The International Journal of Plant Reproductive Biology 10(2) July, 2018, pp.178-183
DOI 10.14787/ijprb.2018 10.2.

Standardization and validation of a modified method for RNA isolation from anther, pistil and developing seed of *Brassica rapa*

Saurabh Anand, Mukund Lal and *Sandip Das

Department of Botany, University of Delhi, Delhi 110 007, India

*sandipdas04@gmail.com; sdas@botany.du.ac.in;

Received: 10.04.2018; Revised: 02.05.2018

ABSTRACT

Studies on molecular aspects of reproductive organ development in *Brassica rapa*, member of one of the largest familyies Brassicaceae, has largely been neglected in spite of being an important vegetable crop. A major cause for this negligence maybe because of technical challenges related to identification of proper developmental stages of reproductive parts, lack of simple and reproducible method of RNA extraction methodologies from small amount of tissue, and use of this RNA in downstream applications (e.g. semi-qRT-PCR, NGS technologies etc.). To overcome these challenges, we first identified pre-meiotic and post-meiotic stages of pollens and correlated these to anther development stages. This would allow identification and collection of appropriate stage of anther under field condition for further analysis, and will permit maintaining uniformity in selecting the stages. We successfully modified and formulated simple RNA extraction protocols for anther, pistil and storage-cum-RNA extraction buffer for developing seeds. The modified protocols allowed extraction and purification of high quality DNA-free RNA with increased yield when compared to previously reported protocols. Finally, we identified *MYB21*, *FAE1* and *ACTIN* genes from *Brassica rapa* and tested the suitability of the RNA for downstream application by cDNA synthesis and performing semi-quantitative RT-PCR (semi-qRT-PCR) based expression analysis. The methods reported in the present investigation will enable researchers to understand molecular basis of reproductive organ development in various species of *Brassica*.

Key words : *Brassica*, anther, pistil, seeds, RNA extraction

INTRODUCTION

Identification and characterization of genes and associated genetic elements involved in development of reproductive organs such as anther, pistil, and seed are critical. Insights gained from identification and molecular characterization of genetic elements involved in development of reproductive organs can unravel novelties in reproductive biology, and are necessary in crop plants towards improving yield.

Brassica species, member of Brassicaceae, are an economically important group of plants because of their high value as vegetable and oil seeds (Rakow *et al.* 2004). Among the six economically important species of *Brassica* and part of the famous U's triangle (U N 1935), *Brassica rapa*, a meso-polyploid, is a genetic diploid with AA genome, and is a genome donor of the allo-tetraploids *Brassica juncea* (AABB) and *Brassica napus* (AACC) (Wang *et al.* 2011). All three, viz. *B. rapa*, *B. juncea*, and *B. napus* are highly valued as oilseed crops world over (Rakow *et al.* 2004).

Strategies towards yield improvement of oilseed *Brassica* species rely on generating hybrid seeds through cytoplasmic male sterile (cms) lines (Yamagishi *et al.* 2014). In spite of the efforts of the *Brassica* breeders, India still is a net importer of rapeseed and mustard oil implying a huge gap in production and demand (from Dept. of Agriculture Cooperation and farmers welfare; Ministry of Agriculture and farmers welfare; Govt. of India, May 2017). It is therefore important to identify and understand the role of various genes involved in floral organ development such as anther and pistil, and that of fruit and seeds in *Brassica* species in order to devise strategies towards yield of seed, and seed oil.

Often, a major challenge towards molecular analysis is the limitation imposed by availability of high quality RNA obtained in a cost effective manner, and a detailed RNA isolation protocols. Some earlier studies have performed transcriptome analysis in *Brassica napus* from anthers (Shen and Hsu 1992), from leaves of different *Brassica* species such as *Brassica napus*, *Brassica nigra*, *Brassica oleracea*, *Brassica carinata*, *Brassica juncea*, *Brassica nigra* (Chen *et. al.* 1997), floral buds of *Brassica rapa* (Braynen *et al.* 2017), seedlings in *Brassica oleracea* (Jeon *et al*. 2018), and mature pods of *Brassica napus* (Xu *et al.* 2015). However, such studies rely extensively on commercially available expensive molecular biology kits for RNA extraction and / or have analyzed mature reproductive stages or vegetative stages.

