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Genetic Differentiation Caused by Chromium Treatment in *Leersia hexandra* Swartz Revealed by RAPD Analysis

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Abstract

Randomly amplified polymorphic DNA (RAPD) technique was applied to assess the genetic variations and phylogenetic relationships in genetic differentiation within 4 Chromium-treatment *Leersia hexandra*. The fresh leaves of *Leersia hexandra* cultivated on the condition of chrome pollution and exogenous organic acids were used as experimental material. The genomic DNA of *Leersia hexandra* was extracted by using CTAB method. The results showed that different samples of *Leersia hexandra* exhibited DNA polymorphism when using the random primer S43, S51and S55 as the primers in the RAPD reaction. One specific DNA band about 1000 bp was found in the sample which treated with 10 mmol/L concentration EDTA when used the S43 primer to RAPD. The obvious differences between different EDTA-treatment levels suggest that EDTA has certain effects on enrichment to heavy metals of *Leersia hexandra,* it will be more favored to *Leersia hexandra* accumulation of chromium when EDTA concentration increased.

Keywords

Chromium Treatment, Genetic Differentiation, Randomly Amplified Polymorphic DNA (RAPD), *Leersia hexandra* Swartz

1. Introduction

Leersia hexandra Swartz, a perennial marshy plant, has been reported to be a Cr-accumulating plant with high tolerance to Cr. Under nutrient solution culture, it did not show any obvious symptoms of Cr toxicity when Cr concentrations in the leaves reached 5608 mg·kg^{-1} dry weight [1]

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Previous and continuous researches show that environmental factors have a significant impact on the plants [2]. Excessive Cr in soil has negative impact on plant growth. At the same time, it can also accumulate in plants by roots, and enter human and animal bodies and harm them through the food chain [3].

Random Amplified Polymorphic DNA (RAPD) is a kind of molecular marker based on PCR. It uses l0 bp random primers amplifying the different DNA fragments in genome to show the polymorphism [4] [5]. One RAPD amplification is actually a simple PCR reaction, and it suits for a large number of samples for rapid analysis. The required DNA template is very small amounts, generally an amplification of only 10 ng to 50 ng DNA [6]. RAPD was used to determine genetic variability of ten populations of alfalfa [7] and the relationships between RAPD markers and 22 quantitative traits of caraway (*Carum carvi* L.) were analyzed [8]. The RAPD-PCR method was used to describe the pattern of DNA band variation in the samples influenced by the environmental pollution, to describe the level of pollution in an area contaminated with smoke and waste from an iron-steel factory, and to reveal the level of potential [9].

At present, there are not molecular biology reports on heavy metal chromium enrichment of *L. hexandra*, and little molecular ecology study on plants to heavy metal pollutants under the long -term effect. Reports on using RAPD to analyze heavy metal pollution on plant population genetic diversity are increasing recently, Li *et al.* (2007) used RAPD to analyze genetic diversity and genetic differentiation of *Dicranopteris dichotoma* populations and lead-zinc mine tailings in population which grow in nature potential [10]. Wen *et al.* (2001) planted 4 *Datura* seeds in different regions and do analysis of this 4 *Datura* habitats potential [11]. Gu *et al.* (2008) used RAPD to analyze genetic diversity of *clethroides* populations which grow in lead zinc tailings in the storing time of 10 years and 20 years in natural and contrast soil potential [12]. *L. hexandra* is a kind of important heavy metal chromium enrichment plant, so it is important to study its molecular enrichment mechanism. Therefore, this study used *L. hexandra* treated with heavy metal pollution in the laboratory as experimental materials, analyzed the genetic diversity of *L. hexandra* under different EDTA treatment of heavy metal chromium pollution through RAPD, and tried to explore the differentiation and evolution of *L. hexandra* population under long-term persistence of toxic heavy metals pollution from molecular level.

