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Developing an SCAR and ITS reliable multiplex PCR-based assay for safflower adulterant detection in saffron samples

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ABSTRACT

Saffron (*Crocus sativus* L.), one of the most important and expensive medicinal spice products traded internationally, is subject to adulteration by design or default with safflower stamens and corn stigmas, leading to poor quality of saffron samples. The present study aims at the development of specific, sensitive and reproducible PCR-based markers to detect these adulterants in traded saffron. Six putative RAPD markers generated by random primers, OPA-14, MG-11, MG-12 and AJ-05, were identified as saffron specific by comparative RAPD analysis of genuine saffron, safflower and corn. These specific RAPD markers were cloned, sequenced and six pairs of SCAR primers were designed. Specific designed primers were able to amplify reproducible saffron DNA with expected sizes and no amplification in corn and safflower DNA. In this study, a primer pair was also designed based on ITS sequences for specific amplification of safflower DNA. PCR reactions were also specifically amplified 613 bp of ITS region in safflower genome. The multiplex PCR assays were further established for the joint use of some SCAR and ITS markers efficiently. The special feature of this new molecular method was technically rapid and convenient practically and suitable for analyzing large numbers of samples. Thus, the simple rapid PCR-based molecular method could be used as a helpful assistant tool for the identification of adulterant saffron samples. This study described the development of a new SCAR and ITS maker-based multiplex PCR assay for the rapid molecular detection of substitutes in saffron.

1. Introduction

Saffron is a spice obtained from the red-dried stigmas of the purple-flowered *Crocus sativus* L. (Iridaceae). It is used for flavoring and coloring in food preparation and also, in traditional and modern medicine (Madan, Kapur, & Gupta, 1966; Melnyk, Wang, & Marcone, 2010; Negbi, 1999; Rios, Recio, Giner, & Manez, 1996). Saffron stigmas contain important secondary metabolites such as crocin and its derivatives (color factors), picrocrocin (taste factor) and safranal (perfume factor); also it contains other compounds such as carbohydrates, proteins, fats and minerals. The amounts of these metabolites determine the quality of saffron (Carmona, Zalacain, Salinas, & Alonso, 2007; Giaccio, 2004; Melnyk et al., 2010; Rios et al., 1996; Winterhalter & Straubinger, 2000). Saffron, in food applications, can be used as a natural color and flavor additive instead of chemical and artificial colors and flavor additives in the production of drinks, dairies and confectionery (Basker & Negbi, 1983). Recently identified medicinal compounds from saffron have brought pharmaceutical benefits including anti-cancer, analgesic, anti-inflammatory and anti-depressant properties (Abdullaev, 2002; Akhondzadeh et al., 2007; Fernandez, 2006; Mousavi, Tayarami, & Parsaee, 2010; Wang et al., 2010).

Saffron is the most expensive spice in the world and there is always the possibility of cheating in saffron trade (Madan et al., 1966). The compounds often used as adulterants are safflower stamens and corn stigmas and these alternatives are disrupting trade in saffron. Today, there are methods for discriminating the faked saffron from the natural one. The chemical composition of saffron samples, as a method for saffron evaluation, indicates that the values reported are strongly dependent on the methods employed for drying, extraction and analysis (Kanakis, Daferera, Tarantilis, & Polissiou, 2004; Lozano, Delgado, Gomez, Rubio, & Iborra, 2000; Zareena, Variyar, Gholap, Bongirwar, & Wani, 2001). Various analytical methods are based on the quality and quantity of saffron pigments using spectrophotometric measurements (Maggi, Sanchez, et al., 2011; Orfanou & Tsimidou, 1996; Sanchez et al., 2008; Zalacain et al., 2005b; Zougagh, Rios, & Valcarcel, 2005), high-performance liquid chromatography (HPLC) (Ahmad et al., 2010; Caballero-Ortega, Pereda-Miranda, & Abdullaev, 2007; Haghighi, Feizy, & Hemati, 2007; Lage & Cantrell, 2009; Loskutov,

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Beninger, Hoseld, & Sink, 2000; Lozano, Castellar, Simancas, & Iborra, 1999; Moraga, Rambla, Ahrazem, Granell, & Gomez-Gomez, 2009), near infrared spectroscopy (NIR) (Zalacain et al., 2005a), nonaqueous capillary electrophoresis (CE) (Zougagh, Simonet, Rios, & Valcarcel, 2005) and proton nuclear magnetic resonance (NMR) (Assimiadis, Tarantilis, & Polissiou, 1998; Tarantilis & Polissiou, 2004). The method of saffron quality characterization currently recommended by the International Standardization Organization is UV–vis spectrophotometry (ISO/TS 3632, 2003). Unfortunately, the method is non-specific and unable to separate genuine and adulterated saffron adequately; thus it is unable to provide a quality category in the international market (Lozano et al., 1999; Zougagh, Rios, et al., 2005). It has been established that the quality of saffron in different geographic areas is not the same (Anastasaki et al., 2009; Del Campo et al., 2009; Maggi, Carmona, Kelly, Marigheto, & Alonso, 2011; Siracusa et al., 2012); also drying methods stigmas and its maintenance (Maggi et al., 2010; Rains, Agarwal, Bhatia, & Gaur, 1996) influence the quality of saffron. Therefore, detection methods of saffron adulterants which are related to saffron pigments are not sufficient to distinguish accurately natural and misused substitutes.