In *Brassica* species, studies on floral organ development has suffered on account of problems associated with RNA extraction from anther, pistil and immature seed, and some of these problems are :

a. Identification of developmental stages, and collection of sufficient amount of anther and pistil tissues from immature floral buds.
b. Field collection of samples sufficient for RNA extraction from such small tissues such as anthers and pistilsat pre-meiotic stages and quick freezing in liquid nitrogen is often challenging, time-consuming and leads to loss of valuable biological material.

c. Storage for long term usage-*Brassica* being a winter crop, tissue availability is limited to a brief time-window of only six-weeks duration of vigorous flowering.

Floral organs can be categorized as four distinct parts: androecium, gynoecium, sepals and petals. Deeper understanding of organ-specific molecular network requires studies that are based on the analyzing transcript or RNA population in an organ specific manner. To the best of our knowledge, stage-wise study of RNA extraction in *Brassica rapa* reproductive parts has been previously carried out only in mature and entire floral buds, composed of all the four organs listed above, by Braynen *et al.* (2017). This may be on account of the fact that no method exists as yet where high quality RNA extraction from small amount of tissue (<50 mg) is possible in an organ-specific manner.

The present study was therefore undertaken in *Brassica rapa*, a mesopolyploid species, to identify developmental stages based on pollen meiosis. To address these problems, we identified pre-meiotic and post-meiotic stages of pollen grains; and correlated the stages with anther length. We further separated anther, pistil and immature seeds from flowers of different stages to standardize RNA extraction from anther, pistil, and developing seeds. We also identified a few candidate gene sequences from *Brassica rapa* genome and tested the efficacy of our protocol and demonstrate the applicability of the procedure through cDNA synthesis of transcript of two candidate genes, *MYB21* (anther and pistil) and *FAE1* (seeds). Protocol generated here could successfully be employed on all the developing stages of anther, pistil and developing seeds.

MATERIAL AND METHODS

Plant material—Plants of *Brassica rapa* cv. Ragini were grown in the fields of University of Delhi facility at Bawana, New Delhi during October-March of 2016-17 and 2017-18. Anthers and pistils of different stages were excised from flower with the help of forceps sterilized with rectified spirit.

Randomly sampled anthers (one each) from at least 10 flowers of *Brassica rapa* were viewed and photographed using a Carl-Zeiss Stereozoom microscope (Stemi 305). Length of the anther from the base of anther to the tip was measured using a standard scale bar.

Anthers and pistils of appropriate stages were collected and pooled from 15 flowers (90 anthers) or 40 flowers (40 pistils) each (ca. 30mg of anthers, and pistil each) in 200 µl of Trizol™ (Invitrogen) in 1.5 ml of micro-centrifuge tube (MCT) kept on ice, and then flash frozen in liquid nitrogen.

Developing seeds from two stages, one day after pollination (stage 1), and just before dehiscence (stage 2) were collected with the help of sterile forceps by first slitting open the pods and harvesting the tissue in modified CTAB buffer (2%w/v CTAB reagent prepared in DEPC water; 50mM Tris-HCL buffer pH 8.0, 0.2M LiCl, 25mM EDTA pH=8.0; containing 1%PVP and 1% beta-mercaptoethanol added just before use). Seeds (ca. 50 mg) were harvested and stored in the modified CTAB buffer prior to RNA extraction using the same storage buffer.

Samples were stored in -80 °C freezer up to 6 months without any loss in RNA quality or yield.

Identification of meiotic stages through DAPI staining—Pollen meiosis was checked using DAPI (4',6-diamidino-2-phenylindole; Thermo Fischer Scientific cat no. D1306). Anthers were fixed in acetic acid: ethanol (1:3) in field and pollens were released on a glass slide by teasing with needles. The pollens were stained with DAPI (0.1 mg/ml; prepared in sterile MilliQ water) and kept in dark for 10 minutes and slides were then viewed under fluorescence microscope (Zeiss A1-Axioscope) and photographed. Appropriate pre-meiotic stages were then selected for RNA extraction of both anther and pistil.

