Isolation of High Quality RNA for High Throughput Applications From Secondary Metabolite Rich Crocus Sativus L

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Isolation of high quality RNA for high throughput applications from secondary metabolite rich *Crocus sativus* L.

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Abstract

Isolating high quality RNA is a basic requirement while performing high throughput sequencing, microarray and various other molecular investigations. However, it has been quite challenging to isolate RNA with absolute purity from plants like *Crocus sativus* that are rich in secondary metabolites, polysaccharides and other interfering compounds which often irreversibly co-precipitate with the RNA. While many methods have been proposed for RNA extraction that include CTAB, TriZol, SDS based methods, they invariably yield less and poor quality RNA. In the present study we made certain changes in the available protocols including modifications in the extraction buffer and procedure viz-a-viz solutions used for precipitation of RNA. Our method led to the isolation of clear and non-dispersive total RNA with an RNA Integrity Number (RIN) greater than 7.5. The quality of the RNA was further assessed by qPCR based amplification of mature miRNAs such as Cs-MIR166c and Cs- MIR396a. In conclusion, the study describes an efficient method of RNA extraction that is highly ideal for high throughput sequencing of small RNAs.

Key words: *Crocus sativus,* RNA, high throughput sequencing, microRNA and secondary metabolites.

Introduction

Crocus sativus is an economically important perennial geophyte bearing crimson colored trifid stigmas. The carpels bearing stigmas are the repositories of apocarotenoids such as Crocin, picrocrocin and saffranal which impart the characteristic color, taste and aroma to it. Leung [1] Owing to its unique organoleptic properties and the difficulties associated vis-a-vis cultivation, handling and harvesting, it is considered among the most expensive spices in the world[2]. Moreover, there is a compelling evidence which supports the therapeutic potential of saffron [3], particularly its stigmas and due to the growing demand it becomes important to devise a strategy aimed at improving both the quality and quantity of stigma threads [4].

The pivotal role of floral development genes in specifying the identity of different organs entails their thorough study in *Crocus* as it will be instrumental in filling the existing lacunae and furnish a platform for targeted manipulation of key genes which are pivotal to flowering and hence broadening our horizons towards preponing the flowering and shortening the, otherwise, prolonged lifecycle of saffron. The ideal strategy to improve the crop yield would, however, involve an exhaustive study of the molecular mechanisms that are involved in regulating the flowering time, flowering transition and the biosynthesis of apocarotenoids. The existing scientific data supports the crucial role of microRNAa (miRNAs) in regulating some important plant developmental processes such as leaf morphogenesis and polarity [5,6] floral differentiation and development [7,8]. Moreover, the microRNAs identified hitherto, have 50% targets as transcription factors out of which many miRNAs target mRNAs encoding transcription factors that regulate development [9,10].

A stepping stone in the direction of molecular analysis would involve extracting the high quality RNA [11] in order to synthesize cDNA for performing routine PCR experiments, quantitative PCR and various other experiments that rely on cDNA as a starting material. Moreover, isolating high quality RNA becomes particularly important while carrying out post transcriptional studies involving microRNAs, as the preparation of microRNA library entails to meet a specific RIN value for the isolated RNA. In order to isolate RNA from different tissues of *Crocus sativus*, it is important to ensure that there is no interference of secondary metabolites, which co-precipitate with the RNA [12] and significantly reduces the quality of RNA. We isolated high quality RNA from different *Crocus sativus* samples using our modified protocol, that was developed by modifying the already published protocols originated from Chan, et al. [13] and . The proposed modified protocol is robust and ideal for isolating high quality RNA with RIN value greater than 7.5, aimed at performing high throughput sequencing of small non-coding RNAs.

Methodology

Plant Material

Plant material was collected from pampore saffron fields at an elevation of 1573 m (5161 ft) at 34.02° N/74.93° E and stored at -80˚c. Samples were collected after obtaining permission from local Biodiversity Board and all the experiments were carried in accordance with international guidelines. Before RNA extraction, the saffron corms were thoroughly washed with sterile water at least three times and then rinsed with 70% ethanol for 30 sec followed by 3 washes with RNase free water respectively. Subsequently the plant material was dried to remove any water and finally for RNA extraction, 50-100mg of sample was crushed in liquid nitrogen into fine powder and transferred into RNase-free micro-centrifuge tubes.

Buffers and chemicals

Trizol Invitrogen, Extraction buffer containing (2%(w/v) SDS; 0.05M, Tris-HCl (pH = 7.5); 0.25M EDTA; and PVP(4%); NaCl, 20mM) was taken from the protocol proposed by Chan, et al. [13] and Liu, et al. [14] with slight modifications. Aside from that, phenol: Chloroform: Isoamyl alcohol (24:1v/v) (PCI 1:1 v/v) and 100% acetone was used to remove secondary metabolites that are highly soluble in acetone.

