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Construction and analysis of the AFLP reaction system of *J. curcas* L.

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ABSTRACT

The high-quality genomic DNA which was suitable for AFLP analysis was isolated by an improved CTAB method from *J. curcas* L. Some key factors affecting DNA extraction, restriction-ligase reaction, pre-amplification and amplification processes were studied and an optimized AFLP reaction system of *J. curcas* was established. Referring to the AFLP reaction system, clear DNA electrophoresis fragments of *J. curcas* were obtained and the result was well reproductive.

KEYWORDS

J.curcas L; DNA extraction; AFLP.

INTRODUCTION

J. curcas L., also known as Xiaotongxi, Gaotong, etc, belongs to the Euphorbiaceae, is native to tropical America, and is now widely distributed in tropical and subtropical regions across the world and distributed in Yunnan, Sichuan, Guangdong, Guangxi and Hainan, etc, in China[1]. stem, leaf and bark of *J. curcas* L. are rich in milk, contain a lot of toxic protein, ketones and other antivirus, anti-AIDS, anti-diabetes and anti-tumor substances, therefore, *J. curcas* L. is a research hotspot globally, especially in the analysis of chemical composition, toxicity and pharmacological guards[2]-[4]. In addition, *J. curcas* L. has a high oil content up to 60% in seeds, it is a high-quality renewable oil plant resource, and can be directly for biodiesel production. *J. curcas* L. is significant thanks to its fast growth, drought resistance, barren resistance and other advantages in the greening of barren hills, water and soil conservation, returning farmland to forest[1]. Therefore, *J. curcas* L. is FAO-listed as a preferred species for renewable energy and reducing the ecological poverty. However, rare studies have focused on the genetic evolution evaluation of resources, genetic breeding of *J. curcas* L. The *J. curcas* resource has been restricted in promotion and cultivation, together with economic and social benefits.

The amplified fragment length polymorphism (AFLP) has been widely applied such fields as biological diversity, phylogeny, linkage map construction, gene location, cultivar identification and molecular breeding owing to the less DNA dosage, good repeatability, abundant polymorphism, high efficiency and reliability and that the genome sequence information is not known in advance[5][6]. Therefore, the present paper is designed to create a set of more reliable *J. curcas* L. AFLP reaction system through creating and optimizing the AFLP reaction system of *J. curcas* L., and plays a good ground for the genetic diversity, germplasm conservation and breeding of *J. curcas* L.

MATERIALS AND METHODS

Mateials

J. curcas L. leaves were collected from Jinsha River basin, Panzhihua, packed in plastic bag, dried with silica gel particles and saved in a fridge at -80 Celsius degree.

DNA Extraction

The leaf DNA was extracted using modified CTAB method. 0.2 g leaves were ground into power in a mortar with liquid nitrogen. During grinding, add PVP-40, transfer the powder to a 2mL centrifugal tube, add 500μL 2×CTAB preheated extraction buffer at 65℃ and 10 μL 10 mmol L^{-1} β-ME, shake evenly, bathe at 65℃ for 45-60 min, slowly shake every 5min so that the solution is adequately mixed; cool the mixture down to room temperature, add chloroform and isoamylol in equal volume (24:1, V V^{-1}), gently put upside down for about 20 min, centrifuge at 4℃ in 8000 r min^{-1} for 6 min, move the supernatant to a new centrifugal tube; add chloroform and isoamylol in equal volume in the supernatant, put upside down evenly, centrifuge at 8000r min^{-1} for 4 min, and recover the supernatant; add pre-cooled isoamylol in 2/3 volume in the supernatant, and settle the DNA, pick up the flocculent DNA; wash the DNA with 70% alcohol trice and dry the DNA at room temperature; dissolve the DNA with appropriate amount of 0.1×TE buffer; purify the DNA by adding Rnase in 1μL:10μL, and save the purified DNA at -20℃ for use. Determine the purity and concentration of genomic DNA by electrophoresis and SmartSpecTM 3000 nucleic acid protein analyzer.