RNA Extraction method—DEPC water (diethyl pyrocarbonate; 1% v/v) was used to create RNase free environment by soaking and rinsing all glassware followed by baking at 180°C for 4 hours. All buffers and solutions were prepared in DEPC treated water in sterilized glassware (Green and Sambrook, 2012). All plasticwares such as micro-pestle, measuring cylinders were soaked in DEPC-water and dried under laminar flow hood to make the area RNase free Plastic tips and MCTs (micro-centrifuge tubes) were autoclaved prior to use.

Samples in MCTs were taken out of -80 °C freezer prior to RNA extraction.

Protocol for extracting nuclease free RNA from anther and pistil—Samples of anther and pistils, stored in MCTs in 200 µl Trizol, were crushed with the help of a sterile micro-pestle in the tubes itself without transferring them to a fresh tube; these were then allowed to stand at room temperature (25°C) for 5 mins. Subsequently, 50 µl of chloroform was added to the homogenized tissues and the tubes were vigorously shaken till the solution turns milky white**.** The tubes were centrifuged at 12000 rpm for 15 minutes at 4ºC. After centrifugation, the pellet was discarded and the supernatant was transferred to a fresh, sterile MCT. RNA was precipitated using ice-cold isopropanol (as per the method prescribed for Trizol™ based isolation) or modified by adding equal volume of cold isopropanol: 4M LiCl (4:1) to the supernatant and was mixed by gently inverting the tubes. This mixture can be left for overnight at -20◦C, or for 1hr on ice. For overnight stored samples, keep it on ice for 5 min and then proceed. After the incubation, the tube was centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was discarded and wash the pellet with 70% ethanol prepared in DEPC water at 4°C and air-dried the pellet for 30 mins inside laminar airflow hood; finally, the pellet was re-suspended in 30 µl DEPC water.

Protocol for nuclease-free RNA extraction from immature seeds—Seed samples were stored in 200 µl CTAB buffer

(2%w/v CTAB reagent prepared in DEPC water; 50mM Tris-HCL buffer pH 8.0, 0.2M LiCl, 25mM EDTA pH=8.0; containing 1%PVP and 1% beta-mercaptoethanol added just before use) and ground to a fine paste with the help of sterile micro-pestle. This paste was incubated in a water bath at 65◦C for 15 min. During incubation, the tubes were gently inverted for uniform mixing at regular intervals after every 2-3 mins. Equal volume of choloroform:isoamyl alcohol (24:1; 200 µl) was added to the homogenized tissue and thoroughly mixed. The micro-centrifuge tube was then centrifuged at 12000 rpm for 15 minutes at 4°C, and the supernatant was carefully transferred to a fresh micro-centrifuge tube avoiding the cellular and tissue debris. To the supernatant, equal volume of phenol:choloroform:isoamyl alcohol(25:24:1) was added, and the contents of the tube were mixed by gentle inversion. The tube was centrifuged at 12000 rpm for 15 minutes at 4◦C, and the supernatant was carefully transferred to a fresh micro-centrifuge tube. Equal volume of 5M LiCl (200µl) was added to the supernatant and this tube was be left for overnight at 4◦C or for 1hr on ice. Further, the tube was centrifuged at 16000g for 30min at 4◦C.The pellet was then washed with 75% ethanol prepared in DEPC water, without dislodging from the tube by centrifugation at 7500 rpm for 5 minutes at 4°C. The supernatant was discarded, pellet was air-dried for 30 min inside a laminar air-flow hood, and then re-suspended in 30 µl DEPC treated water.

RNA quantification and DNase treatment :

RNA quantification—The yield and purity of RNA was checked using a nanodrop (NanoVue Plus™; GE Healthcare Life Sciences) after DNase treatment. For RNA quantification, 1µl of RNA was loaded onto the NanoVue™ reader. Absorbance ratio was measured at 260/ 280 nm and recorded.

DNase treatment was given by adding 5 µl 10x DNase buffer to 1 µg of total RNA, 1 unit of DNase enzyme (NEB), and nuclease free water (upto 50 µl). The reaction was incubated at 37◦C for 30 mins followed by heat inactivation of DNase at 80°C for 10 mins**.** Yield of Dnase treated RNA was then estimated, and used for cDNA synthesis, or was stored at -80◦C for later use.