2. Material and Methods

2.1. Materials

Plant material handling

L. hexandra collected from Guilin city, China, and artificial cultivated in sunlight greenhouse. 400 mg chrome metal Cr^{3+} ($CrCl_3$) to per kg were added to soil when *L. hexandra* planted. EDTA solution of 0 mmol/L, 2.5 mmol/L, 5 mmol/L and 10 mmol/L concentration were prepared to handle *L. hexandra* plant, 3 repeats for every kind of concentration gradient, joined the appropriate EDTA solutions to *L. hexandra* plant every other week, and added a total of 3 times.

Reagents and solutions

2 × CTAB extracting buffer (2% CTAB, 1.4 mol/L NaCl, and 20 mmol/L EDTA, and 100 mmol/L Tris-Cl, pH 8); phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1), β-mercaptoethanol; 3 mol/L NaAc, (pH 5.2) isopropyl alcohol; TE solution (10 mmol/L Tris-Cl, 1 mmol/LEDTA, pH 8); 75% alcohol; alcohol; 1 × TAE buffer (40 mmol/L Tris, and 20 mmol/L HAc, 1 mmol/L EDTA, pH 8.0), Taq DNA polymerase; agarose; Lambda DNA/EcoR I + Hind III Marker.

Instruments and equipments

General refrigerators; HVE-50 high pressure sterilization pot (Japan Hirayama), LEGEND MICRO17 high speed centrifuge (United States saimofei Fisher); trace moving liquid (Japan Nichipet EX); T1Thermocyler amplification apparatus (Germany BIOMETRA); DYY-12 Sanheng Multiple use Electrophoresis apparatus (Beijing Liuyi); DYCP-34A Electrophoresis tanks (Beijing Liuyi); MRS-1200048U scanner (Shanghai, Zhongjing technology); Furi FR-980 gel imager (Shanghai, Furi technology).

The primer sequences

The primer sequences used in this study are shown in **Table 1**.

2.2. Experimental Methods

Extraction and purification for L. hexandra genomic DNA

Table 1. RAPD random primer of *Leersia hexandra* Swartz

NO	(5′→3′)	NO	(5′→3′)	NO	(5′→3′)	NO	(5′→3′)
S41	ACCGCGAAGG	S51	AGCGCCATTG	S82	GGCACTGAGG	S94	GGATGAGACC
S42	GGACCCAACC	S52	CACCGTATCC	S83	GAGCCCTCCA	S95	ACTGGGACTC
S43	GTCGCCGTCA	S53	GGGGTGACGA	S84	AGCGTGTCTG	S96	AGCGTCCTCC
S44	TCTGGTGAGG	S54	CTTCCCCAAG	S85	CTGAGACGGA	S97	ACGACCGACA
S45	TGAGCGGACA	S55	CATCCGTGCT	S86	GTGCCTAACC	S98	GGCTCATGTG
S46	ACCTGAACGG	S56	AGGGCGTAAG	S87	GAACCTGCGG	S99	GTCAGGGCAA
S47	TTGGCACGGG	S57	TTTCCCACGG	S88	TCACGTCCAC	S60	ACCCGGTCAC
S48	GTGTGCCCCA	S58	GAGAGCCAAC	S89	CTGACGTCAC	S81	CTACGGAGGA
S49	CTCTGGAGAC	S59	CTGGGGACTT	S92	CAGCTCACGA	S93	CTCTCCGCCA

Applied an improved ctab method potential [13] [14], to extract total DNA from leaves of *L. hexandra* dried by silica gel, and used phenol/chloroform/isoamyl alcohol (25:24:1) to extract total DNA, anhydrous ethanol to purified total DNA, 0.8% agarose gel electrophoresis to detect DNA extraction and effects.

Primers screening

Used *L. hexandra* genomic DNA extracted as DNA templates for RAPD random primers screening. Primers are used in this research process all synthesized by Shanghai bio-engineering technology services company limited. Random primers with clear RAPD amplification and stability response were screened for final primers of *L. hexandra* RAPD molecular markers.

