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Season, Environment Stress and Refrigerated Storage Affect Genomic DNA Isolation of Tung Tree

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ABSTRACT

Many metabolites in leaf tissue disturbed plant genomic DNA isolation and they always varied when leaf was harvested from different environments. Objective of this study was to investigate whether season, environment stress and refrigerated storage affect genomic DNA isolation of tung tree leaves. Five types of young leaves and two DNA isolation protocols, the recycling CTAB protocol I and II, were adopted to carry out the experiment. Our results showed that both leaf type and protocol affected DNA isolation of tung tree. Using the recycling CTAB protocol II, though little DNA were obtained from three types of young leaves, though the other two have satisfying results. Whereas the recycling CTAB protocol I could produce high yield genomic DNA from all the five types of young leaves. All the detectable DNA samples in agarose gel electrophoresis were good templates for PCR reaction. Season, environment stress and refrigerated storage had a big effect on genomic DNA isolation of tung tree. The recycling CTAB protocol I was proved to be an effective and universal protocol for DNA isolation of tung tree. Five types of young leaves could all act as the tissue for isolation of genomic DNA, but the summer healthy young leaves without long-time refrigerated storage are the best. The optimal leaf tissue will benefit DNA isolation of plant species.

Keywords: Tung Tree; *Vernicia fordii*; DNA Isolation; Season; Environment Stress; Storage

1. Introduction

Plant often produces many metabolites, such as polysaccharides, polyphenols, protein and tannin. These compounds always interfere with DNA isolation and downstream DNA-based experiments [1-6]. To date, lots of DNA isolation protocols have been published and aimed at eliminating the negative effects of biochemical components on DNA isolation [7-17]. However, DNA isolation is still a challenge for many plant species.

Chemotypic heterogeneity among different species did not allowed the direct application of an extraction protocol for a species to other species [17-19]. Metabolites in plant leaves were not stable, changing with many factors, such as leaf age [19-21], season [22-24], light intensity [25] and environmental stress [26,27]. Leaf age was reported to affect the properties of extracted DNA, which was inferred to be related with the accumulation of defense compounds during the leaf development [19]. Environmental factors such as light intensity, temperature and seasonal variance were reported to affect the production of polysaccharide [25], phenolic compounds [28,29] and other metabolites in leaves. However, up to now, little reports were about the effect of theses environmental factors on DNA isolation of plant species.

Tung tree (*Vernicia fordii*), belonging to Euphorbiaceae, is an oil-producing plant with multiple uses especially its potential in biodiesel production [30-32]. DNA extraction is essential for molecular genetic analyses and marker-assisted improvement of this oil-producing species. In present study, we found that season, environment stress and refrigerated storage all had big effect on genomic DNA isolation of tung tree. Besides, the recycling CTAB protocol I was proved to be a universal method for DNA isolation of tung tree.

2. Materials and Protocols

2.1. Materials

Young leaves were harvested from healthy tung tree at different seasons, in the autumn (early November in 2009), spring (early April in 2010) and summer (July in 2010), respectively (hereafter called autumn healthy leaves, spring healthy leaves and summer healthy leaves for short). Besides, leaves were also harvested from the tung tree that suffered from both transplant and waterdrown stress in the summer of 2010 (hereafter called

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summer stressed leaves for short). These four types of leaf tissues were ground into fine powder in liquid nitrogen, and then 0.3 g of powders for each leaf tissue type were immediately used to isolate genomic DNA. Besides, some other summer healthy leaf tissues were stored in freezer for three months and then was used to isolate genomic DNA. Tissue powder was a mixture of several genotypes. Unless state, the leaf referred to the young leaf without the refrigerated storage in the text below.

