Efficient Isolation of High-quality Total RNA from Strawberry

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Abstract. Sufficient yields of high-quality RNA are needed for next-generation sequencing and high-throughput real-time polymerase chain reaction analyses. In the case of strawberry (*Fragaria × ananassa*) fruits, successful RNA isolation requires removal of abundant inhibitory substances (polysaccharides and polyphenols) that greatly reduce quality and yield. In this study, we applied various combinations of RNA isolation protocols directed at reproductive organs. The best manual isolation method involved nonionic polymer and modified acid guanidinium thiocyanate-phenol-chloroform treatments followed by phenol/chloroform/isoamyl alcohol extraction. Compared with other methods, this approach gave significantly higher yields [84.0 µg/g fresh weight (FW)] of RNA of greater purity (A260/A280 = 1.99; A260/230 = 1.51). Better-quality RNA (A260/230 = 2.11) was obtained using an automated method, but the yield was lower (18.1 µg/g FW) than that obtained manually. This automated method consisted of pretreatment with nonionic polymer followed by a silica-based system extraction. Although RNA of sufficient quality [RNA Integrity Number (RIN) ≥ 6.5 and 28S/18S ≥ 1.0] for RNA sequencing was obtained from receptacles using both automated and manual methods, the manual method yielded high-quality RNA from achenes and anthers. The automatic method features 6-fold faster high-throughput capacity, whereas the manual method has wider applicability to different tissues.

Strawberry is a popular and important crop worldwide, and contains various food compositions like secondary metabolites (Severo et al., 2015). Therefore, it has been selected as a research material for genome

projects in the horticultural field. Following whole-genome sequencing of strawberry species *Fragaria vesca* (Shulaev et al., 2011) and *Fragaria × ananassa* (Hirakawa et al., 2014), post-genome research is now under way. One of the most important post-genome approaches, transcriptome analysis, has been greatly accelerated by RNA sequencing (RNA-seq) with the advent of next-generation sequencing (NGS) technologies. Selection of suitable methods for isolating and purifying RNA is thus dependent on the type of downstream assay(s) that will subsequently be applied (Davis et al., 2006; Nuyts et al., 2001; Wang et al., 2012).

Strawberry fruits contain abundant polysaccharides and polyphenols (Giampieri et al., 2014) that greatly reduce the efficiency of RNA isolation. Several methods have been used to remove these inhibitory substances during isolation of RNA from strawberry, including removal by 2-butoxyethanol (2-

BE; Manning, 1991), proprietary methods of various extraction kits (Bianchi et al., 2011), and the use of alkaline buffer and phenol-chloroform-isoamyl alcohol (Christou et al., 2014). In addition, Mazzara and James (2000) have developed a protocol for extraction of RNA from strawberry leaf petioles. However, the protocol has not been optimized for RNA quality and yield with fruits and anthers. Although these methods are compatible with subsequent reverse-transcription and/or real-time polymerase chain reaction (PCR) applications, their usefulness when followed by NGS like RNA-seq has not been reported. In cases in which RNA-seq has been applied to various strawberry organs (Hollender et al., 2014; Kang et al., 2013; Sánchez-Sevilla et al., 2014, 2017), they use spin column with silica membrane, or differential precipitation with 2-BE; however, extraction protocols have not been optimized for RNA quality and yield. An additional development that needs to be considered is the recent introduction of automated instrumentation that eliminates repetitive and time-consuming manipulations. It is important to evaluate combinations of methods from different companies for developing the best protocol by a disinterested viewpoint. The study described here is the first-reported evaluation of RNA isolation protocols from different strawberry organs for use in NGS and high-throughput analyses.

Materials and Methods

Materials. Receptacle, achene, and anther organs of strawberry (*F. × ananassa*, cultivar ‘Akihime’) were used. Ripe fruits were immediately frozen in liquid nitrogen and achenes were separated from receptacles by using a scalpel. Anthers were removed from open flowers and frozen in liquid nitrogen. The hard frozen strawberry organs wrapped in aluminum foil were roughly crushed into small pieces by a rigid plastic hammer. All pieces were ground with a mortar and pestle (methods A and D in Table 1) or pulverized with a multi-beads shaker (MBS; Yasui Kikai, Osaka, Japan). When using the MBS (methods B, C, E, and F in Table 1), an ≈3-mL aliquot of pieces was put into a 50-mL volume tube with a metal cone and were shaken vigorously at 2000 rpm for 10 s twice. This process was repeated until all aliquot powders were gathered in a storage tube for every fruit. In all steps, the samples and instruments were kept at low temperature by frequent immersing in a liquid nitrogen. The powdered samples were stored at –80 °C until use.