First strand synthesis and complementary DNA (cDNA) synthesis was performed using iScript cDNA synthesis Kit (BIO-RAD; catalogue no. 1708891). For first strand synthesis, 1µg of RNA was taken as template to which 4µl 5x iScript reaction mix, 1 µl Script reverse transcriptaseand nuclease free water was added up to a final reaction volume of 20 µl. Reaction mixture was then incubated at 25◦C for 5 m, followed by at 42°C for 30 m, 85◦C for 5 m and final hold at 4°C for 10 m.

Second strand (cDNA) synthesis and amplification of target genes—2 µl of first strand product out of a total of 20 µl was taken as template in a 0.2ml thin-walled PCR tube. To the template, 1µl each of forward and reverse primers (from a stock of 10 µM; see table 1 for primer sequence) for *MYB21*, or *Fatty Acid Elongase 1* (*FAE1*) or β-actin (as internal control) was added, along with 0.4 µl of dNTP mix (from a stock of 10mMdNTP mix), and 0.2 µl Taq DNA polymerase (3U/µl); Sterile MilliQ water was added to a final volume of 20 µl. Second strand synthesis was performed using the PCR cycle condition : initial denaturation for 94°C for 3 minutes, then 28 cycle at 94°C for 30 sec, annealing temperature for 45 sec, 72°C for 1 min and final extension of 72°C for 5 min; and hold at 4°C (optional).

RESULTS AND DISCUSSION

Floral buds representing the entire spectrum contained in an inflorescence were divided and labeled as stage 1 (smallest) to stage 14 (before anthesis). We first identified the pre-meiotic and immediate post-meiotic stages in pollen grains through DAPI staining of pollen grains harvested from stage 3 and stage 4, such that the meiotic stages can be correlated to the phenotype of anther under laboratory condition. Cytological analysis of developing microspores from at least 10 anthers collected from different flowers treated as biological replicates (one anther from each flower) revealed that anthers that are approximately 501.18± 2.04 µm in length correspond to pre-meiotic stage of microspores in the anthers; whereas anthers that are at least 712.18±1.48 µm long contain pollen grains at post-meiotic stages (Fig. 1; Table 2).Six anthers from one flower of *Brassica rapa* at pre-meiotic stage (500 µm in length) weigh only 2 mg; and one pistil weighs 1 mg, approximately. The corresponding pre-meiotic and post-meiotic stages in *Arabidopsis thaliana* have been identified as stage 7 (bud size of <0.3mm) and stage 8 (bud size of 0.3–0.4mm) respectively (Armstrong and Jones 2002). This staging was followed in all subsequent studies and allowed us to identify and maintain uniformity in selecting the appropriate stages while pooling anthers or the corresponding pistil from different flowers or plants.

We further standardized isolation of high quality, intact RNA from pre-meiotic and post-meiotic stages of pollen grains and the corresponding pistil using a modified extraction method based on Trizol™(Invitrogen) and measured the purity as absorbance ratio of 260/280 nm and calculated the total yield (Fig. 2). Trizol™ based methods recommend use of only isopropanol for RNA precipitation and using the recommended protocol we were able to obtain total RNA ranging from 2.8µg -3.7µg from a total of 30-40 ng tissue (Table 3). In order to increase the yield of RNA, we modified the protocol by replacing isopropanol for precipitation of RNA with a mix of LiCl (4M): Isopropanol in a ratio of 1:4. This modification allowed us to isolate highly pure RNA ranging from 23.7µg - 25µg from 30-40 mg tissue, an almost 10-fold increase in yield (table 3). Distinct bands of 28S rRNA and 18s rRNA, with almost negligible shearing can clearly be seen after DNase treatment (Fig. 2a). Previously, researchers

have published few reports in various plants that documented attempts to extract RNA from anthers of *Brassica napus* (Shen and Hsu 1992), from anthers of flower stage 11 and 12 of *Arabidopsis thaliana* (Cheng *et al.* 2009), or from entire inflorescence that includes all stages of anther, pistils, sepals and petals of *Brassica* species (Zhang *et al.* 2018). LiCl method of RNA precipitation has widely been used and some examples are use of 2M LiCl for RNA precipitation in maize seeds (Rochester *et al.* 1986), 5M LiCl for RNA precipitation in wheat pistils (Manickavelu *et al.* 2007) and a variety of LiCl and isopropanol concentrations have separately been used for different plant parts (Farrell, 2010). In our study, a 4:1(Isoprapanol:4M LiCl) ratio has been found to give higher yield and purity of RNA (Table 3) as compared to isopropanol (Trizol™ method of extraction of RNA) only. Not only the yield and RNA purity were improved by this method, but also small amount of tissues (30 mg) could successfully be used for higher yield of RNA.

