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Full Length Research Paper

Rapid genomic DNA isolation from corm of *Crocus* species for genetic diversity analysis

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The successful extraction of useful DNA from the plants is associated with all extraction steps for molecular techniques used in the next steps such as PCR amplification, digestion and DNA sequencing. Unlike in nonplant DNA extraction protocols, methods need to be adjusted to each species and even to each tissue because of the wide range of primary and secondary metabolites in the plants. Current methods produce degraded and denatured DNA or give extremely poor yields. To overcome these difficulties, we modified available DNA extraction methods. We have obtained higher quality of DNA from corm of *Crocus* species. This extraction technique provides an extraction procedure that can be completed in less than 50 min. This method yielded high-quality and high quantities of DNA. This technique has a potential to be an effective protocol for DNA extraction using corm for *Crocus* species, and perhaps for other plants, when sufficient young leaf tissue is not available. This could be adopted as a standard method for plants propagated with corm for isolation of DNA for marker analyses.

Key words: DNA extraction, saffron, molecular marker, corm.

INTRODUCTION

Herbal and aromatic plants are attracting more attention among contemporary plant researchers and have been increasing in importance to society continuously for the past 100 years. Saffron is the most expensive spice in the world. It is made from the dried stigmas of the saffron flower (*Crocus sativus* Linn.), a triploid sterile plant that is vegetatively propagated by means of corms. It is mostly used as spice and food colorant and, less extensively, as a textile dye or perfume. However, due to its analgesic and sedative properties folk herbal medicines have used saffron for the treatment of numerous illnesses for centuries (FernЈndez, 2004). Genetic analysis of plants relies on high yields of pure DNA samples. Isolation of purified DNA, from medicinal plants is challenging because of secondary metabolites and other compounds which would interfere with the DNA isolation procedures (Chua et al., 2008; Reddy et al., 2007). A large number of secondary metabolites such as tannins, alkaloids, phenolics and terpenes are responsible for the valuable pharmacokinetic properties of medicinal plants interfere with the isolation process, tend to copurify with DNA and interact irreversibly with proteins and nucleic acids (Katterman and Shattuck, 1983). However, the same substances that make these plants worthy of such intensive study also may hinder molecular approaches. Therefore, identification of genomic structure and application of molecular technology would increase and facilitate production of these substances and help to save natural resources. Extraction of high purity and quantity DNA of the plants is a crucial step for further molecular analyses based on molecular technology (Tanaka and Ikeda, 2002). A number of methods are available and are being developed for the extraction of nucleic acids from leaves (Dellaporta et al., 1983; Saghai-Maroof et al., 1984; Oard and Dronavalli, 1992; Wang et al., 1993; Wang, 2010; Richards et al., 1994; Davis et al., 1995; Porebski et al., 1997; Ikeda et al., 2001). Usually, DNA is extracted from fresh leaves which may not be available in sufficient quantity throughout the growing season or continually. Obtaining of young and fresh leaves

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especially from wild type plants limited to 2 to 3 weeks in spring season, while corms are available in sufficient quantity all year round (Fernandez, 2004). The aim of this study was to establish a new protocol for DNA isolation from corm of plants that can be used for diverse medicinal plants to get high quality DNA that is suitable for generation of molecular works in all seasons. The method presented here was developed for *C. sativus*, but may also be useful for any plant that propagated with corm. The protocol permitted isolation of DNA from diverse plant species in fairly good yields, and the isolated DNA proved amenable to PCR amplification and restriction digestion.

MATERIALS AND METHODS

Plant materials

Corms and leaves of six accessions of *C. sativus* were used for DNA extraction. The corms were dried using a freeze dryer and the powder was stored at -20°C and the leaves freeze in liquid nitrogen, and was store at -70°C until DNA isolation.

DNA extraction

Only the successful procedures of DNA isolation of corm were carried out and purification is reported here.

