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Orthogonal design in the optimization of a start codon targeted (SCoT) PCR system in *Roegneria kamoji* Ohwi

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ABSTRACT. *Roegneria kamoji* Ohwi is an excellent forage grass due to its high feeding value and high resistance to some biotic and abiotic stresses. However, the start codon targeted (SCoT) polymorphism has not been conducted on *R. kamoji*. In this study, an orthogonal L_{16} (4^5) design was employed to investigate the effects of five factors (Mg^{2+}, dNTPs, Taq DNA polymerase, primer, and template DNA) on the polymerase chain reaction (PCR) to determine the optimal SCoT-PCR system for *R. kamoji*. The results showed that the most suitable

conditions for SCoT-PCR in *R. kamoji* included 1.5 mM Mg^{2+}, 0.15 mM dNTPs, 1.0 U Taq DNA polymerase, 0.4 pM primer, and 40 ng template DNA. SCoT primers 39 and 41 were used to verify the stability of the optimal reaction system, and amplification bands obtained from diverse samples were found to be clear, rich, and stable in polymorphisms, indicating that this reaction system can be used for SCoT-PCR analysis of *R. kamoji*. We have developed a simple and rapid way to study the mutual effects of factors and to obtain positive results through the use of an orthogonal design $L_{16}(4^5)$ to optimize the SCoT-PCR system. This method may provide basic information for molecular marker-assisted breeding and analyses of genetic diversity in *R. kamoji*.

Key words: *Roegneria kamoji* Ohwi; Reaction system; SCoT-PCR; Optimization; Orthogonal design

INTRODUCTION

Roegneria kamoji, which is classified under the largest genus in the wheat family, is mainly located in the warm and cold zones of the Northern hemisphere. There are about 120 *R. kamoji* species worldwide (Baum et al., 1991), of which 70 are located in the northwest, southwest, and north of China. *R. kamoji* is a fine forage grass because of its high feeding value, and high resistance to some biotic and abiotic stresses such as diseases caused by gibberella (*Fusarium graminearum*) and low-lying wet stress (Jiang and Liu, 1990; Yang et al., 2001). A number of different molecular marker systems have been developed to study the genetics of *R. kamoji*, including simple sequence repeats (Peng et al., 2012; Yang et al., 2016), gliadin (Xiao et al., 2008), RAMP (Zhang et al., 2005), and polymerase chain reaction (PCR)-RFLP (Marson et al., 2005; Zhang et al., 2006) markers. However, start codon targeted (SCoT) polymorphisms have not been applied to *R. kamoji*.

The SCoT polymorphism is a new method to sign target gene, which was proposed by Collard and Mackill (2009) in rice based on the single primer amplification reaction. In single primer PCR, SCoT uses single 18-mer primers to amplify the genomic region based on flanking sequences and conservation of the ATG translation start site in plant genes (Joshi et al., 1997; Collard and Mackill, 2009). SCoT markers are reproducible, suggesting that annealing temperature and primer length are not the sole factors determining reproducibility. These are dominant markers, similar to inter-simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD). In addition, they can be used for genetic analyses, quantitative trait loci (QTL) mapping, and bulk segregation analysis. In short, the SCoT polymorphism method is similar to RAPD and ISSRs because of the use of a single forward and reverse primer (Bhattacharyya et al., 2013). There are many advantages of this method, including its high polymorphism, simple operation, low cost, good universality, good reproducibility, and simple primer design (Hu et al., 2009). SCoT markers have been confirmed to be highly effective for the evaluation of genetic variation and population structure in rice (Collard and Mackill, 2009) and some other species including, mango (*Mangifera indica*) (Luo et al., 2011), grape (*Vitis vinifera*) (Guo et al., 2012), peanut (*Arachis hypogaea*) (Xiong et al., 2011), Lonicera Flos (*Lonicera macranthoides*) (Chen et al., 2015), and dendrobe (*Dendrobium nobile*) (Bhattacharyya et al., 2013). In addition, SCoT markers that are developed from transcribed

regions may be related to gene function. For example, the oligo-dT anchored cDNA-SCoT technique was used to exploit differentially expressed genes in mango (*M. indica* L.) under several stress conditions (Luo et al., 2014).