Manual RNA extraction. To extract RNA using Fruit-mate for RNA Purification (Takara Bio, Shiga, Japan), 700 µL Fruit-mate solution (Takara Bio) was added to 50 to 100 mg powdered sample in all methods except B. The mixture was vortexed and then centrifuged at 13,000 g_n for 5 min at 4 °C. An equal volume of Sepasol RNA Super G (Nacalai Tesque, Kyoto, Japan) was added to the supernatant, and the RNA was extracted according to the kit instructions. After incubating the RNA with DNase I (Takara

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Bio) at 37 °C for 30 min, the DNase was inactivated using a heat treatment in method A, or by adding Tris-buffered phenol (pH = 8.0)–chloroform–isoamyl alcohol [25:24:1 (v/v/v)] in other methods (B, C, D, and E) according to the DNase I instruction manual. RNA was then precipitated with ethanol and resuspended in 50 µL RNase-free water. As an additional step in method E, resuspended RNA was added to ethanol and RLT buffer and then purified using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the instructions in the “RNA cleanup” section of the manufacturer’s protocol.

Automated RNA extraction. A Maxwell 16 Automated Purification system (Promega, Madison, WI) was used for automated RNA extraction. As a pretreatment, 50 to 100 mg of powdered sample was mixed with 500 µL Fruit-mate. The mixture was then vortexed, followed by centrifugation at 13,000 g_n for 5 min. To 400 µL supernatant, 200 µL lysis buffer was added to prepare the sample solution for extraction using a Maxwell 16 LEV Plant RNA kit (Promega). RNA extraction procedures, including a DNase treatment, were conducted according to the Maxwell purification system “RNA-PLANT” protocol.

Determination of RNA yield and quality. Absorbance was determined with a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Electropherograms, RINs, and 28S/18S ratios of RNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The resulting RNA samples were stored at –80 °C.

RNA-seq analysis. Total RNA was isolated from receptacle, achene, and other tissues by different methods: the automated method described previously, and manually using an MBS, Fruit-mate, Sepasol, or an RNeasy kit, followed by phenol-chloroform extraction. Each extracted total RNA was submitted to a custom service (BGI Japan, Kobe, Japan) for complementary DNA (cDNA) library preparation and sequencing reactions. Messenger RNA (mRNA) was isolated by magnetic beads with Oligo (dT) and fragmented. cDNA is synthesized from mRNA fragments followed by adenine attachment. After the adapter ligation, fragments were amplified by PCR to make cDNA library. The cDNA libraries were sequenced on the HiSeq4000 platform (Illumina, San Diego, CA) using paired-end chemistry with 100–base pair reads. Bioinformatic analysis was carried out using scientifically recognized algorithms according to Ishibashi et al. (2018). Briefly, sequencing reads were filtered by SOAP-nuke (Chen et al., 2018). Filtered short-sequence reads were mapped to the *F. ×ananassa* reference database; FAN_r1.1 (Hirakawa et al., 2014) by bowtie/BWA (Langmead et al., 2009). The numbers of expressed genes were estimated using RSEM (Li and Dewey, 2011).

cDNA synthesis and real-time PCR. A ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) was used for reverse transcription according to the manufacturer’s protocol. The cDNA was synthesized by reverse-transcribing 100 ng total RNA using both oligo (dT) and random primers. The cDNA solution was stored at –20 °C. In real-time

PCR, a LightCycler Nano (Roche Diagnostics, Basel, Switzerland) was used with THUNDERBIRD SYBR quantitative PCR (qPCR) Mix (Toyobo), based on the SYBR Green I dye intercalation method. The PCR assay contained 10 µM specific primers, 2 µL cDNA solution, and 10 µL THUNDERBIRD SYBR qPCR Mix. Primers were designed for the strawberry allergen genes *Fra a 1.01* (F: CACACCAAGGGAGATGTCG, R: GGGTGGTCCTTGAGGTATCC) and *Fra a 1.02* (F: ACACCAAAGGTGACGTGGA, R: ATTAGGATTGGCCAAGAGGTAG), and for the elongation factor 1 α gene (*EF1 α*) as the control referred to by Amil-Ruiz et al. (2013) (F: TGGATTTGAGGGTGACAAACATGA, R: GTATACATCCTGAAGTGGTAGACG GAGG). The thermal cycling conditions were as follows; 95 °C for 30 s, three-step amplification with 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, pre-melt hold at 95 °C for 10 s, and melting at 60 to 97 °C at 0.1 °C/s. All data were normalized against each *EF1 α* transcript level.