Solutions and solvents

(1) Extraction buffer (25 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, SDS 0.5%, RNase 10 mg/ml, 0.2% β- mercaptoethanol).
(2) Chloroform: Isoamyl alcohol (24:1).
(3) Phenol-Chloroform- Isoamyl alcohol (25:24:1).
(4) Isopropanol.
(5) Ethanol, 70 and 96%.

DNA isolation protocol

(1) 100 mg corm tissue was grounded to a fine powder after freeze drying.
(2) 1000 µl pre-warmed extraction buffer was added to the sample and grounded once more in the buffer.
(3) The solution was incubated in 58°C for 15 min.
(4) Samples were centrifuged at 12,000 rpm for 12 min, and the supernatant was transferred to a fresh tube.
(5) Equal volume of chloroform-isoamyl alcohol (24:1) was added to the supernatant and mixed gently, inversion for 5 to 10 min.
(6) After centrifugation for 10 min at 14,000 rpm, supernatant was transferred to a fresh tube and equal volume of phenol-chloroform-isoamyl alcohol was added.
(7) After centrifugation for 5 to 10 min at 13,000 rpm, supernatant was transferred to a fresh tube and equal volume of isopropanol or 2 times volume absolute alcohol was added.
(8) The samples were then centrifuged for 6 min at 12,000 rpm and 76% ethanol was added to the pellet.
(9) The pellet was once more washed with 70% ethanol optionally and air-dried until all ethanol was removed.
(10) The obtained nucleic acid pellet was dissolved in an appropriate amount of TE buffer (50 µl).
(11) The nucleic acid dissolved in TE buffer was treated with ribo nuclease (RNase, 10 mg/ml) and stored at –20°C until use.

Isolated DNA from corm and leaf tissues of *Crocus* accessions using this procedure compare to three DNA extraction methods; Method #1 (Doyle and Doyle, 1990), method #2 (Dellaporta et al., 1983) and method #3 (Ikeda et al., 2001). The quality of extracted DNA by the all methods was evaluated based on the electrophoresis on agarose gel and RAPD-PCR products amplified from these DNA templates.

DNA quantification

Genomic DNA was quantified by measurement of absorbance ratio at 230 and 260 nm with UV- Spectrophotometer (Shimadzu, Kyoto and Japan). DNA was quantified by agarose gel electrophoresis. Two microliters of extracted DNA (200 to 500 ng) was electrophoresed on 0.8% agarose gels, stained with ethidium bromide, and visualized by UV fluorescence. The quantity and quality of DNA were measured by comparing band intensity with that of standard amounts of DNA.

Polymerase chain reaction amplification

Amplification of RAPD fragments was performed according to Williams et al. (1990) using arbitrary primers (Operon Technologies Inc, USA; SIGMA-D, USA). Amplifications were performed in a 25 µl reaction volume containing 5 µl DNA (50 ng), 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 unit of Taq polymerase, 2.5 µl 10× Taq buffer, and 10 pmol primer. Amplification was performed in a programmed MJ Research PTC-100 thermal cycler (MJ Research Inc., USA) within initial denaturation at 94°C for 4 min, 45 cycles of denaturation at 94 °C for 30 s, primer annealing at 36°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. Amplified products were electrophoresed in 1.2% agarose in 1× TBE buffer (Beiki et al., 2010; Grilli Caiola et al., 2004). The gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, Hercules, and California).

RESULTS AND DISCUSSION

Plant tissues are among the most difficult materials for high quality DNA extractions. The key is to use an appropriate technique for extraction of DNA from a given tissue (Llanes, 2002). Usually, DNA is extracted from fresh leaves (Murray and Thomson, 1980; Dellaporta et al., 1983; Saghai-Maroof et al., 1984; Lange et al., 1998; Maltas et al., 2011), which may not be available in sufficient quantity throughout the growing season or all the time. *Crocus* species including cultivated saffron produce a small quantity of leaf tissue in a very short period of time, while corms are available in sufficient quantity all year round (Fernandez, 2004). The DNA extraction method, by using the corm, was not reported in any plant. We attempted to extract DNA from corm by previously reported methods and also modified protocols including those of Saghai-Maroof et al. (1984) and Dellaporta et al. (1983) but DNA obtained was not suitable for manipulation and analysis. These methods produce degraded and denatured DNA or give extremely poor yields. To overcome these difficulties, we modified