However, SCoT has some limitations. For example, 1) SCoT is based on the PCR method, but many factors including primer, dNTPs, Mg^{2+}, and Taq DNA polymerase concentrations influence the stability of PCR; 2) the low stringency PCR conditions may limit polymorphism detection; 3) PCR conditions may diverge between species (Collard and Mackill, 2009). Therefore, it is critical to establish a stable and optimized reaction system for use with SCoT molecular markers (Zeng et al., 2015).

In this study, we employed an orthogonal design referred to as $L_{16}(4^5)$ (four levels of five factors: Mg^{2+}, DNA template, Taq DNA polymerase, dNTPs, and primer) to optimize the SCoT-PCR system for *R. kamoji* and to provide a basis for the future study of genetic relationships, genetic diversity, construction of molecular linkage genetic maps, gene localization, variety identification, QTL analysis, and molecular marker-assisted breeding in *R. kamoji*.

MATERIAL AND METHODS

Materials

The samples used in this study were obtained from the Teaching and Research Center at Southwest University (Rongchang), Chongqing in China in May 2013 (Table 1). Fresh leaves were collected from the field and placed in an ultralow temperature refrigerator at -80°C.

Table 1. Names and types of *Roegneria kamoji* samples used in the experiment.

No.	Material type (name)	Source
1	Cultivar, Du Jiang Yan (ZY)	Sichuan, China
2	Cultivar, Japan (88)	Japan
3	Cultivar, Gansi One (CK)	Jiangxi, China
68	Wild material	Sichuan, China
69	Wild material	Sichuan, China
70	Wild material	Sichuan, China
71	Wild material	Sichuan, China
72	Wild material	Sichuan, China
73	Wild material	Sichuan, China
74	Wild material	Sichuan, China
75	Wild material	Sichuan, China
76	Wild material, 004	Chongqing, China
77	Wild material, 005	Chongqing, China

DNA extraction and PCR program

The DNA of 13 fresh young leaf samples was extracted using a genomic DNA extraction kit (ComWin Biotechnology Co., Ltd., Beijing, China). The concentration and quality of the DNA were confirmed by electrophoresis on 0.8% agarose gels and spectrophotometric analysis with the NanoDrop 2000 nucleic acid/protein analyzer (Nanodrop Technologies, Wilmington, DE, USA). Samples were stored at -20°C and DNA was diluted to 20 mg/μL prior to conducting the experiment, and then stored at -4°C.

The SCoT primer sequences (SCoT S16: 5'-ACCATGGCTACCACCGAC-3'; SCoT

S39: 5'-CAATGGCTACCACTAGCG-3', and SCoT S41: 5'-CAATGGCTACCACTGACA-3') were previously described by Luo et al. (2011), and synthesized by Shanghai Shenggong Biological Engineering Technology Services Ltd. (Shanghai, China). Taq DNA polymerase, 6X buffer, 10X buffer, dNTPs, Mg^{2+}, and DL2000 marker were provided by Takara Biotechnology (Dalian) Co., Ltd. (Shiga, Japan). PCR amplification was performed on an Eppendorf Mastercycler (Hamburg, Germany) and included the following thermal profile: 94°C for 3 min, followed by 36 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The final extension was set at 72°C for 10 min, followed by storage at 4°C. The PCR amplification products were then added to 2 μL 6X buffer, and a 8-10 μL sample from each treatment was separated on 1.5% agarose gels in 1X Tris-borate EDTA buffer, and then stained with GoldView™ dye (Beijing Bioteke Biotechnology Co., Beijing, China). Finally, a gel documentation system (Bio-Rad, Hercules, CA, USA) was used to visualize the DNA fragments under UV light.

Orthogonal array design

The optimum concentrations of template DNA (Gansi One), Taq DNA polymerase, dNTPs, Mg^{2+}, and primer (SCoT S16) were determined using an orthogonal design L_{16} (4^5). We selected four concentrations based on experience. Further details about these five factors, and detailed information on the experimental concentrations, are provided in Table 2. The L_{16} (4^5) orthogonal experimental design is shown in Table 3. The amplification conditions were as follows: 2.0 μL 10X buffer and other components, and ddH_2O was added to obtain a final volume of 20 μL. A scoring system was applied to determine the variance between patterns of SCoT-PCR fingerprints obtained under different treatments. A scoring criterium was used as described by He et al. (1998), and the generated DNA amplification patterns were scored from the best (16 points) to the worst (1 point), including the number of amplified fragments and the clear degree of the PCR amplification results (Table 3).