RAPD amplification reaction of L. hexandra

We used *L. hexandra* genomic DNA extracted as DNA templates for RAPD amplification reaction. Optimized reaction system: 25 μl total volume, 18.5 μl double distilled water, 2.5 μl 10 uffer liquid, 2 μl Mg^{2+} concentration for 20 mmol/L, 0.5 μl dNTP concentration for 200 μmol/L, 0.5 μl primer concentration for 0.4 μmol/L, 1 μl template DNA originated from DNA extraction diluted 4 times with TE buffer liquid, 2U/25 μl Taq enzyme. Circulation system after optimized: for 5 min 94˚C predegeneration, then for 40 cycles, followed by 1 min 94˚C predegeneration, 1 min 36˚C annealing, 1 min 72˚C stretch. Last, 7 min 72˚C complete the extension potential [15]. All RAPD reactions were in T1 Thermocyler amplification a paratus (Germany BIOMETRA). Results of PCR amplified products detected by 0.8% agarose gel electrophoresis, and used Lambda DNA/Hind III + EcoR I Markers as molecular standard, and electrophoresis in 120 V constant-voltage for 40 min then do EB dyeing for 10 min, electrophoresis buffer of 1 × TAE, observed by Furi FR-980 gel imaging device, and then taken pictures by the gel image analysis system.

3. Results and Discussion

3.1. *L. hexandra* DNA Extraction Results

Agarose gel electrophoresis results of *L. hexandra* leaves genomic DNA is shown in **Figure 1**. Test combined with common CTAB method for genomic DNA extraction, and extracted genomic DNA respectively for 12 samples, high quality DNA have great influence on stability and reliability experiment results. Seen from **Figure 1**, the primary belt of *L. hexandra* genomic DNA is at about 21,226 bp. After agarose gel electrophoresis for DNA extracted from *L. hexandra* leaves, its band isn't very bright and clear but with much tail, which showed that process of DNA extraction degradated in severe, proteins and RNA degradation was not very thorough. To illustrate, extraction of DNA purity in this experiment was not high, but in little amount of DNA in RAPD experiment, generally, expansion just need 10 ng to 50 ng DNA, purity requirements were not very high. The RAPD results showed that the extraction genomic DNA from *L. hexandra* can be applied to RAPD reaction.

3.2. *L. hexandra* RAPD Primers Screening Results

Used *L. hexandra* genomic DNA as a template, for screening of the 46 UBC random primers, results showed

that 29 UBC hadn't amplified products or obscured and couldn't tell, other 17 UBC random primers amplified a strip of clear and stable, that account for 36.9% of total primers. They varied approximately 200 bp or 3000 bp size, each primer amplification of fragments between changes in 1 - 7. Part *L. hexandra* leaves DNA RAPD primers screened results are as shown in **Figure 2**.

3.3. *L. hexandra* RAPD Amplification Results

Screened 46 random primers (see **Table 1**) preliminary, and used primers that can amplify stability and clear DNA bans as DNA amplification primers of *L. hexandra* polluted by chromium, and screened random primers S43, S51, S55 from tests results for RAPD reaction in this study. RAPD analysis was used for different samples of *L. hexandra* which under heavy metals chromium of 400 mg/kg concentration pollution and then added different concentration exogenous EDTA, sample RAPD results detected by agarose gel electrophoresis as shown in **Figure 3**, **Figure 4** and **Figure 5**.

3.4. RAPD Amplification Results of Primer S43

Figure 3 shows the amplification results of S43 RAPD random primers of *L. hexandra* samples. EDTA concentration of the first sample was 0 mmol/L (contrast). EDTA concentration of the second and third sample was 2.5

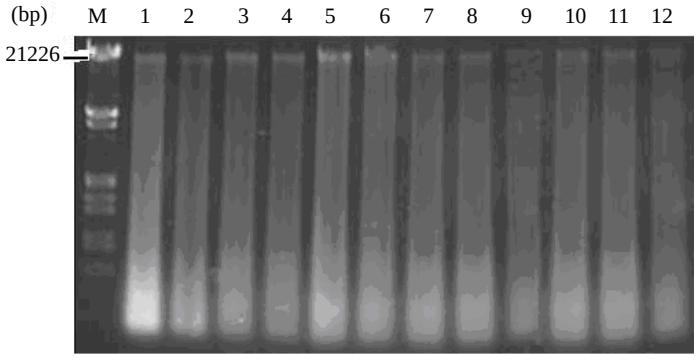

Figure 1. Leaves general DNA electrophoresis spectrums of *L. hexandra*, 1 - 3: EDTA 0 mmol/L; 4 - 6: EDTA 2.5 mmol/L; 7 - 9: EDTA 5.0 mmol/L; 10 - 12: EDTA 10 mmol/L. M is Lambda DNA/Hind III + EcoR I Markers.