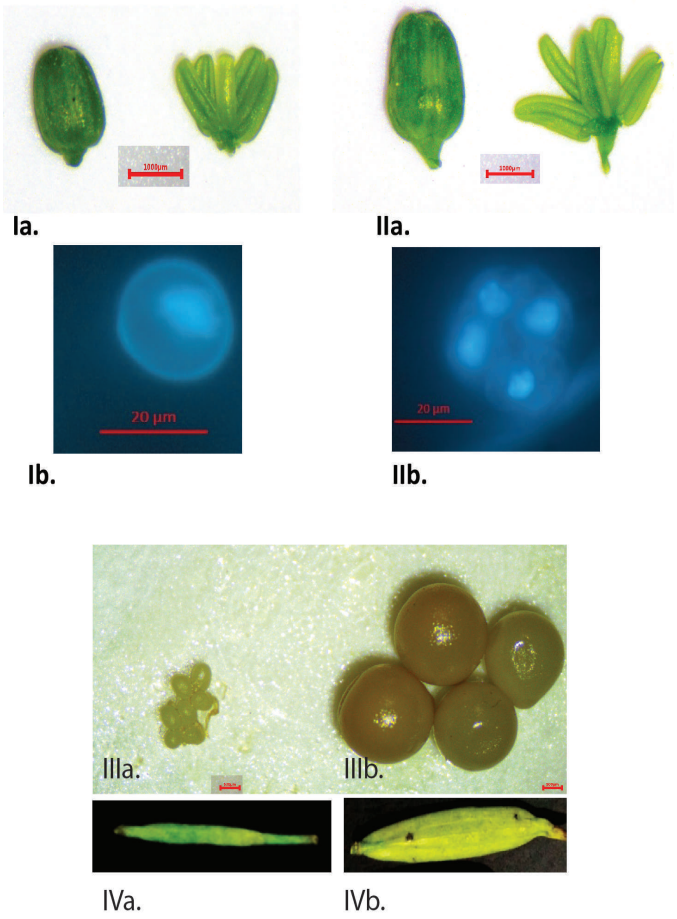

Fig. 1— Stages of floral buds and anthers of *Brassica rapa* CV Ragini used for determining pre-meiotic (Ia) and post-meiotic stages of pollen grains (Ib). Scale bar represents 1000µm. Pre-meiotic (IIa) and post-meiotic (IIb) stage of pollen identified after DAPI staining (scale bar represents 20µm). Developing seeds and silique 1 Day after pollination (IIIa and Iva, respectively; DAP), and 30 DAP (IIIb and IVb, respectively).Scale bar represents 500µm.

RNA extraction from developing seeds of *Brassica* is challenging on account of presence of large amount of fatty acids. In order to standardize RNA extraction, we attempted RNA extraction from immature seeds of *Brassica rapa* based on methods that has been reported earlier for transcriptome studies (Lee *et. al.* 2008). Collecting samples from field grown plants and storing in a proper medium for long term usage for RNA extraction is a limitation for *Brassica rapa* and other species of *Brassica*. RNALater™, a commercially available product that has been successfully used for several other tissues and organs, did not prove useful in our laboratory, as repetitive washing after storing in RNA later caused RNA degradation. Extraction of RNA from immature seeds of *Brassica rapa* using Trizol method that was previously standardized for anther and pistil was also unsuccessful. We observed 260/280 nm ratio between 2.3-2.9 (Table 3) which are outside the recommended ratio of 2.0±0.1 (Farrell 2010) which is indicative of contamination of excess salt, organic solvent, proteins carried over during isolation process. Electrophoresis of RNA on denaturing agarose gel also revealed sheared and degraded RNA (Figure 2b, lanes 1, 3) and unsuccessful attempts in making cDNA (data not shown) led us to devise a modified protocol to store and extract RNA.

