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Research Article

Successful Amplification of DNA Using Recombinase-Aided Amplification (RAA) and its Application to Seven Horticultural Crops

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Abstract

The amplification of nucleic acid is essential for many analyses requiring DNA. Current techniques require sophisticated equipment or complex experimental procedures whose implementation may be limited to outsourcing to other laboratories, or a continuous electricity supply that is expensive and inaccessible in many regions. In this study, a novel approach, recombinase-aided amplification (RAA), was used to rapidly amplify DNA. Since primer length is one factor that can influence the success of DNA amplification, in this assay, 18 primer pairs were tested between 18 and 28 base pairs in length. Four of these were applied to seven horticultural crops: mango (*Mangifera indica* L. var. 'Tainong no.1'), Manchurian walnut (*Juglans mandshurica* Maxim.), Chinese pear (*Pyruspyrifolia* (Burm.) Nak), grape (*Vitisvinifera* L. var. 'Thompson'), Cattley guava (*Psidium cattleianum* Sabine var. 'Littorale'), lychee (*Litchi chinensis* Sonn. var.'Bobaitangbo') and Chinese sugarcane (*Saccharum sinense* L.). RAA could successfully and consistently amplify the DNA of all seven horticultural crops.

Keywords: Amplification; Deoxyribonucleic acid; Primer length; Recombinase.

Introduction

Deoxyribonucleic acid (DNA) is essential for downstream applications. Its amplification is one of the most basic and important tools for molecular biologists [1], and usually applied in molecular biology, recombinant DNA technology, and medical and forensic tests [2,3]. Established DNA amplification techniques rely on sophisticated instrumentation such as temperature-regulating equipment or complex sampling handling procedures, and even though these are not problematic for specialized laboratories, their requirements have hampered their use in the field [4]. Recombinase-aided amplification (RAA) is a relatively recent isothermal amplification technique in which the classical thermal stable enzyme such as Taq polymerase has been replaced by recombinase, a DNA-binding protein and DNA polymerase [5]. The RAA reaction can be optimized at 37°C or at room temperature without any specialized heating system [5]. There are several isothermal nucleic acid amplification methods, including transcription-mediated amplification, nucleic acid sequence-based amplification, rolling circle amplification, signal-mediated amplification of RNA, strand displacement amplification, loop-mediated isothermal amplification of DNA, single primer isothermal amplification, helicase-dependent amplification, circular helicase-dependent amplification and isothermal multiple displacement amplification [2]. RAA overcomes the technical difficulties posed by these DNA amplification methods because it does not require thermal denaturation of the template and operates at a low and constant temperature. In combination with a novel probe-based detection approach, RAA is a significant advance in the development of portable and widely accessible nucleic acid-based tests. In RAA, the isothermal amplification of specific DNA fragments is achieved by binding opposing oligo nucleotide primers to template DNA and their extension by a DNA polymerase [5]. For the successful and consistent amplification of DNA by any method, there are some dependent factors, including the amount or quality of DNA, temperature, primer length and the proportion of reaction mixtures. The objective of this study was to assess a suitable primer length for the successful and consistent amplification of DNA over a wide range of horticultural species by RAA.

Materials and Methods

Plant Material

The mature leaves of mango (*Mangifera indica L. var. 'Tainong* no.1), Chinese pear (*Pyruspyrifolia* (Burm.) Nak), Cattley guava (*Psidium cattleianum* Sabine var. *'Littorale'*), lychee (*Litchi chinensis Sonn.* var. 'Bobaitangbo') and Chinese sugarcane (*Saccharum sinense L.*) were collected from Guangxi province, China. The mature leaves of Manchurian walnut (*Juglans mandshurica Maxim.*) and grape (*Vitisvinifera* L. var. 'Thompson') were collected from around

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Beijing. Harvested leaves were washed with tap water, blotted dried with filter paper and immediately stored in small sealed plastic bags to prevent the loss of transpiration. No ice boxes or liquid nitrogen were used during transportation to the laboratory, where they were frozen at -70°C until use. Standardization of the RAA protocol was carried out in the laboratory of the Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China.