Since DNA markers can detect differences in the level of DNA and they are not affected by environmental conditions, they are more reliable than morphological traits (Kumar, 1999; Kumar, Gupta, Misra, Modi, & Pandey, 2009). Among different DNA markers, RAPD is a fast and cheap technique with short and random primers which need no previous sequence information (Welsh & McClelland, 1990; Williams, Kubelic, Livak, Rafalski, & Tingey, 1990). Nevertheless, due to the high sensitivity of the reaction conditions, RAPD repeatability is low, and it's difficult to exchange the results among the laboratories. To overcome this problem, RAPD polymorphism bands are converted to a more specific and reliable marker known as SCARs (sequence-characterized amplified regions) (Paran & Michelmore, 1993). SCAR primers have larger lengths which are designed on the sequence obtained from random markers and they can reproduce specific bands with less sensitivity to reaction conditions (Hernandez, Martin, & Dorado, 1999). Many studies have demonstrated that SCAR markers derived from RAPD fragments have been successfully used to identify plant species and cultivars and detect adulterants in commercial species (Busconi, Sebastiani, & Fogher, 2006; Kiran, Khan, Jabeen Mirza, Ram, & Abdin, 2010; Marieschi, Torelli, Poli, Bianchi, & Bruni, 2010; Scheef, Casler, & Jung, 2003).

One of the most popular sequences for interspecific and intergenic level divergences in plants is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron. The high copy number of this region allows easy amplification from total DNA (Baldwin, 1993; Baldwin et al., 1995). The ITS region has been proposed as a useful region to gain insight into DNA sequence evolution and interspecific/intergeneric-level phylogenetic analyses among angiosperms and other eukaryotes (Hershkovitz & Zimmer, 1996). Pairwise comparisons between taxa revealed that the divergence values ranged from 0 to 39% (Baldwin et al., 1995). This feature can be used to design specific primers for differential amplification of ITS region in different species.

Due to high economic value of saffron, substitution of it with orange-flowering plants such as safflower is the most common cheating method in saffron trade and it is necessary to develop a simple and accurate method with the least possible time and minimal cost for distinguishing the safflower impurity in saffron samples. Recently, a method based on SCAR markers developed from RAPD specific for seven common bulking agents was used as saffron adulterants (*Arnica montana* L., *Bixa orellana* L., *Calendula officinalis* L., *Carthamus tinctorius* L., *Crocus vernus* L. (Hill), *Curcuma longa* L., and *Hemerocallis* sp.) to authenticate their presence in commercial saffron samples (Marieschi, Torelli, & Bruni, 2012). Therefore, our aim was to develop a new SCAR and ITS marker-based multiplex PCR assay as a simple and efficient PCR-based method for the rapid molecular identification of saffron and the detection of safflower, as the main misused substitute saffron, in adulterant samples.

2. Materials and methods

2.1. Plant materials and DNA extraction

The dried stigmas of five saffron genotypes from different geographical regions of Iran (Khorasan, Isfahan, Yazd, Hamadan and Shiraz), dried stigmas of five corn cultivars (single cross 647, 700, 704, 716 and back cross 666) and also, dried stamens of five safflower genotypes including three *Carthamus* species (*C. palestinus*, *C. oxyacanthus* and *C. tinctorius*: C_{111} and IL_{111} cultivars) were obtained from the Agricultural Research Center of Isfahan, Isfahan, Iran.

Total genomic DNA was isolated from dried saffron, corn stigmas and safflower stamens according to modified CTAB procedure, which was followed by using LiCl (Ribeiro & Lovato, 2007). The quality and quantity of DNA samples were determined by 0.7% (w/v) agarose gel electrophoresis and verified by spectrophotometric measurements. DNA samples were diluted to approximately 20 ng/μl and stored at −20 °C.

2.2. RAPD analysis

Twenty five RAPD primers (Operon Technologies Inc., USA) were tested on saffron, safflower and corn DNA samples. The RAPD reactions were carried out in a volume of 15 μl containing 0.4 μM of primers, 1.5 μl 10X PCR buffer, 0.3 mM dNTPs, 1.5 mM $MgCl_2$, 2 units *Taq* DNA polymerase and approximately 30 ng of genomic DNA. RAPD amplifications were performed in a thermo cycler (Eppendorf mastercycler gradient, Germany) with the following program: 94 °C for 3 min; 40 cycles at 92 °C for 1 min, 35 °C for 1 min and 72 °C for 2 min in order to denature, anneal and extend the reaction; and the final extension at 72 °C for 5 min. PCR products were resolved in 2% agarose gel in 0.5X TAE buffer, followed by electrophoresis at 6 V/cm. The gels were stained using 0.5 μg/ml(w/v) of ethidium bromide and the bands were visualized under UV light.

2.3. Cloning and sequencing of the bands

The RAPD specific bands amplified in saffron rather than safflower and corn DNA samples were selected and excised from the gel using Silica Bead DNA Gel Extraction Kit (#K0513, Fermentase) and cloned in pTG19/T vector (#TA010, Vivantis Technologies Sdn. Bhd., Selangor Darul Ehsan, Malaysia). The transformants were selected through rapid screening and enzymatic digestion (*BamH*I) method (Sambrook & Russell, 2001).

DNA inserts were sequenced on both strands by universal M13 primers. The quality of sequences was evaluated by Chromas ver 2.13 (Technelysium, 2004) and MEGA ver 4 softwares (Tamura, Dudley, Nei, & Kumar, 2007). The prepared sequences were aligned for homology searches in the NCBI (www.ncbi.nlm.nih.gov) database by the BLASTn (Altschul et al., 1997) program.

2.4. SCAR design and analysis

Eight SCAR primer pairs in different sizes were designed using the OLIGO software ver 7.0 (Rychlik, 2008) (Table 1). Through specific designed primers, PCR reactions were conducted on approximately 10 ng of DNA template in 15 μl volume containing 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase, 1X PCR buffer, 0.3 mM dNTPs, and 5 pmol of each specific forward and reverse primers. Amplifications were performed using a thermal cycler (Eppendorf