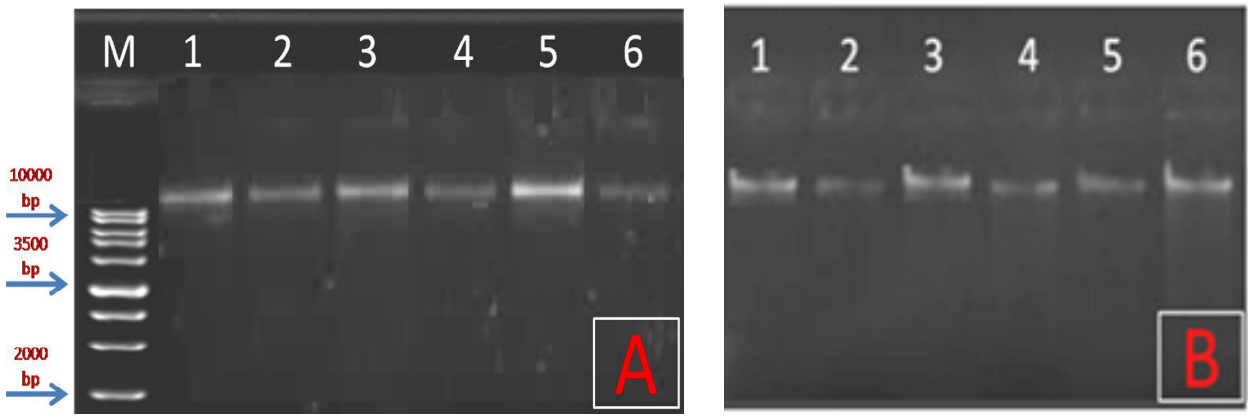

Figure 1. Comparison of quality and quantity of extracted DNA from corm (A) and leaf (B) by using the protocol outlined above in six *Crocus* accessions.

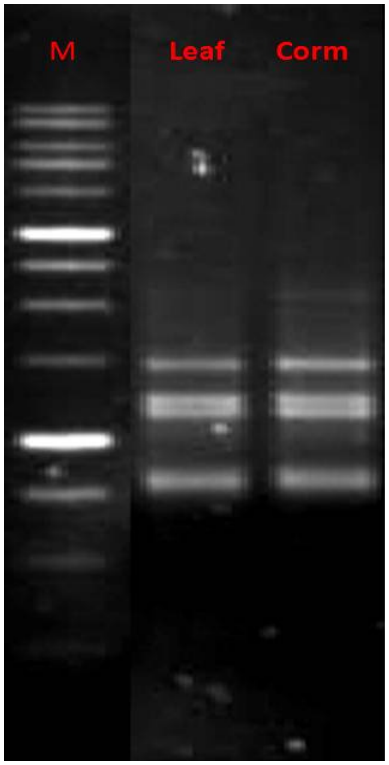

Figure 2. RAPD-PCR amplification products of Leaf and corm by using OPM3 Primer.

available DNA extraction methods. We have obtained good quality of DNA from corm of *Crocus* species as well as leaves (Figure 1) by using the protocol outlined previously. This extraction technique provides an extraction procedure that can be completed in less than 50 min. This method yielded high-quality and high quantities of DNA. The DNA was further analyzed in agarose gel, there was no DNA degradation and the average size of bands was about 25 kb or so. The corm extracted DNA quality was assessed by spectrophotometry. The A260/A280 absorbance ratio ranged from 1.8 to 2.0.

