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DNA and metabolite based profiling in *Crocus sativus L.*

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Abstract- Saffron derived from *Crocus sativus* is the most expensive, flavored and colorful spice with worldwide importance and utilities in many disease conditions. Many efforts have been made to detect variability in this plant. We used sequence specific amplified polymorphism (SSAP) and retrotransposon microsatellite amplified polymorphism (REMAP) markers for analyzing the genetic diversity among thirty accessions of *Crocus sativus.* Besides, we also attempted metabolite profiling in these accessions. This work aimed at assessing the molecular and biochemical variability in saffron since it has been reported to be highly stable in other parts of the world. Various primer combinations were used in SSAP and REMAP for PCR amplification and the primers giving the best pattern were used for further analysis. The retrotransposon based molecular marker failed to find any variation at molecular level inferring the inability of retrotransposon mobility to cause polymorphism in geographically different cultivars of *Crocus sativus*. However, the HPLC profiling of samples collected from 17 selected locations among the thirty samples, revealed variations in metabolite concentrations of crocin and safranal. The results obtained in this study supported the monomorphic nature of Kashmir saffron at genetic level.

Index Terms- Crocin, High Performance Liquid Chromatography, , REMAP, Safranal, SSAP

I. Introduction

Saffron, *Crocus sativus* is a triploid (3n=24) sterile plant belonging to the family Iridaceae. Saffron is considered to be the most expensive spice of the world. The major saffron producing countries are Iran, Greece, Spain, Turkey, India, and Morocco. Recently, Europe has also started cultivating saffron successfully in various areas (Caballero & Miranda, 2007). In India, saffron is cultivated in Kashmir valley and Kishtwar region of Jammu and Kashmir State. Although, saffron has remained under cultivation various stress conditions resulting in mutations, but the sterility and vegetative propagation of the plant has limited these mutations from being redistributed in the plant (Ahmad, Zaffar, Mir, Razvi, Rather & Mir, 2011).

During the past few years, molecular markers such as RAPD (Random Amplified Polymorphic DNA) (Caiola, Caputo & Zanier, 2004), ISSR inter-simple sequence repeats (Tanyolac, 2004), AFLP (Amplified Fragment Length Polymorphism) (Ozkan , Kafkas, Ozer & Brondolini , 2005 and IRAP (Inter Retrotransposon Amplified Polymorphism) (Alavi , Mohammadi, Aharizad & Moghaddam, 2008) have been used extensively for detection of genetic variation among various accessions of *C. sativus* but failed to find polymorphism in *C. sativus.* However, molecular markers based on transposable elements i.e., retrotransposons (REMAP and SSAP) that have proved to be promising for elucidating genetic diversity in other plants have not been used in saffron. Because of the irreversible nature of retrotransposon insertions, they are considered particularly useful in phylogenetic studies. Present work is the first report on the application of SSAP and REMAP on *C. sativus*, especially of Indian accessions.

Saffron owes its importance to the presence of apocarotenoid that are characterized by a bitter taste, yellow orange colour and hay like fragrance because of apocarotenoids crocin, picrocrocin and safranal (Negbi, 1999). Metabolite profiling of saffron may help in identifying the clones with high quantity of apocarotenoids. Application of molecular markers and metabolite profiling may help in establishing a relationship between genetic and metabolic variation, if any. Moreover, the variations in the contents of bioactive active components greatly influence the quality and beneficial health effects of this herb. Therefore, the simultaneous quantification of bioactive compounds can play an important role in the effective quality evaluation for *C. sativus*.

II. Materials and Methods

Plant material

For studies on molecular markers leaves were collected from saffron plants growing at 30 locations in Jammu and Kashmir. However, among these 17 locations (having significant difference in altitude) were selected for metabolite study (Table 1). Plant tissues were independently harvested, frozen in liquid nitrogen and stored at -80°C until required.

DNA extraction

DNA was extracted from 400 mg of leaf material using the protocol given by Doyle and Doyle with some modifications (Doyle and Doyle, 1991). The extracted DNA was quantified spectrophotometrically and stored at -20°C till further analyses.

REMAP analysis

For REMAP analysis, LTR based primers MKD-4, MKD-5, MKD-9, MDK-11 were used in combination with B1, B2, B3, B4, B5, B6, B7 and B8 ISSR based primers (Table 2). Several combinations of primers were used and the combinations resulting in large number of bands were selected for further analysis. The reactions were performed in 25 µl reaction volume containing 0.25 mM dNTPs, 1X buffer, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase and 5 pmol primers. PCR thermal cycler conditions consisted of initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation for 30 S at 94°C, annealing at 46°C for 30 S, extension for 2 min at 72°C, and the final extension of 10 min. The PCR-amplified fragments were

electrophoresed on a 4% agarose gel in TAE and were visualized by ethidium bromide staining.

