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Instant genomic DNA extraction method from safflower leaf for PCR Analysis

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Abstract
In spite of the possibility of analysing large samples and the rapidity of Polymerase Chain Reaction (PCR) technology, the usual protocols for DNA plant extraction remain time-consuming, slow and even hazardous. To lessen labor, time or cost of DNA extraction, a simple and instant method for genomic DNA extraction from leaf tissue of Safflower (*Carthamus tinctorius*) leaf is established. Small quantity of tissue materials (typically 3-5 mg) was ground in a centrifuge tube using plastic pestles in extraction solution. Extracted DNA was suitable for PCR analysis, without centrifugation. The feasibility of this method was confirmed by testing molecular markers and transgene detection. This method requires less than 1 mg of plant tissue stored frozen or used fresh and is useful for molecular marker, transgene detection, and other experiments.

Keywords: safflower (*Carthamus tinctorius*), DNA extraction, PCR analysis, RAPD, ISSR

Introduction
Safflower (*Carthamus tinctorius* L.) is an important rabi oilseed crop and produces high quality oil rich in polyunsaturated fatty acids, which helps in reducing the cholesterol level in the blood (Popov and Kang, 2011) [10]. India is the largest producer of safflower (2 lakh tonnes) in the world with highest acreage of 4.3 lakh hectares. Unfortunately, the area and production of safflower in India has experienced a downward trend for the last 4-5 years. The full potential of the crop is far from being exploited and the yield levels of the country are the lowest in the world due to several reasons such as occasional adverse climatic conditions, poor agronomic methods of cultivation, biotic and abiotic stresses. Therefore, it is needed to improve the yield potential of safflower varieties/hybrids to increase safflower production of the country. Substantial effort has been directed toward molecular analysis of this crop for various purposes, such as genetic enhancement for qualitative and quantitative traits.
Marker assisted selection is of increasing importance in plant breeding programs, where large numbers of genotypes are screened for numerous traits in a short time period. While the availability of DNA markers linked to traits of interest has increased, available DNA extraction procedures have limited the number of samples able to be processed (Rehman *et al.*, 2007) [11]. The genomic DNA extraction methods for PCR-quality DNA from Safflower are not time efficient, since they require several steps, like the tissues be ground in liquid nitrogen, followed by precipitation of the DNA pellet in ethanol, washing and drying the pellet, etc. However, several DNA extraction procedures have been published in several plants and although some of these are undoubtedly rapid, many require the use of expensive, often environmentally hazardous chemicals, and specialized laboratory equipment (Rehman *et al.*, 2007; Kang and Yang, 2004; Amani *et al.*, 2011) [11, 8, 2].
In recent years commercial manufacturers have developed kits that allow rapid and efficient isolation of high quality DNA from a wide variety of plant species. However, the disadvantage of any commercial kit is the high per-sample cost. The need for a rapid and simple procedure is urgent, especially when hundreds of samples need to be analyzed. Here, we describe an instant, cheaper and efficient genomic DNA extraction method for PCR amplification. In this method, no liquid nitrogen, no centrifugation, no DNA precipitation and washing steps were used.

Materials and Methods
Plant materials
Leaf samples (young immature to mature leaf) of safflower were used DNA extraction.

DNA extraction Protocol

Small quantity of tissue materials (typically 3-5 mg) was ground in a centrifuge tube using plastic pestles in warm extraction solution (Tris-Cl- 10 mM, pH 8.0; EDTA- 1 mM, pH 8.0; SDS- 0.1 %). The solution was mixed by vigorous vortexing followed by incubation of tubes at 50-60^{0}C for 15 min. Lysate were diluted (1:10) with nucleases free water. Approximately 2µl of diluted lysate were used in 20 µl for PCR analysis. The PCR was done for different molecular marker analysis [Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISR)] and transgene detection.

Molecular marker analysis

RAPD amplification was performed in 20 µl volume containing 10 µl of Premix Taq® Version 2.0 (Xcelris Lab Ltd. Ahmedabad, Gujarat), 0.2 µM primer and 2 µl of diluted lysate DNA in a thermal cycler. The reagents were mixed thoroughly and then placed on a Thermalcycler (Agilent Technologies) for cyclic amplification and the conditions for amplification was programmed as follows: The thermal profile set comprised a denaturation step of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 30 s at 35°C, an extension at 72°C for 2 min and a final extension at 72°C for 10 min. Amplification products were then subjected to electrophoresis in 1.2% agarose gel using 1 X TBE and detected by ethidium bromide staining, viewed under UV light and photographed with Geldocumentation system.

ISSR analysis was carried out in 20µl volume containing 10µl of Premix Taq® Version 2.0 (Xcelris Lab Ltd. Ahmedabad, Gujarat), 0.2µM forward primer, 0.2µM reverse primer and 2 µl of diluted lysate DNA in a thermal cycler (Agilent Technologies). The amplification reaction involved an initial 94°C for 5 min for denaturation followed by 35 cycles of 1 min at 94°C, 30 sec at 42°C, and 1 min at 72°C and final extension at 72°C for 3 min. Agarose gel (1.2%) was prepared to separate the amplified product gel documentation system was used for visualization of amplified DNA fragments. Each experiment was repeated three times with each primer to test the reproducibility of ISSR primer.

Transgene identification

For transgene identification analysis, leaf of transgenic safflower transformed with vector having double CaMV 35S promoter (unpublished data) were used for DNA analysis. PCR amplification of the transgene from WT and transgenic lines using CaMV35S specific primer pair (CaMV35S F: CTCGGATTCCATTGCCCAGCTAT & CaMV35S R: TTGCGAAGGATAGTGGGATTGTGC) was performed in a reaction volume of 20 µl containing the 2 µl of diluted lysate DNA, 10 µl of Premix Taq® Version 2.0 (Xcelris Lab Ltd. Ahmedabad, Gujarat) and 0.5 µM of each primer with an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 53°C), and extension (40 s at 72°C). After the last cycle, a final extension was carried out for 5 min at 72°C. Amplification products were separated on 1% agarose gel and gels were visualized under UV using gel documentation system.