Reagents Required

In this study a new protocol consisting of two solutions for genomic DNA extraction was used. Solution 1 consisted of 0.4 M glucose, 20 m-Methylenediaminetetraacetic acid (EDTA) (pH 8.0), 3% (w/v) polyvinylpyrrolidone (PVP-40; MW 40,000), and 0.2% (v/v) β-mercaptoethanol (v/v). Solution 2 consisted of 2% (w/v) cetyltrimethyl ammonium bromide (CTAB), 100 mMTris (pH-8.0), 20 m-Methylenediaminetetraacetic acid (EDTA) (pH 8.0), 1.4 M NaCl, and 0.15% (v/v) β-mercaptoethanol. In both solutions, β-mercaptoethanol was added just prior to use. In addition, the following reagents and buffers were prepared: chloroform: isoamyl alcohol (24:1, v/v); 70% alcohol; 100% alcohol; sodium acetate 3M (pH 5.2); a Tris-EDTA (ethylenediaminetetraacetic acid) (TE) buffer consisting of 10 mM Tris (pH8.0) and 1 mM EDTA (pH8.0). RNase a (0.01μg/ul) was used to remove RNA.

Genomic DNA Isolation and Purification and RAA Reaction

The protocol used was previously optimized for mango and several other horticultural crops [6]. In this study, 18in-house designed primers were synthesized by Beijing Sun biotech Co. Ltd. (Beijing, China; Supplementary Table 1) and tested. Reactions were performed in triplicate in a 50 μl reaction volume containing 17.5 μl of water, 25 μl of 10% polyethylene glycol (PEG), 2 μl forward primer,2 μl reverse primer, 1 μl genomic DNA template and 2.5 μl MgAc(RAA kit Taijing Sci. & Tech. Co. Ltd, Zhejiang, China). Tubes were mixed by flicking for a few seconds and then incubated at37°C for 40 min. An equal volume of phenol:chloroform (25:24, v/v) was then added and mixed well by vortexing. The mixture was centrifuged at 12,000 rpm for 5 min and the upper aqueous phase was collected into a new tube. The amplified products were separated by electrophoresis on a 1.5% agarose gel in 1X TAE buffer at 120V for 35 min and visualized by staining the gel with 0.5 μg/mL of ethidium bromide. Bands were photographed with a Gel doc system (Bio-Rad Laboratories, Hercules, USA) and the results were stored as digital pictures at 300 dpi. The size of each fragment was estimated with a Trans 2k Plus DNA ladder (Trans Gen Biotech, Beijing, China).

Results and Discussion

Even though there was some variability in the application of primers of different lengths, the DNA of seven horticultural crops could be successfully amplified by RAA. Only four primers (28, 24, 20 and 18 bp, which generate 174-, 250-, 208- and 244-bp fragments, respectively) could successfully amplify the DNA of seven horticultural crops after triplicate trials (Table 1). Mango, Manchurian walnut, Chinese pear, lychee and Cattley guava DNA could be amplified using the 20-, 24- and 28-bp primers (Figures1A, 1B, 1C, 1D, and 1E, respectively), but not by the 18-

Table 1: Primer sequences andprimer lengths used for RAA

Primer name	Sequence (5´-3´)	Primer length (bp)	Product size (bp)
Man (d)	F: cgtatatttaagttgttgcagttaaaaagc	30	174
	R: agcactctaatttcttcaaagtaacagc	28	
Man (g)	F: atcagataccgtcctagtctcaac	24	250
	R: agctctcagtctgtcaatccttac	24	
Man (n)	F: ttctgccctatcaactttcg	20	208
	R: tccaattaccaagctcgaag	20	
Man (t)	F: aataccgggctcttcgag	18	244
	R: gccagttaaggccaggag	18	