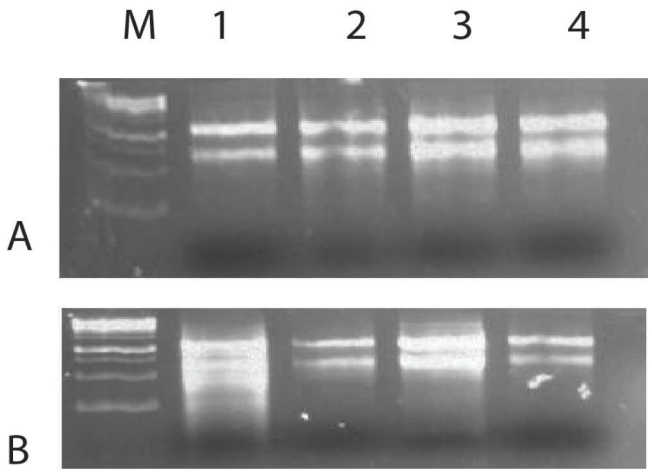

Fig. 2—Evaluation of RNA quality and integrity through agarose gel electrophoresis (2% agarose gel in TBE buffer).

A : 2µl of RNA isolated from anther (A1) with pre-meiotic pollen grains, and pistil at pre-meiotic stage of pollen (A3); anther (A2) with post-meiotic pollen grains, and pistil at post-meiotic stage of pollen (A4) after DNase treatment was electrophoresed.

B : 2µl of RNA isolated from immature seeds using TRIzol (B1-1DAP and B3-30DAP) or modified CTAB buffer (B2-1DAP, and B4-30DAP) after DNase treatment was electrophoresed. Lane M represents 100bp ladder as molecular weight marker

We devised a modified storage-cum-extraction/lysis buffer using a combination of LiCl with CTAB for immature developing seeds (1 DAP and 30 DAP). This was designed keeping in mind that LiCl in homogenization medium can help in extracting RNA in buffer solution within 60 secs (Farrell 2010), which is helpful where secondary metabolites and glucosinolate accumulation hinders isolation of pure RNA. The second modification that we standardized was to use only 5M LiCl instead of a mixture of isopropanol and LiCl for RNA precipitation. These two modifications allowed us to improve the quality of RNA as the 260/280 nm ratio ranged from 1.9-2.03 thus confirming that all the RNAs extracted using the modification is free from contamination (Fig. 2b, lanes 2, 4). The modification also allowed us to increase the yield from only 3.2-3.3 µg to 21-22 µg from 30-40 mg tissue (700-850 ng/µl; table 3). Use of phenol : chloroform : isopropanol is known to improve the efficiency of deproteinization of the sample and facilitating removal of lipids from the homogenate (Farrell 2010).

Previous to our study, NaCl with CTAB has been used in extraction buffer (Chang *et. al.* 1993; Barman *et al.* 2017) and LiCl with SDS (Onate-Snachez et al., 2008). To determine the utility of our modified protocol for further downstream studies, we performed semi-qRT PCR to amplify three candidate genes- *ACTIN* (house-keeping gene), *MYB21* (anther and pistil), and *FAE1* (developing seed) using locus specific primers (table 1). Primers for *ACTIN* were designed spanning the introns to check for genomic DNA contamination, if any. *MYB21* has been reported to be involved in stamen development in *Arabidopsis thaliana* (Cheng *et al*. 2009), and *FAE1*is involved in erucic acid metabolism in developing seeds of *Arabidopsis thaliana* (Mietkiewska *et al*. 2004). Homologs of *MYB21* (Bra025300) and *FAE1* (Bra034635) in *Brassica rapa* were retrieved from Brassica Database (BRAD) using standard methods (Singh et al., 2017). Synthesis of first strand and cDNA was prepared to evaluate the transcript abundance, and RNA quality. Amplification products were checked on 2% agarose gel. We were able to successfully amplify the housekeeping gene *ACTIN* (400bp), the transcription factor *MYB21* (291bp) and an intron-less gene *FAE1* (425bp) (Fig 3A, B and C). Levels of *MYB21* increase in anthers from pre-meiotic to post-meiotic stages, whereas in pistils *MYB21* levels showed a downward trend. A comparison of cDNA levels of *FAE1* revealed that the levels increase in developing seeds from 1 DAP to 30 DAP (Figure 3: B2 and B4). We used genomic DNA as control and comparison of amplicon size between the *ACTIN* cDNA (400 bp) and genomic DNA (550 bp) revealed absence of any genomic DNA contaminants in RNA samples (Figure 3, lane V, lower panel) using intron spanning primers).