One prerequisite to reliable molecular biology work is that the genomic DNA of a sample should be of good quality. The isolation of intact, high-molecular-mass genomic DNA is essential for downstream analysis. In order to check the suitability of extracted DNA, RAPD-PCR analysis was done with OPM3 primer. As shown in Figure 2, the DNA isolated from corm was also suitable for amplification by PCR. The amplified PCR products of corm DNA showed identical band patterns and similar intensity to that of leaf tissue.

We successfully isolated DNA from *Crocus* species using the four protocols described earlier. As compared to other methods, our procedure produced a higher quality DNA from corms (Figure 3a).

The quality of the extracted DNA through this method was relatively higher, based on the intensity and the resolution of randomly amplified polymorphic DNA fragments (Figure 3b).

Conclusion

In conclusion, extraction of DNA from herbal and aromatic plants is difficult owing to the presence of large amount of secondary metabolites (Vural and Değeri, 2009; Wang, 2010; Maltas et al., 2011). Furthermore, *Crocus* species including cultivated saffron produce a small quantity of leaf tissue in a very short period of time, while corms are available in sufficient quantity all the time. However, our protocol facilitates sufficient access and high quality DNA continually. The development of a simple DNA extraction protocol from corms can constitute a breakthrough in applying molecular techniques to

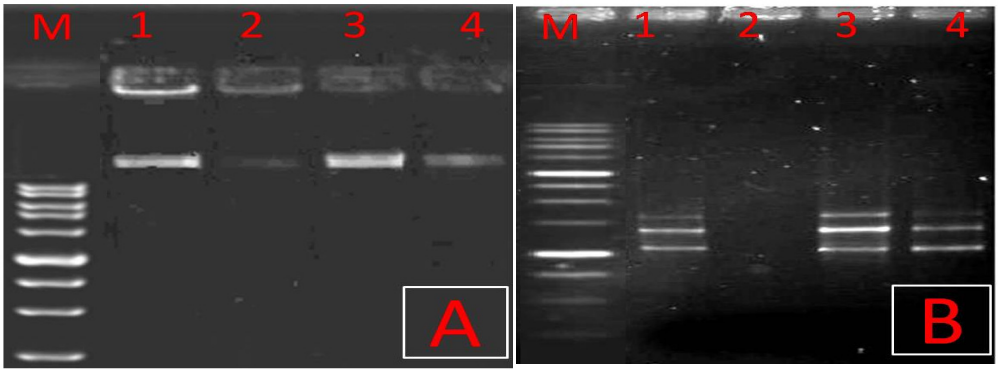

Figure 3. Comparison of extracted DNA (A) and RAPD products (B) by different DNA isolation methods. Lane 1: Method #1 (Doyle and Doyle, 1990), Lane 2: Method #2 (Dellaporta et al., 1983), Lane 3: Our method and Lane 4: Method #3 (Ikeda et al., 2001).

saffron breeding. Current studies indicate that extraction of DNA is not always routine and simple, and conventional methods are not necessarily reproducible for all species (Doyle and Doyle, 1987; Dellaporta et al., 1983; Saghai-Maroof et al., 1984; Oard and Dronavalli, 1992; Wang et al., 1993; Richards et al., 1994; Davis et al., 1995; Porebski et al., 1997; Ikeda et al., 2001; Maltas et al., 2011; Wang, 2010; Zidani et al., 2005), especially for extraction of DNA from corms of *Crocus* species. Also DNA must be of high quality, sufficient quantity and stable during storage. Here we report, for the first time, a fast, simple, low cost, high-throughput DNA extraction procedure, from corms. All DNA was stable and could be amplified by PCR, requiring only 0.5 ng DNA in 25 µl reactions. No further purification step or any additional treatment was required for obtaining a suitable DNA prep for ordinary amplification by PCR.

This procedure eliminates the need for a time consuming and tedious stage of corm regeneration and isolates DNA of substantial quality for use in reproducible PCR reactions.

This technique has potential to be an effective protocol for DNA extraction using corm for *Crocus* species, and perhaps for other plants, when sufficient young leaf tissue is not available. This could be adopted as a standard method for plants propagated with corm for isolation of DNA for marker analyses.

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