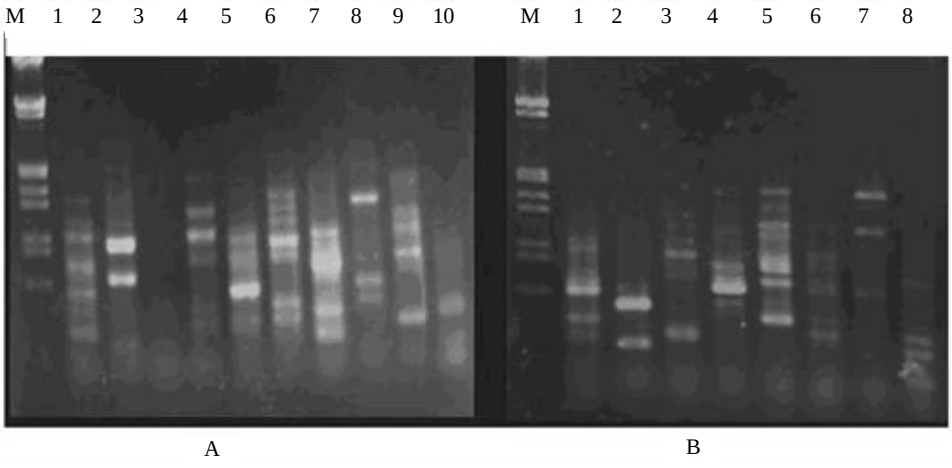

Figure 2. Filtered results of RAPD part primers in *L. hexandra* leaves DNA, A: Lane 1 - 10 Respectively Express Primer S51, S52, S57, S59, S60, S93, S94, S95, S96 and S97 amplification results; B: Lane 1 - 8 Respectively Express Primer S4, S9, S98, S99, S43, S44, S55 and S56 amplification results; M is Lambda DNA/Hind III + EcoR I Markers.

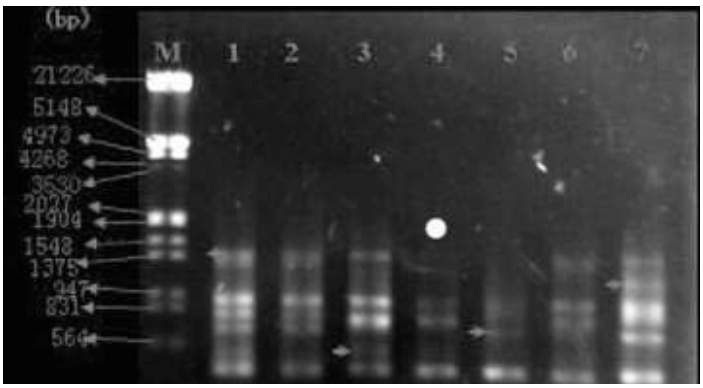

Figure 3. Amplification results in primer 43(GTCGCCGTCA) RAPD, 1: EDTA 0 mmol/L (Contrast); 2,3: EDTA 2.5 mmol/L; 4,5: EDTA 5.0 mmol/L; 6,7: EDTA 10 mmol/L; M is Lambda DNA/Hind III + EcoR I Markers.

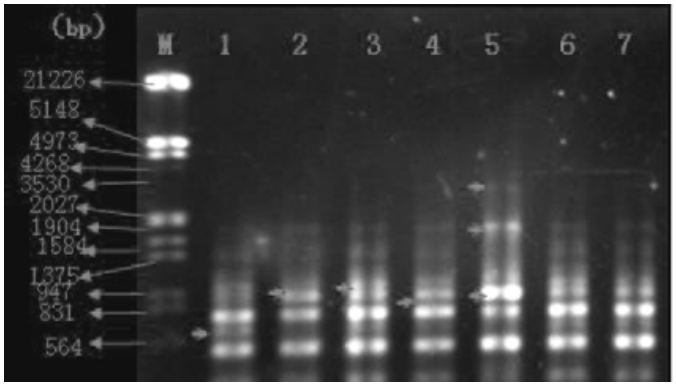

Figure 4. Amplification results in primer 51(GTCGCCGTCA) RAPD. The treatment of samples 1 - 7 is the same as **Figure 3**.