SSAP Analysis

For SSAP analysis, the protocol of Kour *et al*. (Kour , Kour and Dhar, 2009) was followed with slight modifications. The purified DNA was digested with *Mse*I restriction enzyme in 100 µl reaction volume. This was followed by ligation with 2pmol of *Mse*I adaptors (ASW1 and ASW2) using 0.2 U of T4 DNA ligase. The samples were incubated at 37˚ C for 3 h and the PCR reaction was performed using primers against LTR region and RNaseH motif. The primers selected for the SSAP analysis enlisted in table 3 with their sequences, were MKD-4, MKD-5, MKD-9, MDK-11, ASW1, ASW2, ASW-8, ASW-9 and ASW-10. The reactions were performed in 25 µl reaction volume containing 0.2 mM dNTPs, 1x buffer, 0.25 mM MgCl2, 0.5 U *Taq* DNA polymerase and 5 pmol primers. PCR thermal cycler conditions consisted of initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturation for 30 S at 94°C, annealing at 46°C for 30 s, extension for 2 min at 72°C, and the final extension of 10 min. The PCR amplified fragments were electrophoresed on a 4% agarose gel and visualized by ethidium bromide staining.

HPLC Analysis

Saffron stigmas collected from plants cultivated at 17 locations were selected for HPLC analysis. Extraction of saffron stigmas was performed according to Lozano *et al*. (Lozano ,Castellar , Simancas &Iborran, 1999) with some modifications. For the estimation of crocins and safranal, 50 mg of saffron stigmas were suspended in 10 ml methanol-water (50:50, v/v) and stirred for 24 h at 4 ^{o}C in the dark. After extraction, samples were centrifuged at 20,000g for 45 min to eliminate plant residues and the supernatant was collected and filtered through a 0.45 µm nylon membrane (Millipore, USA). The samples were stored in dark till analyzed. HPLC analysis was performed with a Waters (USA) HPLC system equipped with 515 quaternary gradient pump, 717 Rheodyne injector, 2996 PDA detector and Empower software (version 3.0). The column used for separation was RP-18 (4.6×250 mm, 5 µm) (Merck) column. The mobile phase consisted of methanol–water (50:50) (v/v) delivered at a flow rate of 0.8 mL/min. The accessions of thirty samples and their geographic origin are listed in Table 1. Standards, safranal 88% and crocins were purchased from Sigma-Aldrich, USA.

III. Results and Discussion

REMAP and SSAP are retrotransposon based markers and are relatively new and efficient techniques which have been used for analyzing the variability in plants. We used twenty different primer combinations for SSAP analysis in saffron. Among these primer MKD9 and ASW8 (figure 1) combination gave a good number of scorable bands. In case of REMAP thirty two different primer combinations were used, primer B5 and MKD9 (figure 2) gave the maximum number of bands but unfortunately were not successful to reveal any polymorphic bands. A total of nine primers, making twenty different combinations were used for SSAP analysis. SSAP and REMAP selected primer combinations were able to give scorable number of bands but were not successful to give any polymorphic band. IRAP, RAPD and ISSR **(**Ozkan , Kafkas, Ozer & Brondolini , 2005; Moraga, Castillo Lopez, Gomez-Gomez & Ahrazem, 2009**)** analysis of *Crocus* has been already conducted and all these genetic markers have shown no polymorphism in *C. Sativus* which corroborated well with the present study of ours.

