer_0	AAGCATAGGTTGCCTTCCCA	124	61.875	50.000
		CGAGGAATCATCTCCAACCA		61.004	50.000
71	Fe_cs_0225_primer_3	TCCATCGTCATCCACCAAAC	178	61.749	50.000
		ATTGCTCGCGATTCCTCACT		61.318	52.632
73	Fe_cs_0227_primer_1	TGCACACCAAATTCACCAAG	138	60.552	45.000
		AAAGGAAAGGAGTAATGAGGAAGTG		60.375	40.000
88	Fe_cs_0272_primer_1	GGCAATCACATAAATGCAACC	100	60.,210	42.857
		GGTCGAATAATGAATACGCCAA		61.027	40.909
89	Fe_cs_0275_primer_0	TGGATTCCCAGATCAAGCAC	299	61.016	50.000
		TCCTTCAATCCCTAACCGAAA		60.778	42.857
90	Fe_cs_0279_primer_2	CACATACCAACCAGAACTCAATACA	184	60.208	40.000
		GCCCGAGTATCGTTTGCTCTC		63.371	57.143
91	Fe_cs_0287_primer_0	TCACAGACGGTATCTCCCCA	61	61.487	55.000
		GGGCATTGAGAAGGAAGGAC		60.970	55.000

Tm, Melting temperature.

RESULTS AND DISCUSSION

The four lines of common buckwheat (Kyusu, Canada, Miyazaki and Botansoa) are self compatible and inbred. Therefore, EST band should be single if the annealing temperature (Ta) of primer is optimum. Annealing temperature is based on the T_m (melting temperature) of the oligonucleotides chosen for PCR amplification. Whenever unwanted bands were observed, the annealing temperature was raised by 2°C-5°C in subsequent optimization runs. We started Ta from 62°C with 40 cycles of PCR for optimizing these parameters. Single and clear band in all four lines were considered as indicator to know the optimum Ta and number of cycles. The PCR results tested in different Ta are given in Table 2. Optimum Ta of primer pairs, 3 and 57 was known by a single experiment (Figure 1). On an average we conducted PCR 6.5 times for the same primer pair to know the optimum Ta. PCR had to be carried out 10 times to find out Ta of primer pair, 88.

As an example, some PCR results are given in Figure 2. Primer 3 showed single clear band in all four lines at Ta of 62°C. Therefore its optimum Ta is 62°C. But in other cases, some showed multiple bands, some were faint, some were missing and in some case no band was observed. For these conditions we had repeated experiments at different Ta. Among 26 primer pairs we could optimize the Ta of only 10 and others 16 primers were discarded due to difficulty in optimizing PCR conditions (Table 2). Three primer pairs performed best at Ta of 54°C. The optimum concentration of $MgCl_2$ was found to be 1.5mM for all primer pairs. Similarly the number of optimum PCR cycles was found to be 40 for all 10 primers except for primer pair 57 for which optimum cycle number was 30.

Table 2. Optimized PCR conditions of primers

SN	Primer	Ta	Cycle	$MgCl_2$	Product length, bp
1	3	62	40	1.5mM	300
2	7	58	40	1.5mM	600
3	8	66	40	1.5mM	900
4	28	60	40	1.5mM	200
5	57	62	30	1.5mM	400
6	65	60	40	1.5mM	200
7	71	54	40	1.5mM	200
8	88	52	40	1.5mM	100
9	89	54	40	1.5mM	300
10	90	54	40	1.5mM	200

The purity and yield of the reaction products depend on several parameters, one of which is the annealing temperature (Ta) (Rychlik et al 1990). At both sub- and super-optimal Ta values, non-specific products may be formed, and the yield of products is reduced (Coyne et al 2001). If the Ta is too low, non-specific DNA fragments are amplified, causing the appearance of multiple bands on agarose gels (Nakao et al 2001). If the Ta is too high, the yield of the desired product, and sometimes the purity is reduced due to poor annealing of primers (Rychlik et al 1990). Optimizing the Ta is especially critical when long products are synthesized or when a total genomic DNA is the substrate for PCR. The optimum annealing temperature is a function of the melting temperatures of the less stable primer-template pair and of the product (Rychlik et al 1990).

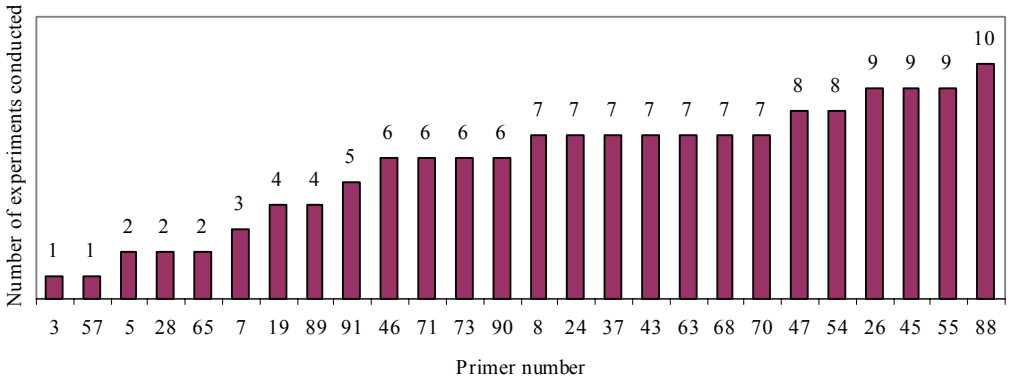

Figure 1. Number of experiments conducted for 26 primers to optimize PCR condition.