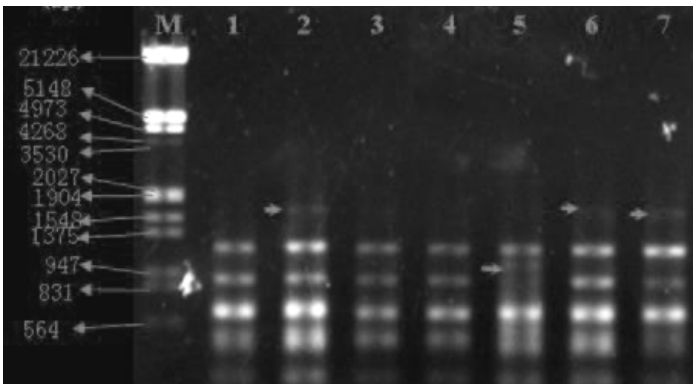

Figure 5. Amplification results in primer 55(GTCGCCGTCA)RAPD. The treatment of samples 1 - 7 is the same as **Figure 3**.

mmol/L and the third sample appeared DNA polymorphism at about 564 bp (as shown by the arrow in the 3rd sample). EDTA concentration of the fourth and fifth sample was 5 mmol/L, and the fifth sample appeared DNA polymorphism at about 700 bp (as shown by the arrow in the 5th sample). EDTA concentration of the sixth and seventh sample was 10 mmol/L, and the 7th appeared DNA polymorphism at about 1000 bp (as shown by the arrow in the 7th sample).

EDTA concentration of the first was 0 mmol/L (contrast). Comparison between the second and third and the

first samples, the third appeared DNA polymorphism at about 564 bp (as shown by the arrow in the 3rd sample). Comparison between the fourth and fifth sample and the first sample, both appeared strip absence at about 1375 bp (absence strip as shown by the arrow in the first sample), in addition, the fourth sample appeared strip absence at about 700 bp (absence strip as shown by the arrow in the first sample). Comparison between the sixth and seventh and the first sample, the sixth sample appeared strip absence at about 700 bp (as shown by the arrow in the first sample), and the seventh sample appeared DNA polymorphism at about 1000 bp (as shown by the arrow in the 7th sample).

*RAPD amplification results of primer S*51

Figure 4 shows the amplification results of S51 RAPD random primers of *L. hexandra* samples. EDTA concentration of the first was 0 mmol/L (contrast). EDTA concentration of the second and third sample was 2.5 mmol/L and compared with each other, the third sample appeared DNA polymorphism at about 1000 bp (as shown by the arrow in the second sample). EDTA concentration of the fourth and fifth sample was 5 mmol/L, and compared with each other, the fifth sample appeared DNA polymorphism at about 3530bp. EDTA concentration of the sixth and seventh sample was 10 mmol/L, and compare result shown that both were almost no difference.

EDTA concentration of the first was 0 mmol/L (contrast). Comparison between the second and third and the first sample, both appeared DNA polymorphism at about 947 bp (as shown by the arrow in the 2nd and 3rd sample). Comparison between the fourth and fifth sample and the first sample, both appeared DNA polymorphism at about 947 bp (as shown by the arrow in the 4th and 5th sample), in addition, the 5th sample appeared DNA polymorphism at about 1904 bp and 3530 bp (as shown by the arrow in the 5th sample), and both the samples appeared strip absence at about 700 bp (strip absence as shown by the arrow in the first sample). Compared to the sixth and seventh sample, the contrast showed no difference.