TABLE 1 : AFLP reaction system of *J. curcas*

酶切体系Digestion system	连接体系Ligation system	预扩增体系Pre-amplification system
2μL 10×NEB buffer	2μL 10×T_4 ligase buffer	2μL 10×PCR buffer
0. 2μL 100×BSA	0.5μL *EcoR*I adaptor (10 pmol/μL)	2μL Mg^{2+} (25mmol/L)
3 U *EcoR* I	5μL *Mse*I adaptor (10 pmol/μL)	2μL dNTPs(2.5mmol/L)
3 U *Mse* I	1U T_4 DNA Ligase	1U Taq polymerse
2μL DNA (200 ng/μL)	Digestion reaction solution 10μL	1μL ligation template
		2.4 μL Primer E-A00 (10 pmol/μL)
		2.4 μL Primer M-C00 (10 pmol/μL)
Ad ddH_2O up to 20 μL	Add ddH_2O up to 20 μL	Add ddH_2O up to 20 μL

Creation and optimization of AFLP system

J. curcas AFLP system is created according to the methods described by Vos et al[7] and optimized about the key factors.

Setting of template DNA concentration

Dilute the extracted leaf DNA with 0.1×TE up to 100 ng/μL as template, compare the influence of different template concentrations (100, 150, 200, 250 and 300 ng/μL) on AFLP reaction.

Digestion and ligation

Digest the template DNA using Mse I and EcoR I enzyme, set 4 digestion times 3, 4, 5 and 6h, and digestion temperature 37℃, keep the temperature at 70℃ for 20min before the reaction is stopped; and then add the ligation solution for overnight ligation at 16℃. Test the digested products by 1.2% agarose gel electrophoresis. See TABLE 1 for digestion and ligation system.

Preamplification

Dilute the ligated products by 10 times as the pre-amplification template. See the TABLE 1 for pre-amplification system. The PCR amplification procedure is done under the reaction conditions as below: predenaturate at 94 ℃ for 2 min; denaturate at 94℃ for 3s, anneal at 56℃ for 30s and extend at 72℃ for 1min in totally 20 cycles. After the preamplification reaction is finished, measure 5μL pre-amplified products for 2% agarose gel electrophoresis to test the preamplification effect. 5μL reaction product to dilute with 0.1*TE buffer in 1: 50 was measured

Selective amplification

The key factors that affect the results of selective amplification system were optimized in the reaction system. See TABLE 3 for gradient design of each factor in the reaction system. The best selective amplifications system was optimized through the single factor and orthogonal experiment. With the gradient PCR amplification technique, the reaction conditions are as follows: predegenerate at 94℃ for 2 min; denaturate at 94℃ for 30s, anneal at 65 ℃ for 30s and extend at 72 ℃ for 1min; afterwards, reduce the annealing temperature by 0.7℃ in each cycle till 56℃ after 13 cycles; carry on 24 cycles without any change to the rest conditions.

TABLE 2 : Adapter and Primer sequences of AFLP analysis for *J. curcas*

Name	Code	Sequence
EcoRI adapter		5’-AAT TGG TAC GCA GTC TAC-3’ 3’-CC ATG CGT CAG ATG CTC-5’
MseI adapter		5’-TAC TCA GGA CTC AT-3’ 3’-G AGT CCT GAG TAG GAG-5’
EcoRI Primer	E-A00	5’-GTA GAC TGC GTA CCA ATT C A-3’
MseI Primer	M-C00	5’-GAT GAG TCC TGA GTA A C-3’
EcoRI +3- ACT	E-ACT	5’- GTA GAC TGC GTA CCA ATT C ACT-3’
MseI +3- CTT	M-CTT	5’- GAT GAG TCC TGA GTA A C CTT-3’

Electrophoretic analysis of selective amplification products

TABLE 3 : Concentration gradients design of different ingredientsfor AFLP selective amplification

Factor	Unit	Concentration ingredient
Mg^{2+}	mmol/L	1.5, 2, 2.5, 3, 3.5, 4
dNTP	mmol/L	0.1, 0.2, 0.3, 0.4, 0.5, 0.6
*Taq*E	U	0.5, 0.75, 1, 1.25, 1.5, 1.75
Primers	μmol/L	0.2, 0.3, 0.4, 0.5, 0.8, 1.0

Test the selective amplification products first by 2% agarose gel electrophoresis, and then measure and mix 3μL products with equal volume of 2*AFLP sampling buffer, denature at 95℃ for 5min, conduct 6% polyacrylamide gel electrophoresis on Model DYCE-20C sequencer(Beijing Liuyi Factory) at 50W constant power for 2h. After electrophoresis, conduct the silver staining step: fix with fixing liquid containing 20% methanol and 10% acetic acid for 20min; wash with ion-free water trice for 5min each; adequately oscillate for 45min in the staining solution (2g $AgNO_3$, 3mL 37% formaldehyde, and 2000mL ultra-pure water); wash the gel with ultra-pure water for 9s; adequately oscillate in the developing solution (60g Na_2CO_3/2L ultra-pure water, added 3mL 37% formaldehyde, 400μL 10g/L sodium thiosulfate solution prior to use) till all bands appear; finally stop develop in the fixing solution (containing 20% methanol and 10% acetic acid) and wash in the ion-free water; place the glass plate vertically overnight at room temperature, dry and scan the images and record the data.