Major components of saffron are crocin and safranal along with picrocrocin and crocetin. Identification and content analysis of these components could be a discriminative key for the characterization of *crocus sativus* of different geographical locations. Chemoprofilng of saffron is gaining attention because of the promising therapeutic roles shown by this spice in number of clinical conditions and disease. Higher the content of saffron components better is the quality of the spice. Chemoprofilng of saffron from different parts of the world have shown that concentration of crocin in saffron varies in the range of 0.85% to 32.4% dry weight of saffron from different countries (Alonso, Salinas, Garijo, Sanchez-Fernandez, 2001). The variation in the components was further supported by the findings of other workers (Li , Lin & Kwan Min 1999; Caballero & Pereda-Miranda 2007). In case of Indian sample concentration of crocin was found to be 67.3 mg/g and of safranal 8mg/g (Sujata, Ravishankar & Venkataraman, 1992). In our HPLC profile study of the samples collected from different geographical areas the concentration of crocins and safranal was found to be variable. The crocin content ranged between 30.74±0.19 mg/g to 79.84±0.29 mg/g (Table 4). Accession CR16 showed highest crocin content (79.38±0.29 mg/g) followed by accession CR19 (78.53±0.39mg/g) (Table 4). The KP accession in Kashmir showed the lowest crocin content. Quantification of safranal has shown a significant variation was with accession KWP from of Kishtwar area having lowest safranal content (0.13±0.016 mg/g) and sample from Samboora area of Kashmir showing highest (0.92±0.051) (Table 4). The concentration of crocin and safranal for the standard sample (Sigma Aldrich, USA) was observed to be 12.5mg/±0.08 and 0.13mg/g 0.016 respectively (Table 4).). In our study the crocin content was found higher in the samples collected from fields of Kishtwar (Table 4). The representative chromatograms of crocin and safranal are shown in figure 3 and 4 respectively.

Although the biochemical analysis of saffron collected from 17 geographical locations of Jammu and Kashmir (India) showed variations in the content of crocin and safranal and also reveals that overall quality of Indian saffron (in terms of content composition) is better than the saffron of the other parts of the world. However, PCR-based approaches i.e. SSAP and REMAP (first of its nature to be used in *C. sativus*) did not reveal any variation at molecular level in samples collected from 30 geographically different locations. This study reinforced the results of monomorphic nature of this plant that has earlier been documented in other parts of the world. Also it is revealed that insertional polymorphism via retrotransposon movements that has been considered one of the major causes of variability in other plants is possibly lacking in *C. sativus*. However, the exploration of advanced markers is required and suggested to detect molecular variation in *C. Sativus* if any.

IV. Conclusion

This study concludes that retrotransposon mobility has not been able to insert any variation in saffron at molecular level and reveals it to be monomorphic at genetic level. However, chemoprofilng reveals significant variation in the content of bioactive components of saffron in addition to better quality of Indian saffron that could be an important aspect for considering saffron to be used as a functional food because of the diverse therapeutic applications of saffron components.

Conflict of interest

The author (s) have not declared any conflict of interests

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Table Legends:
Table 1: Geographical locations of sample collection.
Table 2: List of primers used for REMAP analysis.
Table 3: List of primers used for SSAP analysis.
Table 4: Metabolite (Crocin & Safranal) concentration of different accessions.

Figure Legends:
Figure 1: SSAP analysis of *Crocus sativus* germplasm collected different areas of Jammu and Kashmir using primer pairs MKD-9 and ASW-8.
Figure 2: REMAP analysis of *Crocus sativus* germplasm collected different areas of Jammu and Kashmir using primer pairs B5 and MKD9.
Figure 3: HPLC chromatogram of crocin, (a) standard, (b) accession KP lowest crocin content , (c) accession CR16 highest crocin concentration & (d) accession R19, highest crocin content from Kashmir division.
Figure 4: HPLC chromatogram of safranal, (a) standard s and (b) accession KWP with lowest concentration of safranal

Table 1: Geographical locations of sample collection.

Accession code	Geographical areas	Accession code	Geographical areas
CR1	Khunmoh A	CR16	Puchhal kishtwar
CR2	Khunmoh B	CR17	Chrar A
CR3	Wuyan A	CR18	chadora
CR4	Samboora pampore	CR19	Khrew A
CR5	Namalbal pampore	CR20	Khrew B
CR6	Tulbagh pampore	CR21	Beerwar kishtwar
CR7	Chandhara near batmill	CR22	Hudri Kishtwar
CR8	Patalbagh pampore	CR23	Galander
CR9	Chandhara B	CR24	Zevan
CR10	Konibal A	CR25	Hadyal
CR11	Konibal B	CR26	Androosa A
CR12	Lathipora A	CR27	Androosa B
CR13	Lathipora B	CR28	Dushnar
CR14	Lathipora C	CR29	Nagam A
CR15	Dusu	CR30	Nagam B

Table 2: Primers used for REMAP analysis.