2.2. Protocols

Two protocols (Lingling Zhang *et al.*, unpublished) were adopted to extract DNA from the five types of tung tree young leaves. The main procedures of recycling CTAB protocol I was followed. 1) Add 1.5 mL of wash buffer to 0.3 g of ground leaf sample. Mix well and place at the low temperature with occasional swirling for at least 15 min. Then centrifuge at 12,000 rpm for 15 min. Discard the supernatant. The tissue pretreatment was carried out for three times; 2) Add 0.5 mL of CTAB extraction buffer and re-suspend tissue pellet. Incubate at 65˚C for 40 min. Centrifuge at 12,000 rpm for 15 min. Transfer the supernatant to a new tube; 3) Add 200 μL Chloroform-isoamylalcohol (24:1). Mix well and let stand for several minutes. Centrifuge at 12,000 rpm for 15 min and transfer the supernatant to a new 1.5 mL tube carefully; 4) Precipitate the DNA by adding two volumes of cold absolute ethanol and incubate for 30 min at −20˚C. Transfer the DNA precipitation with a tip and wash it with 70% ethanol for 30 min, better for overnight. Then pour off the ethanol; 5) Dry the DNA and dissolve it in 100 μL $T_{0.1}E$. Add 1 μL of 10 μg/mL ribonuclease. Incubate the DNA solution at 37˚C for 30 min, and then incubate at 65˚C for 15 min. This DNA sample was termed as the first DNA (1st DNA); 6) Similarly, treat the residual pellets in the above step 2) for other three cycles of the step 2) to the step 5). Correspondingly, the resulted DNA samples were termed as the second DNA (2nd DNA), third DNA (3rd DNA) and fourth DNA (4th DNA), respectively. The recycling CTAB protocol II shared all the procedure of the recycling CTAB protocol I except that the tissue pretreatment was only carry out for one time.

2.3. DNA Isolation and Assessment

The quality and yield of extracted DNA were determined by agarose gel electrophoresis. Concentration of DNA samples was assessed as the following. 2 μL of DNA solution for each sample was added into 48 μL of sterilized water (mix well). Then 5 μL of the diluted DNA solution plus 5 μL of 2.5X loading buffer was loaded into 0.8% agrose gel and run in 0.5X TBE buffer (pH 8.0) at room temperature. The gel was visualized with ethidum bormide staining. DNA quantity for each lane/sample was estimated by comparing band intensity with the concentration-known λ DNA standards (Promega, Madison, WI, USA). DNA concentration was calculated using formula, concentration = [quantity (ng) of 5 μL diluted DNA/5 μL × dilution factor (50/2 = 25)].

2.4. Polymerase Chain Reaction Amplification

Coding region cloning of fad2 gene was performed using PCR approach. On the basis of GenBank sequence AF525534, sequences of primers for PCR amplification are 5'-GATGGGTGCTGGTGGCAGAATGTCA-3' and 5'-CCAGAACTTCCAAGCCCTTCACTTTTG -3' [33], which amplify an approximately 1.2 kb fragment encompassing the entire coding region of fad2 gene. The PCR were performed in a 25 μL reaction volume (60 ng template DNA, 0.4 μM specific primers, 1 U Taq DNA polymerase, 0.2 mM dNTP mixture, 1 × PCR buffer, 0.15 mM Mg^{2+}) using BIORAD-My Cycle Thermocyclers (Bio-Rad Laboratories, Inc., California, USA) with the following program: pre-denaturation at 94˚C for 4 min; 30 cycles of denaturation at 94˚C for 45 s, annealing at 65˚C for 45 s, extension at 72˚C for 60 s; and a final extension step at 72˚C for 10 min, then hold on for 15 min at 4˚C. Then PCR products were separated in 1% agarose gels and the gel was visualized with ethidium bromide staining.

3. Results

3.1. Comparisons of DNA Samples Isolated From Five Types of Leaves Using the Protocol I

Using agarose gel electrophoresis and λDNA of known concentration as standards, concentration of DNA samples produced by the protocol I were determined. As shown in table 1, yield of the 1st DNA varied for the five types of young leaves, about 200 ng/μL both for spring healthy leaf and summer healthy leaf, 100 ng/μL for summer stressed leaf, but little DNA for summer healthy leaf with three-month refrigerated storage and autumn healthy leaf (**Figure 1**). As shown in **Figure 1**, the 2nd, 3rd and 4th DNA for each type of leaf were all obviously detectable in agrose gel electrophoresis, the concentration of these DNA samples ranging from 300 ng/μL to 500 ng/μL (**Table 1**). These data indicated that the protocol I could be successfully applied to different types of young leaves without significant differences.