RESULT AND DISSCUSS

DNA extraction of *J. curcas*

As shown in Figure 1 for the electrophoresis result of CTAB-extracted DNA, DNA has no obvious dispersion phenomenon with main clear band, suggesting that the genomic DNA is perfect. OD260/OD280 was tested between 1.7~1.9 in line with the AFLP experimental requirement.

AFLP construction of *J. curcas* L.

Template DNA: 5 DNA template dosage 100~300ng set in the experiment can give the clear of AFLP spectrum, suggesting that the template concentration ranged between 100 and 300ng does not affect the experimental results of J. curcas L. AFLP system.

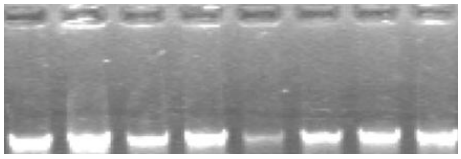

Figure 1 : Agarose gel electrophporesis of genomic DNA

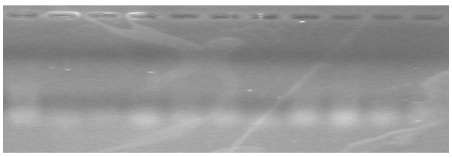

Figure 2 : Restriction figure of the genomic DNA with *EcoR* I and *Mse* I on 2.0 % agarose gel electrophoresis

Digestion and ligation: the digestion pattern of EcoR I and Mse I restriction endonucleases was used. The digestion results in Figure 2 showed that the genomic DNA can be completed digested by EcoR I and Mse I. Analysis on 5 digestion time showed that the result at digestion time 4~5h is best. But if the digestion time is too short, the digestion will be incomplete; on contrary, if the digestion time is too long, the specificity of band will be reduced.

Pre-amplification: pre-amplification results can not only reflect the ligation results, but also directly affects the results of selective amplification. Figure 3 shows the preamplified signal is strong consistently among all samples. This suggests that the pre-amplification system has a better result in the experiment, that can provide a ideal template for the late selective subsequent amplification and that the previous operation steps and results are in line with the requirement.

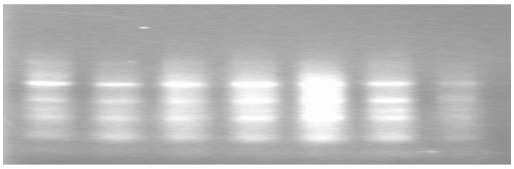

Figure 3 : Preamplification

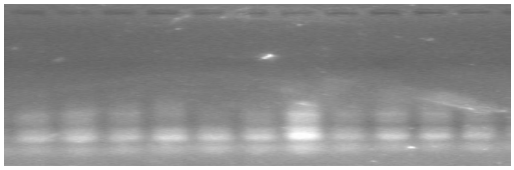

Figure 4 : Selective amplification

Creation of selective amplification system: With comparable studies of several key factors, such as Mg^{2+}, dNTP, Taq and primer concentrations in single factor and orthogonal experiments, it may be created that 20μL selective amplification system is a best combination: 2.5mmol/L Mg^{2+}, 0.3mmol/L dNTP, 1U Taq, 0.4μmo/L primers concentrations. See Figure 4 for the2% agarose gel electrophoresis of selective amplification products in the system. The selective amplification products give a clear polymorphism electrophoresis spectrum through 6% polyacrylamide gel electrophoresis (see Figure 5), suggesting that the reaction system is suitable for the analysis of J. curcas L. AFLP.

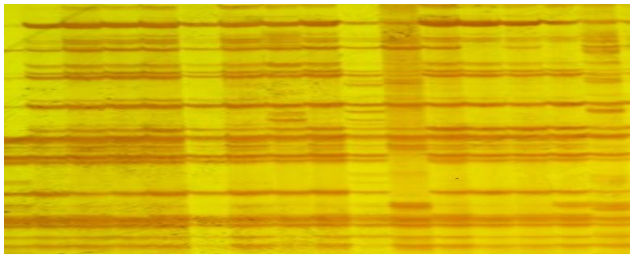

Figure 5 : AFLP fingerprints by primer E-ACT/M-CTT on 6.0 % polyacrylamide gel electrophoresis