F: forward; R: reverse

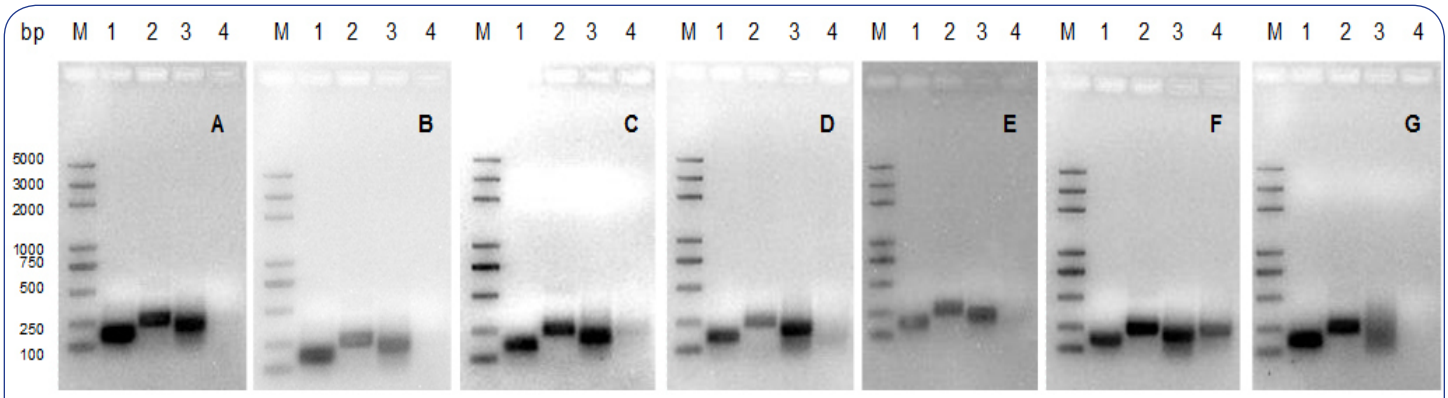

Figure1: Amplification profilesofsevenhorticultural crops using recombinase-aided amplification (RAA) amplified byfour primerslisted in Table 1. DNA marker = Trans 2K plus ladder.A = mango, B = Manchurianwalnut, C = Chinese pear, D = lychee, E = Cattley guava, F =grape, G =Chinesesugarcane. Lanes 1, 2, 3, 4 = 28, 24, 20, 18 bp primers, respectively (generating174-, 250-, 208- and 244-bp fragments, respectively).

bp primer. All four primers could amplify grape DNA (Figure 1F). Chinese sugarcane DNA was only amplified by 24- and 28-bp primers (Figure 1G). Primer length is an important parameter for the successful and consistent amplification of RAA to horticultural crops.

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Supplementary Table 1: 18 primers applied to RAA (tested originally for mango)

Primer name	Sequence (5´-3´)	Primer length	Product length (bp)
Man (a)	F: ttatagtttgtttgaaggtatctgctactc	30	421
	R: atttagattgtactcattccaattaccaag	30	
Man (b)	F: aatcagttatagtttgtttgaaggtatctg	30	427
	R: atttagattgtactcattccaattaccaag	30	
Man (c)	F: ttatagtttgtttgaaggtatctgctactc	30	429
	R: gttaagggatttagattgtactcattcc	28	
Man(d)	F: cgtatatttaagttgttgcagttaaaaagc	30	174
	R: agcactctaatttcttcaaagtaacagc	28	
Man (f)	F: actcggataaccgtagtaattctagagc	28	491
	R: ctttttaactgcaacaacttaaatatacgc	30	
Man(g)	F: atcagataccgtcctagtctcaac	24	250
	R: agctctcagtctgtcaatccttac	24	
Man (h)	F: atcagataccgtcctagtctcaac	24	227
	R: ctatgtctggacctggtaagtttc	24	
Man (i)	F: cattagcatgggataacatcatag	24	240
	R: atcgtttatggttgagactaggac	24	
Man (j)	F: attagcatgggataacatcatagg	24	239
	R:atcgtttatggttgagactaggac	24	
Man (k)	F: atcagataccgtcctagtctcaac	24	241
	R:tctgtcaatccttactatgtctgg	24	
Man (n)	F: ttctgccctatcaactttcg	20	208
	R: tccaattaccaagctcgaag	20	
Man (o)	F:gctaatacgtgcaccaaacc	20	152
	R: gggcagaaatttgaatgatg	20	
Man (p)	F: gctaatacgtgcaccaaacc	20	217
	R: aaccctaattctccgtcacc	20	
Man (r)	F: gctaatacgtgcaccaaacc	20	190
	R: accatggtaggcctctatcc	20	
Man (s)	F: gctaatacgtgcaccaaacc	20	151
	R: ggcagaaatttgaatgatgc	20	
Man (t)	F: aataccgggctcttcgag	18	244
	R: gccagttaaggccaggag	18	
Man (u)	F: accaaaccccgacttctg	18	230
	R: ttctcaggctccctctcc	18	
Man (v)	F: cattggagggcaagtctg	18	181
	R: gccagttaaggccaggag	18	

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