RNA extraction

1. 50 mg of tissue sample was crushed yielding fine powder in the presence of liquid nitrogen and allowed to stand for 5-6 minutes to evaporate any liquid nitrogen.
2. The powdered sample was transfered into 1ml RNAse-free micro-centrifuge tubes, washed twice with 100 percent acetone, and centrifuged at 12000×g.
3. 500 µL of extraction buffer and 200 µL of β-mercaptoethanol were applied to the pellet obtained. After vigorous mixing, the tubes were incubated at 25°C for 10-15 minutes before being centrifuged at 4°C for 10 minutes at 12000×g.
4. The supernatant (~300µL) was decanted into 1.5ml RNAse-free tube and 300µl of (1:1) Phenol: chloroform Isoamyl alcohol (24:1) was added to it and mixed thoroughly for for about 2 minutes.
5. The tubes were centrifuged at 12000×g for 10 minutes at 4^{o}c, then 1/10 volume of 3M sodium acetate (PH 5.2) and 2.5 volume of 100 percent ethanol were applied to the supernatant, and incubated for 1 hour at 4^{o}c.

6. After centrifugation at 4°c for 10 minutes at 12000×g, the pellet was dissolved in 50µL of milliQ.
7. To remove any DNA contamination, the samples were treated with DNase I (Thermo scientific # EN0521) as per the manufacturer's protocol.
8. 300µl of milliQ and 200 µl (1:1) phenol: Chloroform isoamyl alcohol (24:1) was added to the tubes and mixed gently.
9. After centrifugation at 4°c for 5 minutes at 12000×g, the upper aqueous layer (~300µL) was transferred into1.5ml RNase-free tube without disturbing the organic phase.
10. To recover the nucleic acids, 1/10 of 3M sodium acetate (PH 5.2) and 2.5 volumes of 100% ethanol (molecular grade) was added to the tubes and incubated at 4°c for 1hr.
11. The samples were centrifuged at 4°c for 10 minutes at 12000×g and pellet was dissolved in 200 µL of RNase-free water.
12. 500µL of 10M LiCl were added to the tubes and incubated on ice for 1 hr followed by centrifugation at 4°c for 10 minutes at 12000×g to recover the nucleic acids.
13. The pellet was washed twice with 500µL of 70% ethanol and allowed to dry in speed vac concentrator (thermo scientific) for 2-5 minutes and re-suspended in 100 µL RNase-free water or 50% deionised formamide for long term storage at -20°C/-80°C.

Analysis of RNA

RNA isolated from different tissue samples of *Crocus sativus* (corm, tepal, and scarlet stigma) was analysed on 1.5% (w/v) agarose gel. The quality and quantity of RNA samples was assessed by Nanodrop & Agilent TapeStation. The RNA samples with RIN (RNA Integrity Number) value greater than 7 were used for further experiments.

cDNA synthesis and PCR

To analyse the quality of RNA, the cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific). The primers specific to *Crocus* tubulin gene were designed using primer 3 online bioinformatics tool. For miRNA specific cDNA synthesis, total RNA was used as template in a reaction comprising of Reverse transcriptase, reverse tubulin primer and specific miRNA stem loop RT primers that bind to 3' end of a mature miRNA, reverse transcribing the miRNA to corresponding cDNA. The cDNA was then used to amplify the product in a PCR with reaction mix comprising of 5µL 10× PCR buffer, 0.5µL 10mM dNTP Mix, 0.5µL of 10µM forward, 0.5µL of Reverse Primer 15.3µL of ddH2O. The PCR program included: Initial denaturation at 94 ^{0}C for 5min; 35 cycles at 94 ^{0}C 30s, 57 ^{0}C for 30s and 72 ^{0}C for 40s last extension at 72 ^{0}C for 10 minutes.

Small RNA library preparation

NEBNext Multiplex Small RNA Library Prep kit New England (Biolabs) was used to prepare small RNA libraries. The kit is supplied with adaptors, primers, enzymes and buffers required to convert small RNAs into indexed libraries for next generation sequencing on the Illumina platform.

qPCR analysis

To evaluate the miRNA expression profile, cDNAs specific to tubulin and miRNAs were used to amplify the tubulin and candidate miRNA using two primers, one specific to stem loop RT primer (universal primer) and other specific to mature miRNA. (**Table S1**). Tubulin was used as an internal control to normalise the expression of miRNAs. The primer efficiency was calculated using 10 fold dilution. Fold change was calculated by $2^{-\Delta\Delta CT}$ method.