S. No.	Primer code	Primer sequence
1	B1	5´GCCTTGAAACTTCTTCTTGTATC3
2	B2	5´CCCTTCCAGTTAAATCAGTCG3´
3	B3	5´GAGTGCGGAAGCGAGTAGAG3
4	B4	5´TCCGTGTCCTCTGTCTGTG3´
5	B5	5´GGGAAATGAACAGAGGAAA3’
6	B6	5´ATGTTGGGAACTTTTAGTGTG3’
7	B7	5´ACCACTGCAATCCTTCATCTTTG3
8	B8	5´CCCTCATCCTCCATTCTTATCG 3’
9	MKD4	5’ACTGCCTTTTTACATGG 3’
10	MKD5	5-AACATATCATCAACATA-3’
11	MKD9	5-CTAAGAGGCTACCACCAAAT-3’
12	MKD11	5-ACACTTAGAGAACTTGGCA-3’

Table 3: Primers used for SSAP analysis .

S. No	Primer code	Primer sequence
1	MKD4	5-ACTGCCTTTTTACATGG-3’
2	MKD5	5-AACATATCATCAACATA-3’
3	MKD9	5-CTAAGAGGCTACCACCAAAT-3’
4	MKD11	5-ACACTTAGAGAACTTGGCA-3’
5	ASW1	5’-GACGATGGATCCTGAG-3’

6	ASW2	5’-TACTCAGGATCCAT-3’
7	ASW8	5’-GATGGATCCTGAGTAAC-3’
8	ASW9	5’-GATGGATCCTGAGTAAAC-3’
9	ASW10	5’-GATGGATCCTGAGTAAACA-3’

Table 4: Showing metabolite (crocin & safranal) concentration of different accessions.

Accesion	Location	Total crocin	Safranal
CR 3	Wuyun,Kashmir	55.58±0.42	0.23±0.032
CR 4	Samboor, Kashmir	54.73±0.33	0.92±0.051
CR 7	Pampore, Kashmir	77.46±0.31	0.5±0.067
CR 10	Konibal, Kashmir	47.06±0.22	0.17± 0.011
CR 12	Lathipor A, Kashmir	42.5±0.37	0.22±0.033
CR 16	Puchhal, Kishtwar	79.38±0.29	0.16±0.032
CR 17	Chrar A, Kashmir	40.66±0.35	0.62±0.021
CR 18	Chadora, Kashmir	53.4±0.43	0.4±0.071
CR 19	Khrew A, Kashmir	78.53±0.39	0.19±0.021
CR 22	Hudri, Kishtawar	63.26±0.29	0.26±0.042
CR 24	Zevan, Kashmir	39.6±0.28	0.22±0.063
CR 25	Hadyal,Kishtawar	69.7±0.34	0.26±0.033
CR 26	Androosa	46.9±0.28	0.17±0.019
CR 28	Dushnar,Kishtwar	64.4±0.36	0.15±0.017
KP	Kashmir,Market	30.74±0.19	0.18±0.023
Sigma	Sigma	12.5±0.08	0.15±0.037
KWP	Kishtwar Market	36.5±0.18	0.13±0.016

Values are represented as mean ±S.D

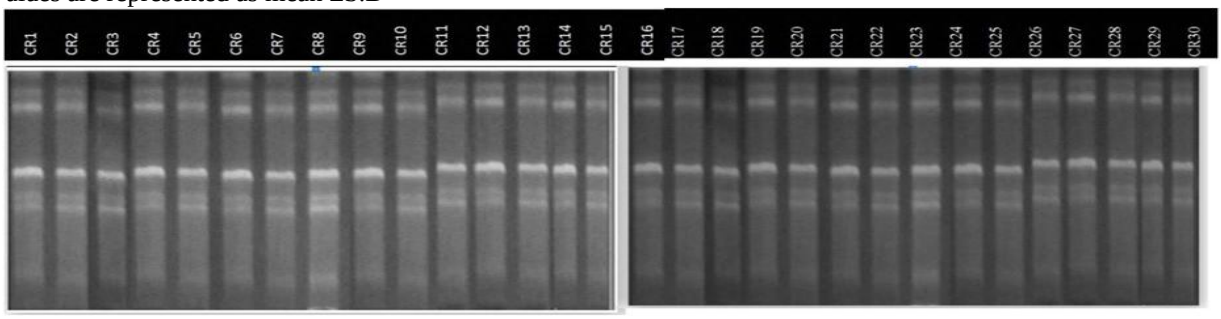

Figure 1: SSAP analysis of *Crocus sativus* germplasm collected different areas of Jammu and Kashmir using primer pairs MKD-9 and ASW-8