DISCUSSION

As AFLP analysis highly requires the DNA template, to extract the high-quality and high-purity DNA is critical to the AFLP's success. Therefore, the present experiment used the modified CTAB method in term of the more substances and complex components in J. curcas L. for total DNA extraction. The test proofed thatadding a small amount of PVP-40 during liquid nitrogen grinding process can greatly improve the DNA extraction quality; and this result was agreed by other plant DNA extraction researches[8][9]. PVP-40 has the ability to strongly bind the polyphenol, can prevent phenolic oxidation into quinines after binding to avoid the solution browning and have antioxidant properties, and can prevent DNA enzyme to degrade the DNA. In addition, adding chloroform/isoamyl alcohol extraction times can play a marked role in removing the protein and polysaccharide, etc in DNA, however, it is also marked that the extracted DNA is lost. Therefore, it is recommended to extract preferably twice.

There are many affecting factors and the procedure is more complex during J. curcas L. AFLP analysis. The concentration is also required in addition to the highly required template DNA quality. If the DNA concentration is too high, more primer or dNTPs will be consumed, the substrate will be amplified excessively, and the unstable illusion of amplification result occurs[9]. During the preamplification and selective amplification process, the Mg^{2+}concentration not only affects the Taq activity, it can also combine with dNTP and DNA template in the reaction solution, affect the binding efficiency between primer and template, chain decomposition temperature between template and product and product specificity and form the primer dimer[7]. Additionally, for the dNTPs as the raw material in PCR reaction, too high concentration will lead to PRC mismatch and the non-specific amplification appears; too low concentration will affect the synthesis efficiency[10]. The samples must be adequately denatured prior to polyacrylamide gel electrophoresis, or otherwise, the background of the run will be darken; too long staining time will lead to darker gel plate background and affect observation. The developing time also serves as a key factor for the silver staining quality. If the band developing time is too long, the band will be relatively blurred; if the band developing time is too short, it is difficult to observe the color and shadow; the developing temperature must also be kept at n 12~15℃.

CONCLUSIONS

The present study creates and optimizes the J. curcas L. AFLP reaction system, provide a ground for the founding of J. curcas L. molecular marker technological platform and researches on the germplasm resource diversity, and has a practical guidance and broad application prospects for J. curcas L. breeding, molecular marker breading, resource utilization and protection.

REFERENCES

[1] Xu Yulan, Cai Nianhui, Xu Hui; Research progress and discussion on breeding of biofuel Jatropha curcas L. [J], Fujian Forestry Science and Technology, **34(3)**, 238-242 **(2007)**.

[2] P.Sirisomboon, P.Kitchaiya, T.Pholpho et al.; Physical and mechanical properties of *Jatropha curcas* L. fruits, nuts and kernels [J], Biosystems Engineering, **97**, 201-207 **(2007)**.

[3] J.Martiner-Herrera, P.Siddhuraju, G.Francis; Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of J*atropha curcas* L. from Mexico [J], Food Chemistry, **96**, 80-89 **(2006)**.

[4] Fan Judi, Yang Song, Song Baoan et al.; Research progress of jatropha curcas L. pesticides and pharmaceutical and biological activity [J], Pesticides, **45(5)**, 298-301 **(2006)**.

[5] Guo Yanyan, Tang Wenyu, Xing Shiyan et al.; Creation and optimization of AFLP reaction system of Ginkgo biloba[J], Jiangxi Agricultural University Journal, **28(3)**, 377-381 **(2006)**.

[6] L.Tatikonda, S.P.Wani, S.Kannan et al.; AFLP-based molecular characterization of an elite germplasm collection of *Jatropha curcas* L., a biofuel plant [J], Plant Science, doi, 10.1016. (available online), **(2009)**.

[7] P.R.Vos, M.Hogers, T.Reijans et al.; AFLP, A new technique for DNA fingerprinting [J], Neu Acid Res, **23(21)**, 4407-4414 **(1997)**.

[8] Huang Wenkun, Guo Jianying, Wan Fanghao et al.; Creation of eupatorium adenophorum DNA extraction and AFLP reaction system, Journal of Wuhan Botanical Research [J], **24(6)**, 498-504 **(2006)**.

[9] Yang Juan, Li Shan, Cao Dongwei et al.; Creation and analysis of dipteronia AFLP reaction system, Acta Botanica Boreali-Occidentalia Sinica [J], **25(11)**, 2173-2177 **(2005)**.

[10] Chen Min, Tao Jing, Ma Chaode et al.; Holcocerus hippophaecolus primers screening and creation of AFLP reaction system [J], Journal of Beijing Forestry University, **30(4)**, 116-120 **(2008)**.