Statistical analyses. Statistical analyses were performed using JMP 13 (SAS Institute, Inc., Cary, NC). The data are means $\pm$ standard errors of four biological replicates, and significant differences were determined by Student’s *t* test or Tukey–Kramer’s honestly significant difference test.

Availability of data and materials. The RNA-seq reads supporting the conclusions of this article are available in the DDBJ (<http://ddbj.nig.ac.jp/BPSearch/>) or NCBI

Table 1. Comparison of quality and yield of RNA extracted from strawberry fruits using various protocols.

Method	MBS ^z	FM ^y	Sep ^x	PCI ^w	RN ^v	MW ^u	A260/A280		A260/A230		Concn (ng/µL)		Yield ^t (µg/g FW)	
A	–	+	+	–	–	–	1.52 $\pm$ 0.05	c	0.63 $\pm$ 0.03	c	61.0 $\pm$ 13.4	b	30.5 $\pm$ 6.7	b
B	+	–	+	+	–	–	1.47 $\pm$ 0.03	c	0.73 $\pm$ 0.08	c	53.8 $\pm$ 12.3	b	26.9 $\pm$ 6.2	b
C	+	+	+	+	–	–	1.99 $\pm$ 0.03	a b	1.51 $\pm$ 0.10	b	167.9 $\pm$ 42.6	a	84.0 $\pm$ 21.3	a
D	–	+	+	+	–	–	1.92 $\pm$ 0.02	b	1.42 $\pm$ 0.08	b	113.3 $\pm$ 22.1	a b	56.6 $\pm$ 11.1	a b
E	+	+	+	+	+	–	2.08 $\pm$ 0.03	a b	1.31 $\pm$ 0.05	b	80.0 $\pm$ 11.4	a b	24.0 $\pm$ 3.4	b
F	+	+	–	–	–	+	2.17 $\pm$ 0.09	a	2.11 $\pm$ 0.09	a	36.2 $\pm$ 6.1	b	18.1 $\pm$ 3.0	b

^zMulti-Beads Shocker (Yasui Kikai).

^yFruit-mate for RNA Purification (Takara Bio).

^xSepasol RNA Super G (Nacalai Tesque).

^wPhenol–chloroform–isoamyl alcohol extraction.

^vRNeasy Plant Mini kit (Qiagen).

^uMaxwell 16 Automated Purification system (Promega).

^tYield [µg/g fresh weight (FW)] were calculated from concentration and total volume of RNA solution, and FW of the original frozen powder; (concentration; ng/µL) $\times$ (volume; µL) $\times$ 10^{–3}/(FW; g).

Different letters following values indicate significant differences ($P < 0.05$; Tukey–Kramer’s honestly significant difference test, $n = 4$).

Table 2. Quality and yield of RNA obtained from different strawberry organs using manual and automated methods.

Organ	A260/A280 ^z	A260/A230 ^z	Concn (ng/µL) ^z	Yield (µg/g FW) ^z	RIN ^y	28S/18S ^y	Clean reads ^x	Genome map rate (%) ^x	Expressed genes ^x
Manual									
Receptacle	2.06	1.60	257.2	128.6	10.0	1.6	– ^w	–	–
Achene	2.14	2.06	1024.9	614.9	8.1	1.8	77277796	64.70	68421
Anther	2.03	1.86	443.2	265.9	8.9	1.7	83108866	63.54	58779
Automated									
Receptacle	2.23	2.11	31.9	16.0	7.4	1.6	97008546	59.72	70689
Achene	2.08	2.09	94.9	94.9	2.1	0.0	–	–	–
Anther	2.09	2.13	107.8	107.8	3.8	0.3	–	–	–

^zAbsorbance at 230, 260, and 280 nm was measured using a Nanodrop1000 spectrophotometer. Absorbance values were used to calculate purity, concentration, and yield.

^yRNA integrity numbers were determined using an Agilent 2100 Bioanalyzer (see also Fig. 1).

^xNumber of clean reads, percentage of mapped reads, and number of expressed genes were based on Illumina HiSeq4000 next-generation sequencing. Clean reads were mapped to the *F. ×ananassa* reference database; FAN_r1.1 (Hirakawa et al., 2014).

^wA dash entry indicates sample was not tested.

Results and Discussion

Establishment of a manual protocol combining various steps for efficient RNA isolation. We investigated a series of manual RNA isolation methods based on different chemical kits. The various combinations, given as method types A to E, are shown in Table 1.