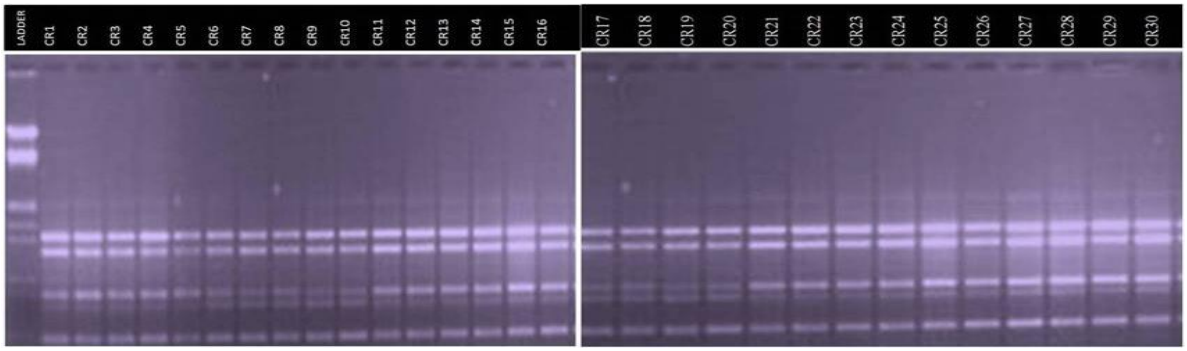

Figure 2: REMAP analysis of *Crocus sativus* germplasm collected different areas of Jammu and Kashmir using primer pairs B5 and MKD9.

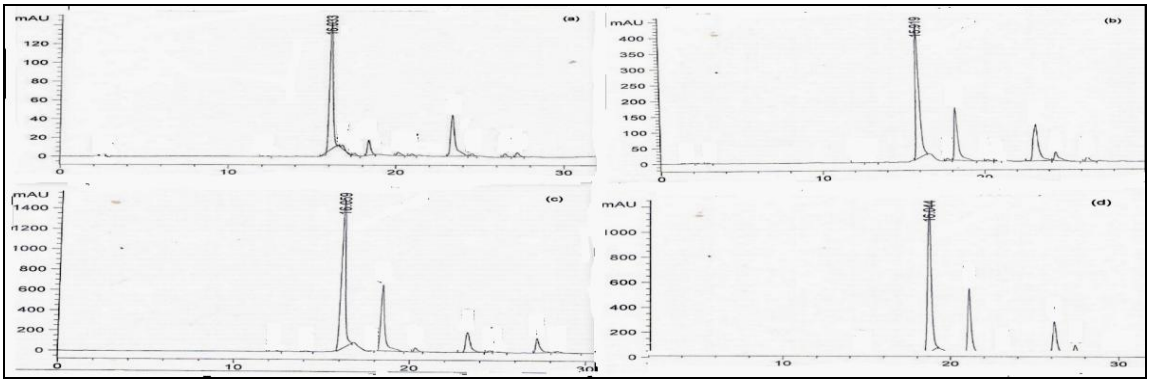

Figure 3: HPLC chromatogram of crocin, (a) standard, (b) accession KP lowest crocin content , (c) accession CR16 highest crocin concentration& (d) accession R19, highest crocin content from Kashmir division.

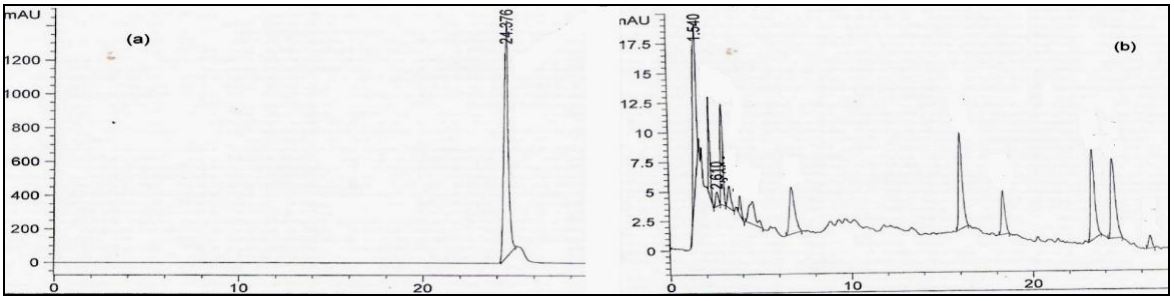

Figure 4: HPLC chromatogram of safranal, (a) standard s and (b) accession KWP with lowest concentration of safranal