Results and discussion

For the purpose of transcriptomics and other RNA based expression studies, extracting high quality RNA with high RNA integrity value is extremely important [15]. Due to the different composition of secondary metabolites and polysaccharides across different plant species, it becomes difficult to isolate high quality RNA free from these interfering compounds, as there is no single protocol that could be universally applicable across different plant species. Moreover, the associated similarity in the chemical and physical nature of secondary metabolites and RNA causes them to co-precipitate rendering the isolated RNA unsuitable for further downstream applications. With the aim to isolate RNA free from polysaccharides and interfering secondary metabolites, we developed a protocol for isolating RNA from different tissues of *Crocus* by modifying the available protocols. [14], [16]. The protocol was efficient in extracting the high quality RNA from Corm, tepal and stigma tissues that was suitable for carrying out high throughput sequencing, viz., small RNA sequencing (**Fig 1**). While performing the extraction using the available protocols such as Trizol method, RNeasy Plant Mini Kit or other methods as described by Liu, et al. [14] and Chan, et al. [13] (**fig 2a-d**). The extracted RNA didn't meet the specifications necessary for high throughput sequencing rendering the isolated RNA unsuitable for high throughput sequencing and other downstream processing. The quality of RNA, based on the intensity of 28S and 18S rRNA, was clearly observed on 1.5 Agarose gel with no obvious degradation (**Fig 3a)**. This was further substantiated by measuring the RIN value using Agilent Bioanalyzer, which was found to be greater than 7.5, ranging between 7.8 and 8.4 **(Fig 3b-d).**

The modified protocol was both efficient and economical in isolating high quality RNA from corm, stigma and tepals compared to other protocols like Trizol, Chan, et al. [13], Liu, et al. [14] and RNeasy Plant extraction Kit respectively that showed markedly blurred and irresolute bands (**fig 2a-d**). Moreover, the RNA extracted using the Trizol method was found to be of good quality as observed on 1.5% agarose gel (**fig 2a**). However, the isolated RNA had RIN value less than 6 (**Table 2**) and hence could not be considered good for high throughput sequencing of small RNA. The spectrometric analysis of RNA extracted using modified protocol had 260/280 ratio raging between1.97 to 2.0 and 260/230 ratio between1.98 to 2.02 (**Table 1**). These ratios were compatible with the expected ratios for the pure RNA sample. The concentration of these samples as obtained using, Qubit and Nanodrop, ranged from 292 to 528 ng/µL. However, when same analysis was performed on RNA samples isolated using TRizol, Chan, et al. [13] and Liu, et al. [14] method had 260/230 ratio deviating from the expected ratio for pure RNA indicating the presence of secondary metabolites/degradation in RNA (**Table 2-4).** The similar results were obtained, with greater deviation in 260/230 and 260/280 ratios, when RNA was isolated using the commercially available kit (Plant RNase mini kit) (**Table 5**). It was therefore concluded that the modified protocol is significantly helpful in extracting high quality RNA from *Crocus sativus*, free from the polysaccharides and other secondary metabolites.

Table1: Spectrophotometric analysis of RNA isolated from different tissues of *Crocus sativus* using modified protocol.

Sample Name	**260/280**	**260/230**	**Qubit concentration (ng/µl)**	**Nano drop concentration (ng/µl)**	**RIN Value**
Corm	1.98±0.08	2.05±0.01	342	528	8.4
Tepal	1.97±0.05	2.01±0.03	393	420	8.0
Scarlet Stigma	1.89±0.03	2.0±0.04	401	441	7.8

Table 2: Spectrophotometric analysis of RNA isolated from different tissues of *Crocus sativus* using Trizol method.

Sample Name	**260/280**	**260/230**	**Qubit concentration (ng/µl)**	**Nano drop concentration (ng/µl)**	**RIN Value**
Corm	1.83±0.03	1.70±0.09	292	230	6.3
Tepal	1.64±0.01	1.88±0.02	210	200	6.4
Scarlet Stigma	1.43±0.06	2.05±0.07	192	188	5.5

Table 3: Spectrophotometric analysis of RNA isolated from different tissues of *Crocus sativus* using Liu, et al. [14] method.

Sample Name	260/280	260/230	Qubit concentration (ng/µl)	Nano drop concentration (ng/µl)	RIN Value
Corm	1.58±0.04	1.05±0.05	109	102	6.0
Tepal	1.50±0.05	1.21±0.03	142	153	5.1
Scarlet Stigma	1.47±0.01	0.98±0.07	187	185	5.8

Table 4: Spectrophotometric analysis of RNA isolated from different tissues of *Crocus sativus* using Chan, et al. [13] method.