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction (Henegariu 2006). Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially template DNA). If the same reaction is performed in the presence of a higher amount of DNA template, the low annealing temperature results in the appearance of many non-specific secondary products. Thus, it appears that by decreasing the amount of DNA template, the number of potentially non-specific sites is also decreased, making possible the drop in annealing temperature (Henegariu 2006, Yap and McGee 1991). Annealing temperature is important in finding and documenting polymorphisms. Slight mismatches, (even 1 base-pair mutations) in one of sequences bound by the two primers used to amplify a DNA locus, can be detected by slight variations in annealing temperature and/or by multiplex PCR (Henegariu 2006). For segments with G + C content significantly greater than 75%, it may be necessary to either increase the denaturation and the annealing / elongation temperatures or to add denaturants such as DMSO or formamide (Dutton et al 1993).

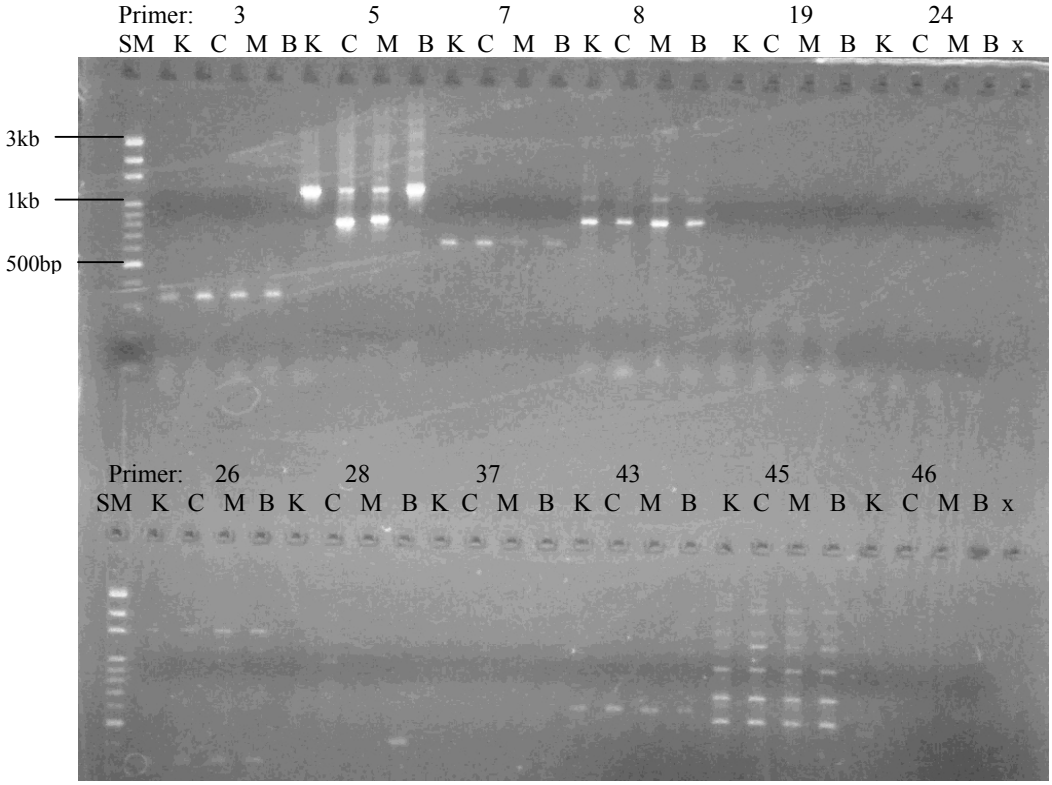

Figure 2. PCR products using different primers (indicated by primer number above the abbreviated buckwheat lines) at PCR 62^{o}C annealing temperature and 40 and 30 cycles *(SM, Size marker, K, Kyusyu, C, Canada, M, Miyazaki, B, Botansoba).*

In general, 30 cycles should be sufficient for a usual PCR reaction (Applied Biosystem 2006, Henegariu 2006). An increased number of cycles will not dramatically change the amount of product. The most obvious variation in the amount of products was around 24 cycles for ethidium bromide stained gels. 28-30 cycles are usually sufficient in a reaction. Little or no quantitative changes (ie relative amounts of PCR products) were observed with increasing cycle number up to 45 (Henegariu 2006). Little quantitative gain was noticed when increasing the number of cycles up to 60.

Generally, larger PCR volumes require lower or longer annealing temperature programs, because the temperature in larger volumes of reaction mixtures changes more slowly than that in smaller volumes (Ishii and Fukui 2001). These results indicate that annealing temperature is an important parameter in PCR for molecular research.

Optimization of PCR conditions is a critical precursor for accurate and robust large-scale marker screening and offers additional benefits for reducing the unit cost of genotyping. These optimized PCR conditions were used for further studies such as transferability of EST primers in other *Fagopyrum* species and construction of linkage map.

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