3.2. Comparisons of DNA Samples Isolated from Five Types of Leaves Using the Protocol II

However using the recycling CTAB method II, DNA samples extracted from the five types of leaves greatly

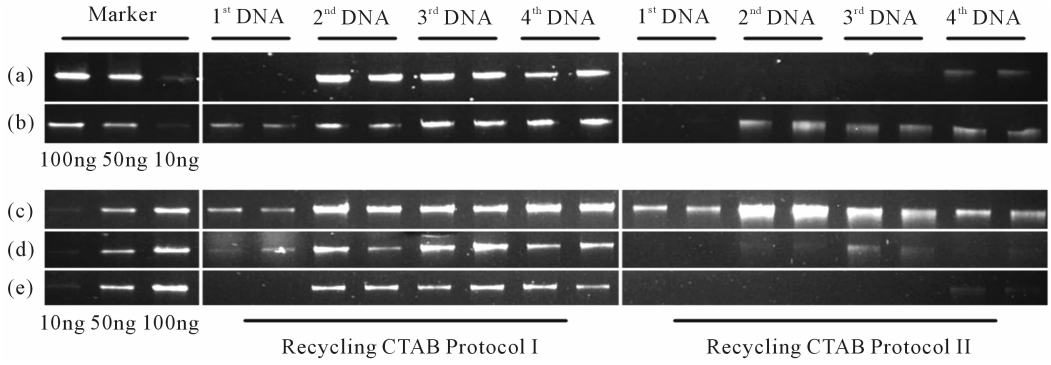

Figure 1. Agarose gel electrophoresis of genomic DNA isolated from five types of young leaves using two protocols. (a) Autumn healthy leaf; (b) Spring healthy leaf; (c) Summer healthy leaf; (d) Summer stressed leaf; (e) Summer healthy leaf with three-month refrigerated storage.

Table 1. Concentration of DNA samples for the five types of young leaves using the two protocols.

Protocol	Leaf Type	C1 (ng)				C2 (ng/μL)			
		1st	2nd	3rd	4th	1st	2nd	3rd	4th
Recycling CTAB Protocol I	a	\	100	100	100	\	500	500	500
	b	40	100	100	100	200	500	500	500
	c	40	100	100	100	200	500	500	500
	d	20	60	100	80	100	300	500	400
	e	\	60	60	60	\	300	300	300
Recycling CTAB Protocol II	a	\	\	\	20	\	\	\	100
	b	\	60	60	60	\	300	300	300
	c	60	150	80	60	300	750	400	300
	d	\	10	20	\	\	50	100	\
	e	\	\	\	10	\	\	\	50

C1, DNA quantity (ng) in 5 μL of diluted solution loaded into agaroe gel estimated by comparison with the standard λDNA of known content, 50 ng; C2, DNA concentration (ng/μL) estimated using agarose gel electrophoresis and calculated as C2/5 μL (volume of the loaded DNA dissolution) × 25 (dilution factor). Four DNA samples were isolated from a single sample. 1st, 2nd, 3rd and 4th represent the first DNA samples, the secondary DNA samples, the third DNA samples and the four DNA samples, respectively. Lowercase letter a, b, c, d and e represent the autumn healthy leaf, spring healthy leaf, summer healthy leaf, summer stressed leaf and summer healthy leaf with three-month refrigerated storage, respectively. The slashes in the tables represent the DNA samples which could not be detectable in agarose gel electrophoresis.