Before extraction, samples were pulverized using either an MBS or a mortar and pestle. Although RNA purity and yield were not significantly increased by using the MBS grinding method (Table 1, methods C and D), this technique was faster and improved reproducibility by increasing particle size uniformity. Sepasol was used as the first extraction step. Sepasol is a commercial reagent containing thiocyanate compounds

based on the AGPC method (Chomczynski and Sacchi, 1987), as well as TRIzol (Thermo Fisher Scientific) or TRI Reagent (Sigma-Aldrich, St. Louis, MO). Although these reagents are slightly different in components, they can be used compatibly. These thiocyanate compounds denature proteins including RNases, and the addition of phenol-chloroform partitions RNA into the aqueous phase and DNA and protein into the phenol-chloroform phase. The simplest method, B, resulted in the lowest quality and yield values. In this case, a red color corresponding to incomplete removal of polyphenols was visible after RNA precipitation. Gehrig et al. (2000) noted that polysaccharides can coprecipitate with RNA in low ionic-strength buffers. This problem can be addressed using Fruit-mate, which contains a nonionic polymer that binds polysaccharides and polyphenols. As shown in Table 1, method C using Fruit-mate was significantly better than

method B, according to our RNA evaluation criteria. After treatment with DNase I, the enzyme was inactivated by heating (Table 1, method A) or phenol-chloroform extraction (Table 1, methods B–E). The purity of RNA obtained using method D was significantly better than that obtained using method A, suggesting that phenol-chloroform is efficient not only for inactivation of DNase I but also for elimination of polyphenols. This finding is consistent with a report that phenol binds to phenolic compounds (Rayani and Nayeri, 2015). An RNeasy Plant Mini spin column was used to further purify RNA. This type of column, which contains a silica-gel-based membrane, is based on the Boom method. The Boom method exploits the binding of nucleic acids to silica in the presence of chaotropic salts, such as guanidinium ions (Boom et al., 1990). The RNeasy column was not very effective for purifying strawberry RNA and significantly decreased

Fig. 1. Electropherograms of RNA isolated from different organs of strawberry. RNA was extracted from receptacles (upper) and achenes (middle) of ripe fruits and anthers (lower) of flowers in full bloom using manual (left) or automated (right) methods. The manual method included Fruit-mate and Sepasol treatments followed by phenol–chloroform–isoamyl alcohol extraction, and the automated method consisted of Fruit-mate pretreatment followed by application of the Promega Maxwell system (see also Table 2). Electropherograms were generated on an Agilent 2100 Bioanalyzer. The x- and y- axes represent nucleotides (nt) and relative fluorescence units (FU), respectively.

yields (Table 1, method E in contrast to method C). We presume that complexes of RNA and impurities, such as polysaccharides, clogged the column or passed through the column before the elution step. According to the instruction manual, normal lysis buffer (Buffer RLT) contains guanidine thiocyanate and coagulates RNA and secondary metabolites depending on plant tissues. Another lysis buffer containing guanidine hydrochloride (Buffer RLC), which is recommended in case of the sample solidification, had no effect on RNA quality and yield (data not shown).

The combination of Fruit-mate, Sepasol, and phenol-chloroform extraction (Table 1, method C) was accordingly the best of tested manual protocols for isolation of RNA from strawberry fruits. This protocol was highly applicable to young leaves in both quality and quantity (831.4 µg/g FW, A260/A280 = 1.89, A260/A230 = 1.85). Compared with the protocol described by Christou et al. (2014), method C resulted in higher yields and superior quality of RNA (23.7 µg/g FW, A260/A280 = 1.78, and A260/A230 = 1.04 in the former). The method C gave almost the same yields and the lower value of A260/A230 in comparison with the procedure reported by Manning (1991) (82.9 µg/g FW, A260/A280 = not determined, and A260/A230 = 2.07). Because Manning's (1991) protocol has many steps, including overnight precipitation with LiCl, the limited steps for

removal of polysaccharides by precipitating with 2-BE is worth trying to incorporate with method C.

Comparison of RNA yield and quality between manual and automated methods and among different organs. The automated method exploited a Maxwell 16 automated purification system. This system, which can purify 16 RNA samples in ≈1 h, is based on the Boom method and uses silica-coated paramagnetic particles. This automated method (Table 1, method F) produced the highest-purity RNA (A260/A230 = 2.11), but had the lowest extraction efficiency among all tested methods. Although we could not determine the cause of the low efficiency, we suspect that the use of the Boom method in the automated protocol may have led to insufficient binding of RNA to silica by contamination with polysaccharides. Such contamination was observed using manual method E, which was also based on the Boom method.