Table 2. Four different levels of factors and volume for SCoT-PCR amplification.

No.	Mg^{2+} (mM)	dNTPs (mM)	Taq DNA polymerase (U)	Primers (pmol/μL)	Template DNA (ng)
1	1.5	0.15	0.75	0.2	20
2	2.0	0.20	1.00	0.3	30
3	2.5	0.25	1.25	0.4	40
4	3.0	0.30	1.50	0.5	50

Determination of an optimal reaction system

According to the score for each treatment, the optimal treatment was calculated (Table 4). The experimental Ki value represents the total score for each factor at the same level, the experimental ki value represents the average of the total score for each factor at the same level, and the experimental R value represents the range of the average total score for the same factors between various levels.

Stability analysis of two reaction systems

The DNA samples (1, 2, 68, 70, 72, 74, and 76) were used to compare the stability of

the highest scored and statistically optimum reaction systems using the SCoT primer S16. The best reaction system was then selected to verify its stability using the SCoT S39 and SCoT S41 primers for 13 samples shown in Table 1.

Table 3. L_{16} (4^5) orthogonal design used for SCoT-PCR amplification.

Treatment No.	Mg^{2+} (mM)	dNTPs (mM)	Taq DNA polymerase (U)	Primers (pmol/μL)	Template DNA (ng)	Score
1	1.5(1)	0.15(1)	0.75(1)	0.2(1)	20(1)	11
2	1.5(1)	0.20(2)	1.00(2)	0.3(2)	30(2)	12
3	1.5(1)	0.25(3)	1.25(3)	0.4(3)	40(3)	13
4	1.5(1)	0.30(4)	1.50(4)	0.5(4)	50(4)	7
5	2.0(2)	0.15(1)	1.00(2)	0.4(3)	50(4)	16
6	2.0(2)	0.20(2)	0.75(1)	0.5(4)	40(3)	10
7	2.0(2)	0.25(3)	1.50(4)	0.2(1)	30(2)	3
8	2.0(2)	0.30(4)	1.25(3)	0.3(2)	20(1)	2
9	2.5(3)	0.15(1)	1.25(3)	0.5(4)	30(2)	8
10	2.5(3)	0.20(2)	1.50(4)	0.4(3)	20(1)	5
11	2.5(3)	0.25(3)	0.75(1)	0.3(2)	50(4)	6
12	2.5(3)	0.30(4)	1.00(2)	0.2(1)	40(3)	14
13	3.0(4)	0.15(1)	1.50(4)	0.3(2)	40(3)	15
14	3.0(4)	0.20(2)	1.25(3)	0.2(1)	50(4)	1
15	3.0(4)	0.25(3)	1.00(2)	0.5(4)	20(1)	9
16	3.0(4)	0.30(4)	0.75(1)	0.4(3)	30(2)	4

Table 4. L_{16} (4^5) orthogonal design for SCoT-PCR amplification.

Results	Mg^{2+} (mM)	dNTPs (mM)	Taq DNA polymerase (U)	Primers (pmol/μL)	Template DNA (ng)
K1	43.00	50.00	31.00	29.00	27.00
K2	31.00	28.00	51.00	35.00	27.00
K3	33.00	31.00	24.00	38.00	52.00
K4	29.00	27.00	30.00	34.00	30.00
k1	10.75	12.50	7.75	7.25	6.75
k2	7.75	7.00	12.75	8.75	6.75
k3	8.25	7.75	6.00	9.50	13.00
k4	7.25	6.75	7.50	8.50	7.50
R	3.50	5.75	6.75	2.25	6.25

RESULTS

Visual analysis of the PCR orthogonal design

Multiple fingerprinting patterns were observed from the PCR amplification products after the orthogonal experiment treatments. Treatment 5 showed the clearest fragments (the score was 16), while the treatment 14 displayed the vaguest fragments (the score was 1) (Figure 1).