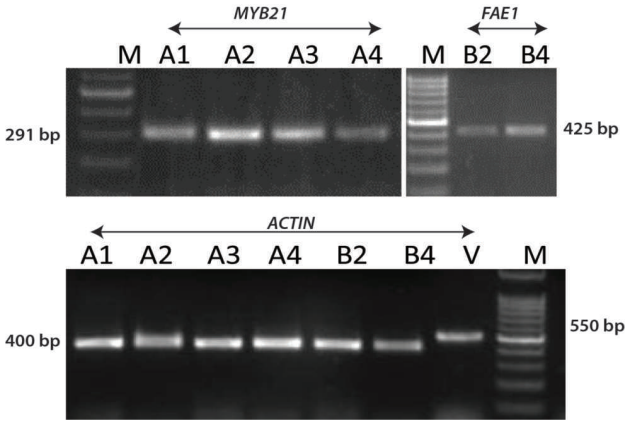

Fig. 3—Expression analysis of MYB21, FAE1 and ACTIN cDNA. MYB21 cDNA synthesized from RNA of anthers at pre-meiotic (A1), post-meiotic (A2) stages, and corresponding pistils (A3, A4) show differential expression. cDNA levels of FAE1 increases between developing seeds at 1 DAP (B2) to 30 DAP (B4). cDNA level of ACTIN was used as internal control (lanes A1-B4, lower panel). Inclusion of genomic DNA as template (lane V) that produces a amplicon of larger size, rules out DNA contamination in RNA samples. Lane M represents 100bp ladder for molecular size estimation.

In conclusion, we, for the first time, report the correlation between the meiotic stages of pollen and the anther phenotype (length) which can be a useful tool under laboratory conditions for easy screening. Previously, protocols reported extraction of RNA from mature stages of anther and pistils, or from entire inflorescence, and not from immature stages such as that reported in the present investigation. This may be because of difficulty in harvesting early stages of floral organ development, non-availability of suitable storage medium, and dependency on expensive commercially available RNA extraction kits. Desired product size for the selected transcripts was obtained from the synthesized cDNA confirming the utility of the modified protocol for isolation and purification of good quality RNA and its use for downstream applications such as qRT-PCR and transcriptome analysis.

Acknowledgements—The study was supported by a DBT grant number BT/PR628/AGR/36/674/2011, and R&D grants from Delhi University to SD. Financial assistance in the form of a DBT project JRF/SRF to SA from DBT and UGC-Non-NET Fellowship to ML is gratefully acknowledged.

REFERENCES

Armstrong SJ and Jones GH 2002. Meiotic cytology and chromosome behaviour in wild–type *Arabidopsis thalina*. *J. Exp. Bot*.**54**(388) 1-10.

Barman P, Choudhary AK and Geeta R 2017. A modified protocol yields high-quality RNA from highly mucilaginous *Dioscorea* tubers. 3 *Biotech*. **7**(2) 150.

Braynen J, Yang Y, Wei F, Cao G, Shi G, Tian B, Zhang X, Jia H, Wei X and Wei Z 2017. Transcriptome Analysis of

Floral Buds Deciphered an Irregular Course of Meiosis in Polyploid *Brassica rapa*. Frontiers in Plant Sci. **8** 768.

Chen ZJ and Pikaard CS 1997. Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in Brassica. *Proc. National Acad. Sci*. **94**(7) 3442-3447.

Cheng H, Song S, Xiao L, Soo HM, Cheng Z, Xie D and Peng J 2009. Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in Arabidopsis. *PLoS genetics*, **5**(3), e1000440.

Farrell RE 2010 *RNA Methodologies*: *A Laboratory Guide for Isolation and Characterization.* Fourth ed., Elsevier Academic Press Inc. USA.

Green MR and Sambrook J 2012. Molecular Cloning: A Laboratory Manual. Fourth ed. Cold Spring Harbor Laboratory Press.