*RAPD amplification results of primer S*55

Figure 5 shows the amplification results of S55 RAPD random primers of *L. hexandra* samples. EDTA concentration of the first was 0 mmol/L (contrast). EDTA concentration of the second and third sample was 2.5 mmol/L and compared with each other, the 2nd sample appeared DNA polymorphism at about 1548 bp (as shown by the arrow in the 2nd sample). EDTA concentration of the fourth and fifth sample was 5 mmol/L, and the 5th sample appeared DNA polymorphism at about 947 bp (as shown by the arrow in the 5th sample). EDTA concentration of the sixth and seventh sample was 10 mmol/L, and compared with each other, result shown that both were almost no difference.

EDTA concentration of the first was 0 mmol/L (contrast). Comparison between the second, the third and the first sample, the second sample appeared DNA polymorphism at about 1548 bp (as shown by the arrow in the 2nd sample). Comparison between the fourth, the fifth sample and the first sample, the fifth sample appeared DNA polymorphism at about 947 bp (as shown by the arrow in the 5th sample). Comparison between the sixth and seventh and the first sample, both samples appeared DNA polymorphism at about 1548 bp (as shown by the arrow in the 6th and 7th sample).

Study on the evolution of pollution can help to understand how plants adapt to the environment and evolve, to understand biological adaptation mechanism under pollution. Knowledge on the genetic diversity can be used in future breeding programs potential [16] [17]. Students concluded that DNA polymorphism detected by RAPD analysis in conjunction with other biochemical parameters could be a powerful eco-toxicological tool in biomonitoring arsenic pollution potential [18].

Heavy metal pollution is an important form of soil pollution, the past researches focused on heavy metal toxic effect and mechanism of plants, and study on migration, accumulation, distribution and other aspects on heavy metals in the plant's tissues, organs and ecological systems potential [19]. The accumulating ability of *Leersia hexandra* Swartz may be due to they live in electroplating waste water pollution in streams for a long time, in the process of adapting to this environment produced a variation, some genetic characteristics have changed, been the emergence of new forms of Chromium patience and Enrichment capacity also increased. And there are few studies on molecular ecology about plant under long-term effects of heavy metals. Through RAPD analysis of genetic diversity of *L. hexandra* under the heavy metals pollution, we can understand fundamental changes that is DNA level changes in the cells under tolerance in heavy metals pollution, so as to reform *L. hexandra* gene by using modern molecular techniques, to make greatly ability on enrichment and accumulation to heavy metals, so as to develop ability of restoring soil .

This study first screen primers of *L. hexandra* RAPD polymorphicing, and screen random primers S43

(GTCGCCGTCA), S51 (AGCGCCATTG), S55 (CATCCGTGCT) from the 46 random primers to be random primers of *L. hexandra* RAPD molecular markers preliminary. And then used S43, S51, S55 as primers on the polymorphism of different samples for research. The combined use of both RAPD and ISSR markers for the genetic analysis of the cultivar varieties have been performed earlier potential [20] [21] (Gupta, *et al.*, 2008, Lu, *et al.*, 2009). Our study utilizes a total of 55 primers to disclose the genetic similarity between the selected varieties.

Compared EDTA concentration of 5 mM/kg, 10 mM/kg, 2.5 mM/kg with the control, the former expansion is larger than that of the latter. Different degrees of difference in comparison between different EDTA concentrations and control of treated RAPD results of *L. hexandra*, this shows that EDTA has certain effects during enrichment of *L. hexandra* to heavy metals. But perhaps, in a certain scope, it will be more favourable to *L. hexandra* accumulation of chromium metal when EDTA concentration increased. *L. hexandra* in the accumulation of chromium metal is also the process of chromium metal tolerance, and cause changes in gene eventually, to make it more resistant chromium metal against.

4. Conclusion

Different *L. hexandra* samples exhibited DNA polymorphism when using the random primer S43, S51 and S55 as the primers in the RAPD reaction. One specific DNA band about 1000 bp was found in the sample which was treated with 10 mmol/L concentration EDTA when used the S43 primer to RAPD. The obvious differences between different EDTA-treatment levels suggest that EDTA has not much effects on enrichment to heavy metals of *L. hexandra*, so we should do more work to research the *L. hexandra* accumulation of chromium mechanism when EDTA concentration increased.

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