For an optimal reaction system, the range, R, reflects the influence of Mg^{2+}, dNTPs, Taq DNA polymerase, primer, and template DNA concentrations. A larger range of R indicates that the factor has a greater influence. The R ranged from high to low, and the factors that affected fingerprinting patterns were in the order of Taq DNA polymerase, template DNA, dNTPs, Mg^{2+}, and primers. The ki value can indicate the optimal concentration of each factor, since it reflects the influence of each factor at a specific level on the reaction system, and a larger ki indicates a better reaction level. To determine the relationship between the five factors, ki was represented as bar charts (Figure 2). Mg^{2+} at a concentration of 1.5 mM performed best in the amplification reaction, while the worst performance was observed at 3.0 mM Mg^{2+} (Figure 2A).

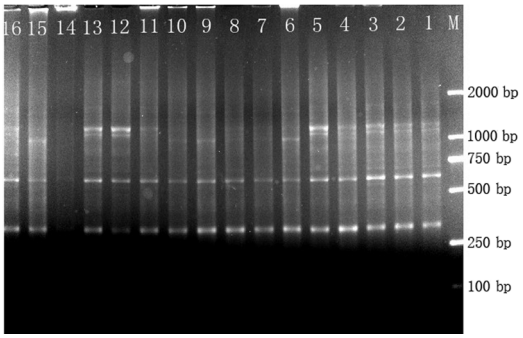

Figure 1. Electrophoresis of samples obtained using an orthogonal design. *Lane M*: marker (DL2000); *lanes 1-16*: treatment numbers are the same as those noted in Table 3.

The concentration of dNTPs will directly affect the results of the PCR, and dNTPs are the substrates of Taq DNA polymerase. As shown in Table 2, the R value of dNTPs was large, which means that changes in the concentration of dNTPs greatly impacted the *R. kamoji* SCoT-PCR. The best amplification results were obtained with 0.15 mM dNTPs (Figure 2B). Taq DNA polymerase, which had the largest R value, is a key factor in the PCR. With increasing concentrations of Taq DNA polymerase, the ki value fluctuated, and the best fingerprint patterns were observed at a concentration of 1.0 U (Figure 2C). The R value of the primers was the lowest, indicating that this factor had the least significant influence. The concentration with the highest score was 0.4 pM (Figure 2D). The R value of template DNA was only less than Taq DNA polymerase, which meant that it was the second most significant factor. An appropriate DNA concentration is a prerequisite for amplification, and the best performance was observed at a concentration of 40 ng, as shown in Figure 2E.

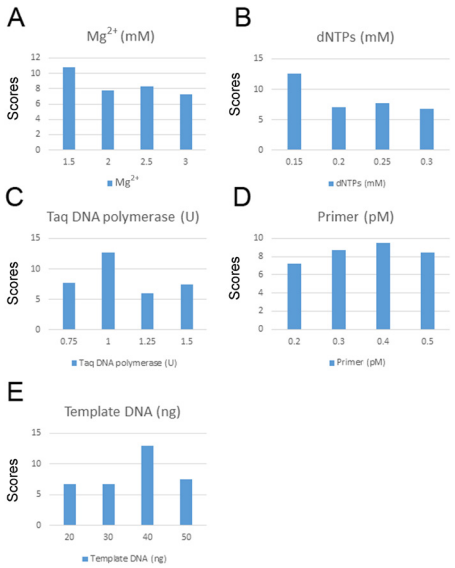

Figure 2. Bar charts showing the relationships between the following factors. **A.** Mg^{2+}; **B.** dNTPs; **C.** Taq DNA polymerase; **D.** primer; **E.** template DNA.