Several RNA samples were prepared from fruit receptacles for further analyses of RIN, 28S/18S ratio, and electropherogram patterns on an Agilent 2100 Bioanalyzer. These parameters indicate the degree of RNA degradation (Schroeder et al., 2006). RINs are evaluated as 10 phases (low number represents more degraded). Other RNA samples were prepared from achene and anther tissues using manual and automated methods. Ex-

traction from receptacles using either manual or automated methods yielded RNA of sufficient quality for NGS (RIN ≥ 6.5 and 28S/18S ≥ 1.0; Table 2). When the electropherograms of RNA samples prepared using manual and automated methods were compared, the RNA obtained using the automated method was somewhat degraded, but its quality and quantity were sufficient for library preparation (Fig. 1). In contrast, RNA isolated from achenes and anthers by the automated method was of insufficient quality for NGS (RIN < 3.8 and 28S/18S < 0.3; Table 2), with many peaks representing impurities and RNA degradation visible on electropherograms (Fig. 1). RNA extracted from achenes and anthers using the manual method had higher RIN and 28S/18S values indicating its suitability for use in NGS. Our results indicate that the selection of the most appropriate RNA isolation method depends on the plant organ used as an RNA source.

Application of RNA isolation methods to downstream assays. To validate the quality of RNA for downstream assays, RNA samples were used in real-time PCR and NGS analyses. Target gene was *Fra a 1*, encoding major allergenic protein in *F. xananassa* because we have already established the evaluation system by real-time PCR (Ishibashi et al., 2018). In both *Fra a 1.01* and *Fra a 1.02* genes, no statistically significant difference was found in relative transcript levels between RNAs isolated by manual and automated methods (Fig. 2). For both genes, a single peak was observed from each sample, with no amplification of multiple homologs (Fig. 3). These results indicate that the tested manual and automated methods of RNA isolation are equally suitable for detecting *Fra a 1* genes in real-time PCR. Accordingly, automated methods are more advantageous for high-throughput expression analyses. For use in high-throughput analyses, 16 samples could be pretreated and processed in 1.5 h by the automated method, compared with 6 samples manually in 3.5 h. The time required of the automated method is six times faster than that of the manual.

Three RNA samples were submitted to a commercial RNA-seq service. After filtering of raw RNA-seq reads, the resulting clean reads were aligned to the reference genome

Fig. 2. Analyses of gene transcript levels of *Fra a 1* in strawberry by real-time polymerase chain reaction. RNA was extracted from receptacles of ripe fruits using manual or automated methods (see legend to Table 1). Relative transcript levels of *Fra a 1.01* (A) and *Fra a 1.02* (B) normalized against that of the elongation factor 1α gene (*EF1α*). Significant differences were not detected between RNA isolation methods as indicated by n.s. ($P < 0.05$; Student's *t* test, $n = 4$).

Fig. 3. Melting peak analyses of *Fra a 1.01* (A) and *Fra a 1.02* (B) conducted after polymerase chain reaction amplifications. RNA was extracted from receptacles of ripe fruits using manual (gray line) or automated (black solid line) methods (see also Fig. 2).

(*F. ×ananassa*, FAN_r1.1; Hirakawa et al., 2014). All samples generated more than 77 million clean reads (>99% of raw reads), comparable to the 30 million clean reads (97% of raw reads) of a previous study (Sánchez-Sevilla et al., 2014). Genome mapping rates ranged from 60% to 65%, which was slightly lower than previously reported values of 68% (*F. ×ananassa* to the *F. vesca* v1.1 reference genome; Sánchez-Sevilla et al., 2014) and 69% (*F. vesca* to the *F. vesca* v1.0 reference genome; Hollender et al., 2014). In contrast, the number of expressed genes, which ranged from 58,799 to 70,689, was higher than the number reported in the two earlier studies (33,458 in Sánchez-Sevilla et al., 2014; and 24,161 in Hollender et al., 2014). The output data, which are summarized in Table 2, confirmed that all submitted RNA samples were of sufficient quality and quantity for RNA-seq analyses.

We have optimized the protocol of RNA isolation from strawberry organs for the practical detection of genes. The advantage of the automated method is its high-throughput capacity, whereas the manual method is applicable to a broader array of plant organs. Both methods produced RNA of sufficient quality and yield for RNA-seq and real-time PCR analyses.

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