Sample Name	260/280	260/230	Qubit concentration (ng/µl)	Nano drop concentration (ng/µl)	RIN Value
Corm	1.49±0.10	1.94±0.09	32	49	6.3
Tepal	1.70±0.02	0.93±0.01	164	151	5.0
Scarlet Stigma	1.03±0.06	1.64±0.03	167	233	4.9

Table 5: Spectrophotometric analysis of RNA isolated from different tissues of *Crocus sativus* using RNasy Plant kit.

Sample Name	260/280	260/230	Qubit concentration (ng/µl)	Nano drop concentration (ng/µl)	RIN Value
Corm	0.64±0.03	0.19±0.07	7	6	3
Tepal	0.77±0.09	0.13±0.01	6	0.98	2
Scarlet Stigma	-0.10±0.07	-0.03±0.01	-0.97	-1.20	1

The quality of RNA isolated through our modified protocol was further validated by amplifying the tubulin gene from cDNA reverse transcribed from RNA. The intense band corresponding to 220 bp (**fig 4a**) indicated that the isolated RNA was of sufficiently high quality to be explored for sRNA library preparation (**fig 4b-d**) and qPCR analysis of mature miRNAs. Moreover, it was observed that the primer efficiency corresponding to tubulin, Cs-MIR166c and Cs-MIR396a obtained from standard curve based on 10 fold dilution, was between 95 and 98 with R value $\geq$ 0.99 as expected for specific primer sequences when annealed to cDNA reverse transcribed from RNA free from any interfering compounds. The qPCR analysis of mature miRNAs including Cs-MIR166c and Cs- MIR396a (**Fig 5a-b**) depicts that the miRNA prepared from the RNA isolated using modified protocol was of good quality.

During the entire course of RNA extraction/ purification, we incorporated several modifications to minimize the RNA degradation and secondary metabolite contamination. Apart from grinding the tissue in liquid nitrogen, necessary for inactivation of RNases, we added β-mercaptoethanol, PVP and SDS in the lysis buffer to ensure inhibition of RNase activity, removal of phenolic compounds including other secondary metabolites and disruption of nucleoprotein complexes respectively [17,18]. The use of PVP proved very effective in removal of polysaccharides, phenolic compounds and other secondary metabolites that would otherwise get oxidised and co-precipitate with RNA and thereby interfering with the downstream applications. In addition, the acetone wash during the grinding process, prior to lysis, significantly reduced the extracellular secondary metabolites in the lysate as acetone offered to be the solvent for dissolution of these secondary metabolites.

Conclusion

As a whole, an efficient RNA extraction method was developed that is highly ideal for high throughput sequencing of small RNAs. The method developed led to the isolation of clear and non-dispersive total RNA with RIN value greater than 7.

Author contribution

ZA, UM and AK carried out the experiments and drafted the manuscript, while AA, BA, and RA conceived and analyzed the data. FF has collected the samples. The manuscript was revised and approved by RA.