These findings suggest that 1.5 mM Mg^{2+}, 0.15 mM dNTPs, 1.0 U Taq DNA polymerase, 0.4 pM primer, and 40 ng template DNA generate the best amplification results. However, these concentrations differed to those applied in the highest scored treatment (2.0 mM Mg^{2+}, 0.15 mM dNTPs, 1.0 U Taq DNA polymerase, 0.4 pM primer, and 50 ng template DNA). To confirm the efficiency of the conditions, different template DNA was amplified. As shown in Figure 3, the fingerprinting patterns of the two systems were quite analogous, indicating that both amplification results were good. However, the amplified fragments obtained with the optimal treatment generated brighter bands than those obtained with the highest scored treatment (Figure 3). Hence, 1.5 mM Mg^{2+}, 0.15 mM dNTPs, 1.0 U Taq DNA polymerase, 0.4 pM primer, and 40 ng template DNA represent the most suitable condition for use in the SCoT-PCR system.

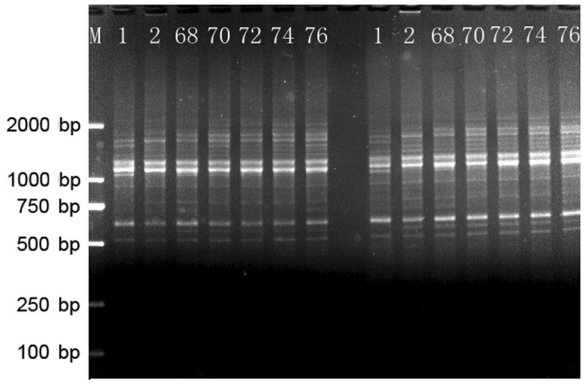

Figure 3. Electrophoresis of the optimal reaction system (right) and the highest scored orthogonal reaction system (left). *Lane M*: marker (DL2000).

Stability of the best reaction system

SCoT primers 39 and 41 were used to verify the stability of the best reaction system. As shown in Figure 4, amplified bands from diverse samples were clear, rich, and stable in polymorphisms. The fingerprinting patterns of the *R. kamoji* samples revealed the genetic differences between samples, and confirmed the genetic stability of each sample, indicating that the best reaction system can be applied in SCoT-PCR analysis of *R. kamoji*.

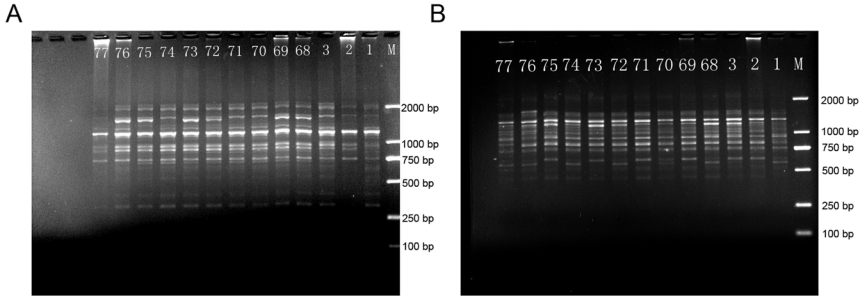

Figure 4. Electrophoresis of start codon targeted (SCoT) S39. **A.** SCoT S41. **B.** *Roegneria kamoji* samples using the best reaction system. *Lane M*: marker (DL2000).

DISCUSSION

The five factors studied here generate different fingerprinting patterns and Taq DNA polymerase is a key factor affecting the PCR. Whether high or low, the concentration of Taq DNA polymerase would lead to poor amplification; therefore, the most effective concentration is 1 U, which is consistent with the result of Yang et al. (2007). There are interactions between each factor in the PCR system. Taq DNA polymerase is an Mg^{2+}-dependent enzyme, which is influenced by the concentration of Mg^{2+}, while Mg^{2+} is also affected by other factors, especially dNTP (Zheng et al., 2008). In the present study, the primer concentration had the smallest influence on the PCR result, which conflicts with the results of Zeng et al., (2015) who used *Dactylis glomerata* as the test material, indicating that samples from different species may generate different results.

Optimizing the SCoT-PCR system for *R. kamoji* is essential. We have developed a simple and rapid method that can be used to study the mutual effects of different factors and obtained good results through the use of an orthogonal design L16 (45) to optimize the SCoT-PCR system. In addition, this method can be used in follow-up studies in *R. kamoji*, especially for molecular marker-assisted breeding and in analyses of genetic diversity.

Conflicts of interest

The authors declare no conflict of interest.

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