Jeon J, Kim JK, Kim H, Kim YJ, Park YJ, Kim SJ, Kim C and Park SU 2018. Transcriptome analysis and metabolic profiling of green and red kale (Brassica oleracea var. acephala) seedlings. *Food Chemistry* 241 7-13.

Lee SC, Lim MH, Kim JA, Lee SI, Kim JS, Jin M, Kwon SJ, Mun JH, Kim YK, Kim HU and Hur Y 2008. Transcriptome analysis in *Brassica rapa* under the abiotic stresses using Brassica 24K oligo microarray. Molecules & Cells (Springer Science & Business Media BV). **26**(6).

Lee YH, Kim KS, Lee JE, Cha YL, Moon YH, Song YS, Jeong EG, Ahn SJ and Park W 2018. Comprehensive Transcriptome Profiling in Relation to Seed Storage Compounds in Tetralocular Brassica rapa. *J. Plant Growth Regulation*. 1-16.

Manickavelu A, Kambara K, Mishina K and Koba T 2007. An efficient method for purifying high quality RNA from wheat pistils. *Colloids and Surfaces B: Biointerfaces*. **54**(2) 254-258.

Ministry of Agriculture and farmers welfare; Govt. of India; http://agricoop.nic.in/sites/default/files/Edible%20oil%20Profile%2C%20May%2C%202017.pdf

Opate-Sαnchez L, and Vicente-Carbajosa J 2008. DNA-free RNA isolation protocols for Arabidopsis thaliana, including seeds and siliques. *BMC research notes*, **1**(1) 93.

Ramalho, AS, Beck S, Farinha CM, Clarke LA, Heda GD, Steiner B, Sanz J, Gallati S, Amaral MD, Harris A and Tzetis M 2004. Methods for RNA extraction, cDNA preparation and analysis of CFTR transcripts. *J. Cystic Fibrosis* **3** 11-15.

Rakow G 2004. Species origin and economic importance of Brassica. In: *Brassica*. Springer, Berlin, Heidelberg. 3-11.

Rochester DE, Winer JA and Shah DM 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp70. *The EMBO J.* **5**(3) 451-458.

Smith AG, Gasser CS, Budelier KA and Fraley RT 1990. Identificaton and characterization of stamen-and tapetum-specific genes from tomato. *Molecular and General Genetics MGG* **222**(1) 9-16.

Shen JB and Hsu FC 1992. Brassica anther-specific genes: characterization and in situ localization of expression. *Mol. General Gene. MGG.* **234**(3) 379-389.

Twell D, Wing, R, Yamaguchi J and McCormick S 1989. Isolation and expression of an anther-specific gene from tomato. *Mol.General Gene. MGG.* **217**(2-3) 240-245.

Wang CS and Vodkin, LO 1994. Extraction of RNA from tissues containing high levels of procyanidins that bind RNA. *Plant Mol. Biol. Rep.* **12**(2) 132-145.

Wang X, Wang H, Wang J, Sun R, Wu J, Liu S, Bai Y, Mun JH, Bancroft I, Cheng F, Huang S, *et al.*2011. *Brassica rapa* Genome Sequencing Project Consortium 2011. The genome of the mesopolyploid crop species Brassica rapa. *Nat Genet*. **43**(10) 1035-1039.

Wang S, Zhou G, Huang X, Hu J, Wang B, Lin C, Li X, Jia Y and Wang A 2017. Transcriptome analysis of non-heading Chinese cabbage under heat stress by RNA-seq and marker identification. *Euphytica* **213**(5) 109.

Xu HM, Kong XD, Chen F, Huang JX, Lou XY and Zhao JY 2015. Transcriptome analysis of Brassica napus pod using RNA-Seq and identification of lipid-related candidate genes. *BMC genomics.* **16**(1) 858.

Yamagishi H and Bhat SR 2014. Cytoplasmic male sterility in Brassicaceae crops. *Breeding Sci*.**64**(1) 38-47.

Zhang Yanfeng, Huang Shuhua, Wang Xuefang, Liu Jianwei, GuoXupeng, Mu Jianxin, TianJianhua, Wang Xiaofeng (2018) Defective APETALA2 Genes Lead to Sepal Modification in *Brassica* Crops. *Frontiers in Plant Sci.* **9** 367.0 June 2017).