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Figures

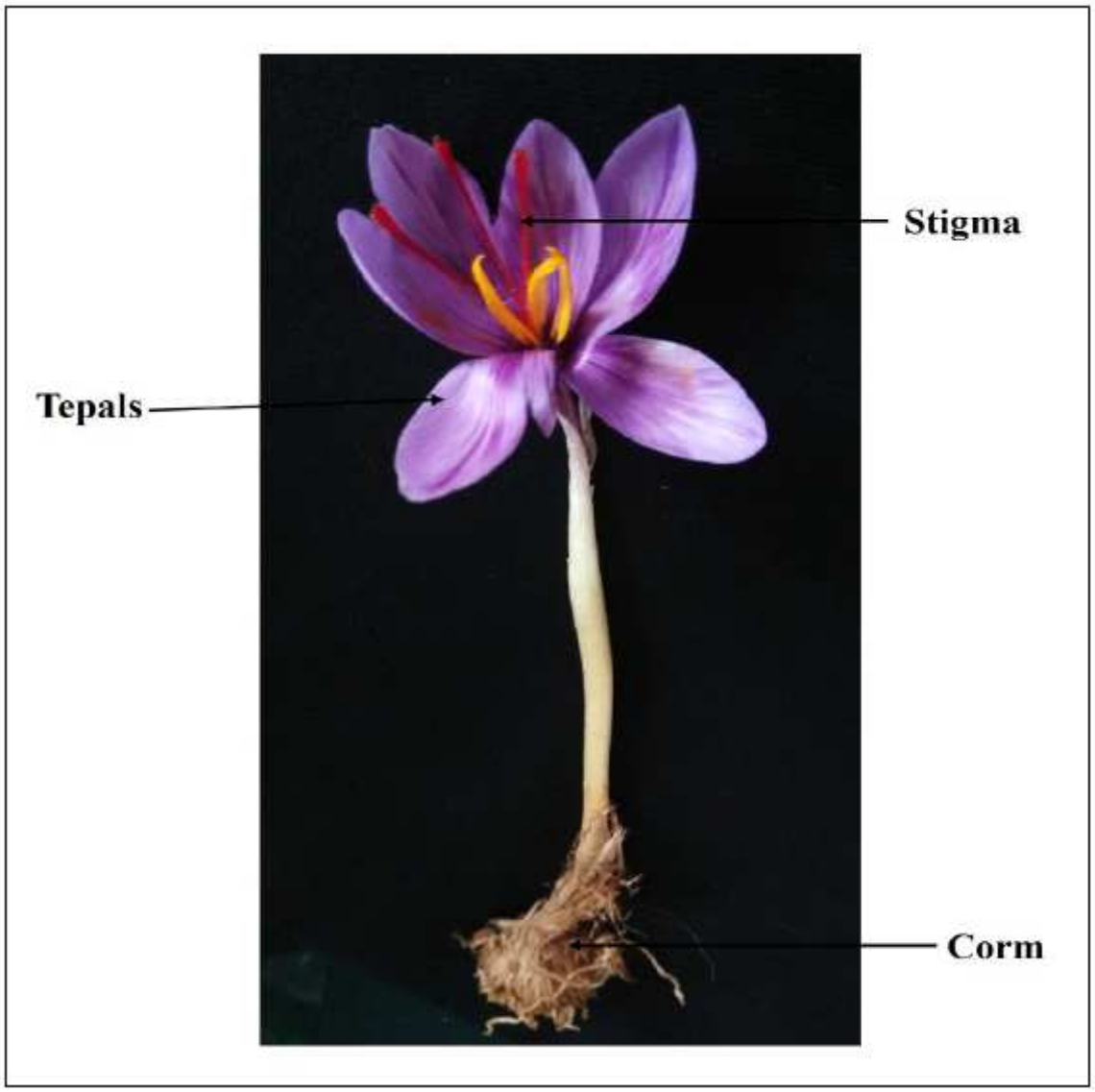

Figure 1

Crocus tissue samples (Corm, Stigma and Tepals) used for RNA isolation

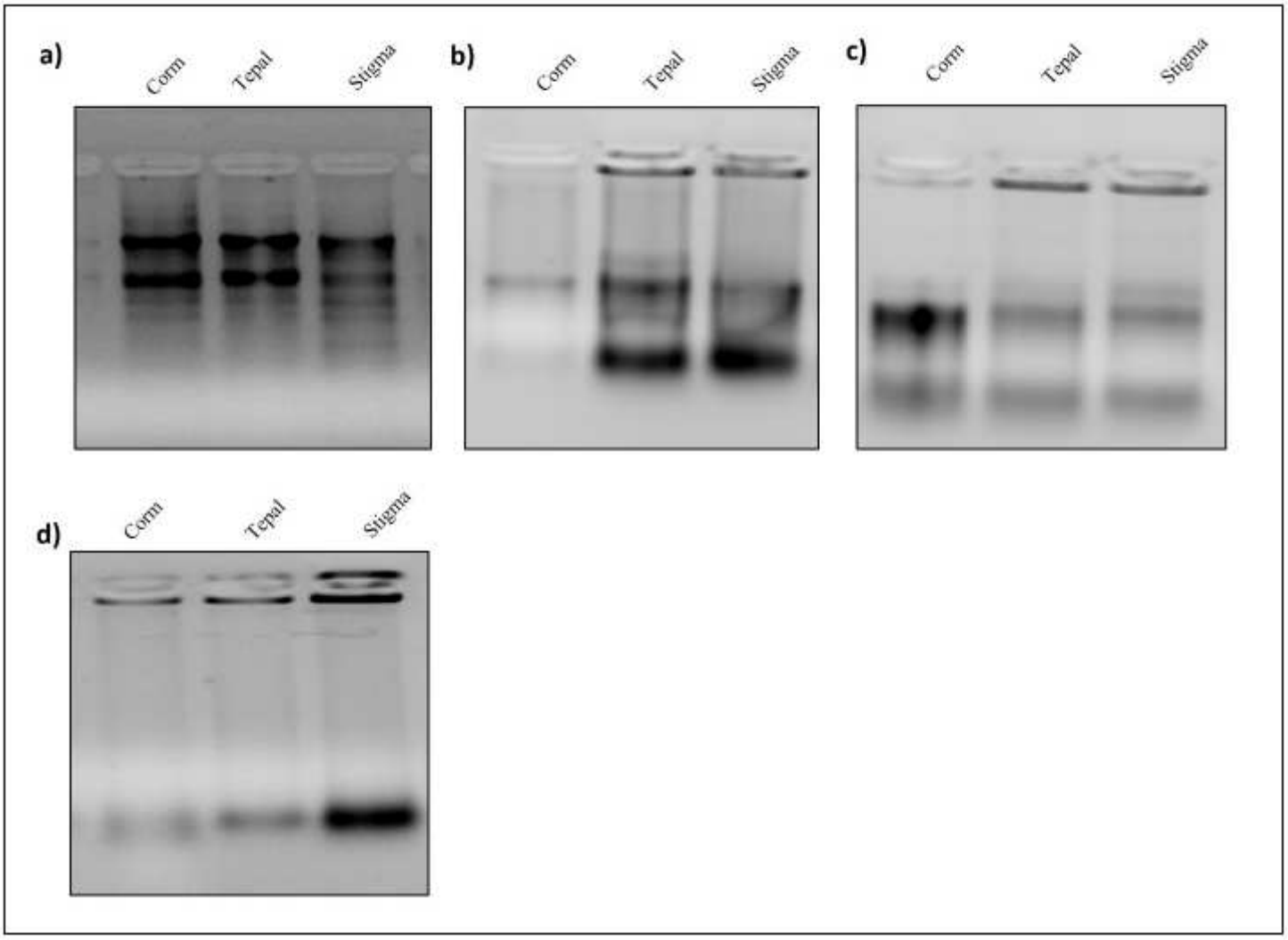

Figure 2

Agarose gel (1.5%) depicting RNA isolated from different tissue samples (Crom, tepal and stigma) of crocus sativus using different protocol (a) Trizol method (b) Liu et al.2018 (c) Chan et al.,2007 (d) RNeasy plant mini kit. Full length gel are provided in supplementary file 2 (Figure S1).