varied in yield. First, for autumn healthy leaf, the first three of the four DNA samples were not detectable and concentration of the 4th DNA was about 100 ng/μL (**Figure 1** and **Table 1**). Secondly, for spring healthy leaf, the 1st DNA was not detectable but 2nd, 3rd and 4th DNA-samples were all detectable, each about 300 ng/μL. Thirdly, all the four DNA samples for summer healthy leaf were detectable, and concentration of 1st to 4th DNA was about 300 ng/μL, 750 ng/μL, 400 ng/μL, and 300 ng/μL, respectively (**Table 1**). Fourthly, for the summer stressed leaf, the 1st and 4th DNA samples was not detected in agarose gel while the band of the 2nd and 3rd DNA samples were very weak, apparently different from the four bright bands of summer healthy leaf above. Last, for the summer leaf with three months refrigerated storage, only the 4th DNA of the four samples was detectable but the concentration was relatively low, about 50 ng/μL. These data indicated that the protocol II could not be applied to the different types of young leaf and also demonstrate the biochemical components for the five types of young leaf tissues were greatly varied.

3.3. Analysis of DNA Quality by PCR Amplifycation of Fad2 Gene

For the four DNA samples of a tissue sample, the DNA-detectable samples were mixed into one DNA sample with an exception for the four DNA samples of the summer healthy leaves. Thus, using the two protocols,

eight DNA-incorporated samples were obtained from the four types of leaves and eight DNA samples were got from the summer healthy leaf. These sixteen DNA samples were all used as the templates to amplify fad2 gene. As shown in **Figure 2**, the target bands for all the sixteen DNA samples were visible and distinct, and were the expected size of fad2 gene fragments (1.2 kbp) targeted by the primers used, indicating that all the sixteen DNA samples had enough purity for fad2 gene cloning.

4. Discussion

4.1. Factors Affecting Genomic DNA Isolation of Tung Tree

Leaves were the main source of DNA isolation. Many metabolites in leaves interfere with isolation of clean DNA. These metabolites in leaves were in mobile droved by the exogenous and endogenous signals. Defense compounds like polyphenols and tannin were reportedly largely produced and accumulated when plants were in the stress [28,29]. Leave development also had a big effect on the composition of biochemical components in leaves [19-22,34]. The matured tree leaves were reported to be difficult in DNA isolation for its thick cell wall, and high content secondary metabolites [18]. Leaf age of Fabaceae (*Dimorphandra mollis*) was reported to affect DNA isolation, in which DNA was successfully isolated from the young but failed from the old leaves, which was inferred to be attributed to the differently cumulative amount of chemical defenses such as tannins and phenols during leaf development [19]. In this study, five types of young leaves of tung tree were harvested in different seasons or environments. Using the recycling CTAB method II, the greatly varied DNA yield indicated the season for leaf harvest, environment stress and refrigerated storage all had big effect on DNA isolation. In conclusion, it will benefit DNA isolation if taking the environments, such as seasons into account at the step of leaf harvesting.

4.2. An Effective and Universal Method for DNA Isolation of Tung Tree

Chemotypic heterogeneity in different species didn't allow the direct application of an extraction protocol for a specific species to other species [19,35]. In present study, chemotypic heterogeneity did not affect the application of protocol I among the five types of young leaf, but seriously disturb the application of the protocol II. It could conclude that the protocol I is an effective and universal method for DNA isolation of tung tree. The only difference between the two protocols is focused on the time of tissue pretreatment, which was carried out for three times for protocol I but one once for protocol II. Hence, it highlighted the importance of efficient elimi-

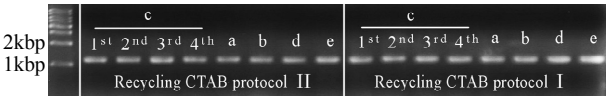

Figure 2. Agarose gel electrophoresis of fad2 gene fragment. Lowercase letter a, b, c, d and e represent the autumn healthy leaf, spring healthy leaf, summer healthy leaf, summer stressed leaf and summer healthy leaf with three-month refrigerated storage, respectively.

nating the secondary compounds on DNA isolation. As discussed above, we choose the optimal leaves for DNA isolation to reduce difficulties in DNA isolation. However, the leaves available were always limited by various reasons and we have to isolate the DNA from the leaves with various backgrounds. Therefore, a universal DNA isolation protocol is expected for the species of interested.

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