Results

The objective of this investigation was to develop a high throughput DNA isolation method suitable for the molecular analysis in safflower. While determining the final protocol for DNA isolation, different combinations of Tris-Cl; EDTA and SDS were tried without used of phenol:chloroform:isoamyl treatment. The optimized protocol works very well for different marker as well as transgene detection in safflower.

Molecular marker analysis

Different types of molecular markers like RAPD and ISSR were checked for the amplification using DNA extracted from our optimized protocol (Figure 1). The reproducibility o was checked by repeating the extraction of DNA and PCR several times. List of primers used in different marker analysis were mentioned in table 1.

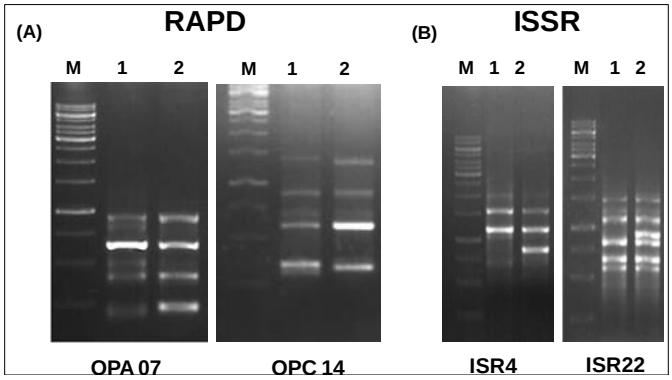

Fig 1: Molecular marker analysis using DNA extracted from safflower plants. (A) RAPD and (B) ISSR analysis. M: molecular weight marker; 1 & 2: two different safflower genotypes

Table 1: Sequences of primer used in RAPD and ISSR analysis.

S. No.	Name of Primer	Sequence
1.	OPA 07	GAAACGGGTG
2.	OPC14	TGCGTGCTTG
3.	ISR4	ACACACACACACACACAG
4.	ISR22	ACACACACACACACACAA

Transgene identification

The transgenic safflower plants (unpublished data) were characterized for the presence of CaMV35S promoter by PCR. The DNA from transformed and wild type (WT) were extracted using our optimized protocol. An amplification product of 400 bp was observed in transformants whereas no amplification was observed in WT plants (Figure 2). These results showed that our one step DNA isolation protocol is suitable for transgenic plant characterization.

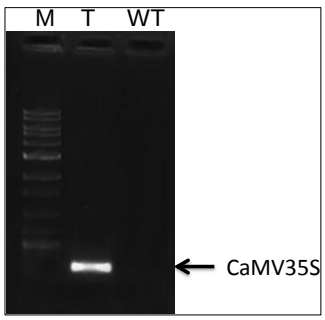

Fig 2: Transgene detection by PCR amplification using CaMV35S F & R primer. M: molecular weight marker; T: transgenic safflower; WT: wild type.

Discussion

A plethora of literatures are available regarding the DNA extraction procedure using plant tissues

(Huang and Sun, 2000; Doyle and Doyle, 1987; Kasajima *et al*., 2017) [6, 4, 9]. The Cetyl Trimethyl Ammonium Bromide

(CTAB) method and its modifications (Huang and Sun, 2000; Doyle and Doyle, 1987) [6, 4] were extensively used in different laboratories, but these methods are time consuming (Cheng *et al*., 2003) [3]. Other conventional DNA extraction protocols, which can remove some contaminants (Jobes *et al*., 1995) [7], require large amounts of plant tissue to be grounded. On the other hand, these methods require long periods for plant growth and are not efficient for screening and analyzing transgenic plants. Other methods use liquid nitrogen and other carcinogenic chemicals (Sharma *et al*., 2002) [12], which are not considered to be safe. There are also a number of protocols which require small quantities of tissues, but these methods have limitations, such as the use of specialized apparatus (e.g. the matrix mill) (Hill-Ambroz *et al*., 2002) [5]. Today, there are numerous DNA isolation kits are available, but the main problem with these commercially available kits, is their high cost per sample (Kang and Yang, 2004; Ahmed *et al*., 2009) [8, 1]. There are several reports of rapid method of rapid DNA extraction. But most of these methods requires treatment of either phenol: chloroform: isoamyl alcohol (Kang and Yang, 2004) [8] or chloroform: isoamyl alcohol (Amani *et al*., 2011) [2]. Some earlier reported methods requires alkali treatment followed by DNA precipitation by cold ethanol (Rehman *et al*., 2007) [11]. This DNA extraction procedure promises simplicity, speed, and efficiency, both in terms of time and the amount of plant sample required. In addition, this method does not require expensive facilities for plant genomic DNA extraction.

This method has the following advantages

1. The quality of the extracted DNA is high enough for PCR.
2. The procedure is simple and rapid, because it does not require any centrifugation steps that pellet the DNA
3. The cost is as low as that of conventional plasmid extraction kits
4. No dangerous organic solvents such as phenol or chloroform are used

The efficiency and the speed of this method together with the use of inexpensive facilities and the absence of toxic chemicals make the present method an attractive alternative for the extraction of genomic DNA. These results show that the DNA produced by this simple, low cost, fast and safe protocol can be used in PCR-based techniques and in laboratories lacking state-of-the-art equipments and technology. In summary, this method will provide a convenient, simple, rapid, low-cost and safe DNA extraction method for molecular biological experiments.

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