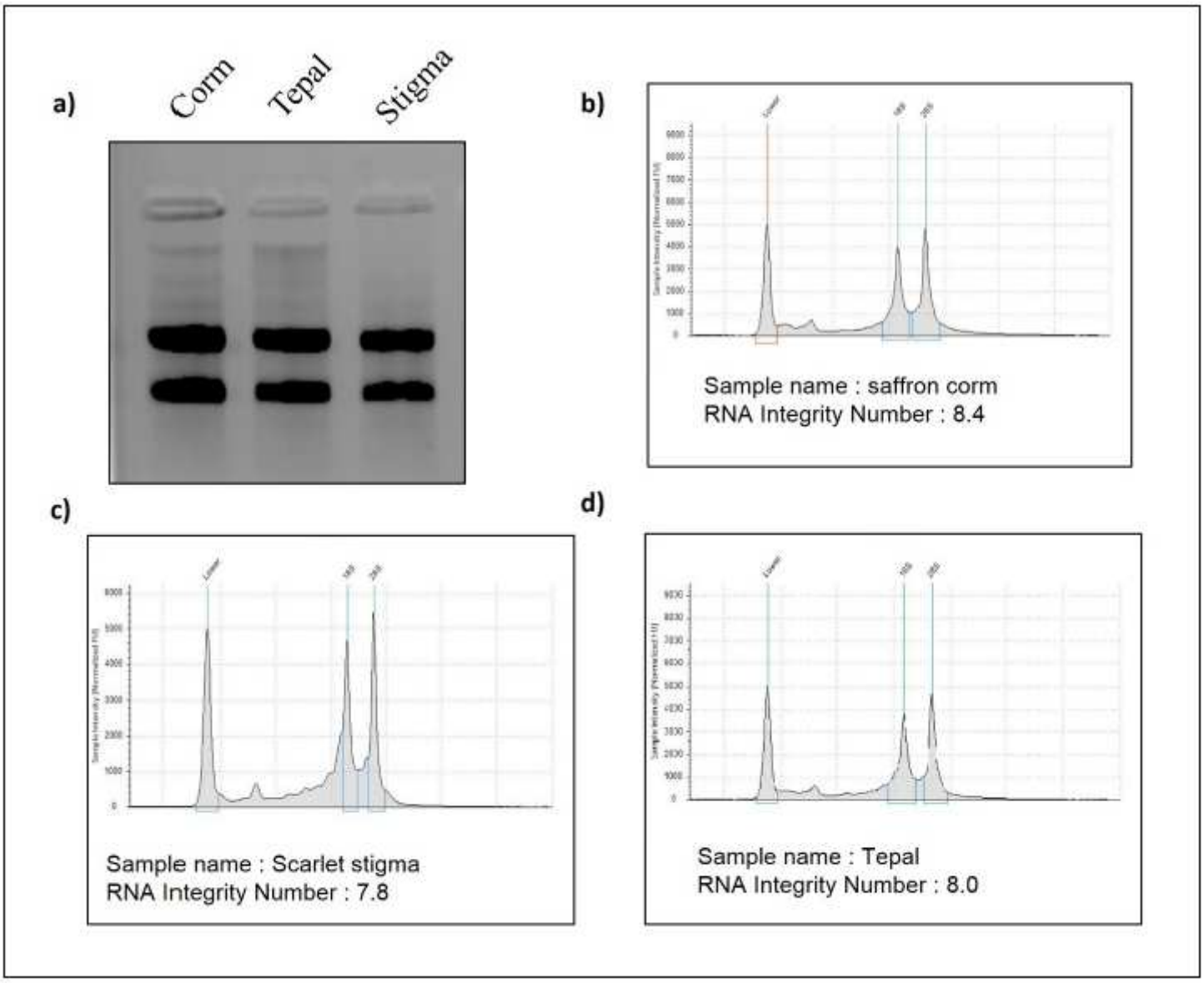

Figure 3

Integrity of RNA isolated through our modified protocol (a) 1.5% agarose gel depicting 28s and 18s RNA bands. Full length gels are provided in supplementary file 2(figure S2). (b-d) RIN value of RNA isolated from corm Tepal and Stigma respectively.

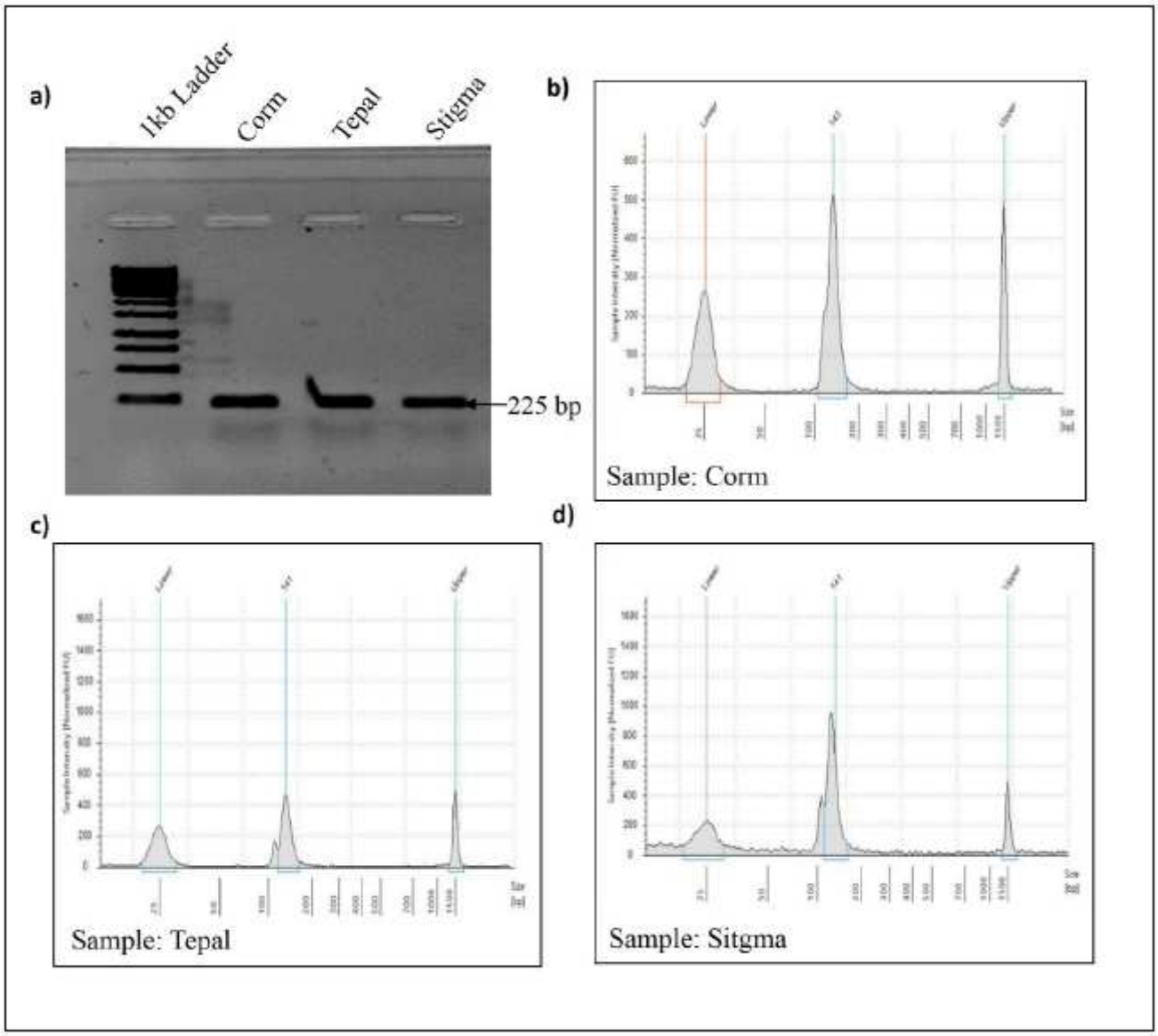

Figure 4

PCR analysis and Small RNA preparation from RNA isolated by modified protocol (a) PCR amplification of 225 bp fragment of tubulin (b-d) quality of small RNA library prepared from RNA isolated from corm, tepal and stigma. Full length gels are provided in supplementary file 2 (figure S3).

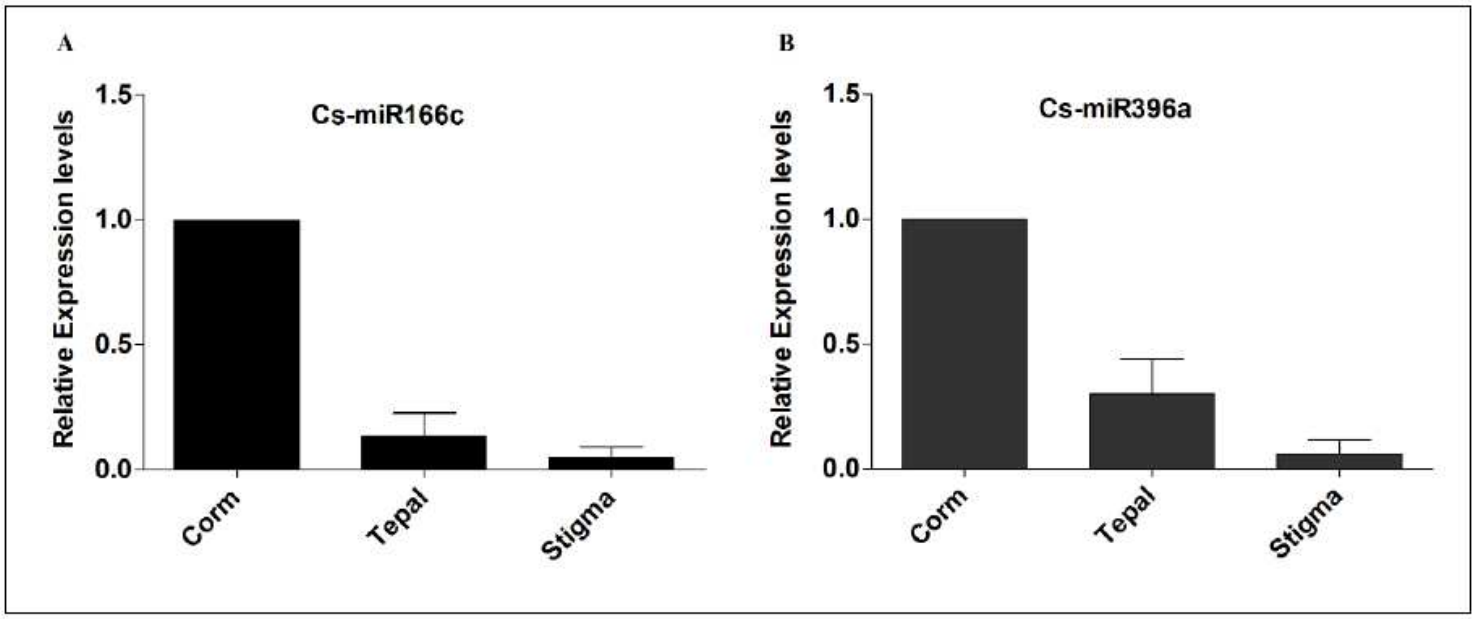

Figure 5

qPCR analysis of small RNA in different tissue samples of Crocus sativus (a) Realtive expression analysis of Cs-miR166c (b) Relative expression analysis of Cs-miR396a isolated corm, Tepal and Stigma respectively

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.pdf
- Supplementaryfile2fulllengthgels.pdf