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Technical Note

Simple and efficient RNA extraction and gene analysis in vegetative organs of Japanese persimmon

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Abstract This examination for simple and efficient RNA extraction methods was conducted in vegetative organs of persimmon, including leaves, which contain inhibitory substances. We applied the result to physiological genetic analysis. Among the four extraction kits and a traditional SDS+PVP method, the combination of FastPure and Fruit-mate was the most suitable, resulting in maximum yield of 3.96 μg with high quality from 20 mg of persimmon mature leaves. Extraction was also possible from several organs other than leaves. The standard curve of quantitative-PCR (Q-PCR) using extracted RNA presented a high correlation coefficient (r^2=0.99). Using this method, the activity of photosynthesis, which is an important role of leaves, was analyzed using *DkrbcL* as a target. The seasonal expression pattern of *DkrbcL* mRNA in leaves which was clearly demonstrated, indicates that this RNA extraction method is very important for the genetic analysis of persimmon vegetative organs.

Key words: *Diospyros kaki*, genetic analysis, RNA extraction.

Recent progress in biotechnology has encouraged various studies to enhance the productivity and usability of food crops of many kinds. In such studies that encompass physiology, analysis at the gene level is indispensable. Especially, RNA is a basic material of the genetic research of plants. Persimmon [*Diospyros kaki* Thunb.], the subject of this study, has also been subjected to physiological investigation at the RNA level. Some studies have examined persimmons' sweetness and astringency and fruit functionality. Many reports have described detailed analyses via RNA extraction from the fruit (Nakano et al. 2003; Ikegami et al. 2005; Zheng et al. 2006; Ikegami et al. 2007; Nakagawa et al. 2008). By contrast, no report describes the conduct of molecular genetic analyses of vegetative organs, including leaves, for elucidation of its physiological mechanisms. This lack of experimental data is considered to be attributable to the difficulty in RNA extraction procedures because substances such as polyphenol and polysaccharides (Duan et al. 2004; Levi et al. 1992; Kameda et al. 1987), which prevent RNA extraction exist in abundance in tissues, especially mature leaves, of persimmon.

Only one report to date, that by Koshita et al. (2002), has described RNA extraction from leaf organs of persimmon. However, that procedure is troublesome: it requires many iterations of centrifugation, which require a long time (Levi et al. 1992). Several reports have described RNA extraction methods to remove polyphenols and polysaccharides, which have also been used in the cases described above. Notwithstanding, these methods are extremely complex, require much work, and are not feasible for high-throughput studies.

For this study, we examined methods comparatively to establish a simple and efficient total RNA extraction that is available for numerous samples. We applied the results for the first time to genetic analysis for physiological studies of vegetative organs (mature leaves) of persimmon.

The cultivar used was 'Maekawa Jiro'. Leaves of Maekawa Jiro located in the Grape and Persimmon Research Station, National Institute of Fruit Tree Science (Hiroshima, Japan) were sampled in October 2008 when maturation had been completed. At that time, RNA was extracted using four kits and one method: RNeasy (RLT buf.), RNeasy (RLC buf.), FastPure, FastPure+Fruit-mate, and SDS+PVP. For kits experiments, samples

Abbreviations: *Dk*, *Diospyros kaki*; PVP, polyvinylpyrrolidone; *rbcL*, ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit; SDS, sodium dodecyl sulfate.

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were crushed by putting 5–50 mg of each sample and a zirconia ball (YTZ-5; Tosoh Corp. Japan) into a 2.0 ml safe-lock tube. Then they were frozen using liquid nitrogen, with subsequent crushing using a mill (MM400 Mixer Mill; Retsch GmbH & Co. KG). Experiments were conducted in triplicate for each method. The RNA extracted using each method was eluted with 50 μl of RNase-free water; 2 μl was used for determination of yield using the NanoDrop 1000 (ThermoFisher Scientific, Corp, Wilmington, USA). SDS+PVP method was conducted according to Levi et al. (1992).

Comparison of five extraction methods showed that FastPure+Fruit-mate was able to extract the largest amount of total RNA of 2.38 μg 40 mg^{-1} fresh weight on average, which is as good or better than that of SDS+PVP method (Levi et al. 1992; Koshita et al. 2002). On the other hand, RNeasy (RLT), RNeasy (RLC) and FastPure were able to recover RNA only slightly, with respective yields of 0.45 μg, 0.38 μg, and 0.34 μg 40 mg^{-1} fresh weight (Table 1). Extracted RNA of all five types were subjected to MCE-202 Multi NA (Shimadzu Biotech Corp, Japan) microchip electrophoresis, yielding two clear bands, 25S and 18S, only for SDS+PVP method and FastPure+Fruit-mate; no band was observable for the other methods (Figure 1). The nanodrop 1000 spectrum showed a distinct curve with a peak at 260 nm. The net time required for extraction was about 40 min. Therefore, it was considered that FastPure+Fruit-mate was the most suitable for the rapid extraction of RNA of high quality from persimmon leaves.

Next, the extraction system used in the study was examined for the optimum sample amount. Results show that only low yields of less than 1.1 μg were obtainable from 5 mg or 10 mg, although the mean yield of 3.96 μg was obtained reliably from 20 mg. In addition, the mean yield of 2.3 μg or greater was obtained reliably from samples that were 30 mg or larger (data not shown). Based on these results, the sample amount of 20 mg was considered to be optimal.

Extraction from multiple organs indicated that RNAs with a certain level of quality (absorbance ratios: 260 nm/280 nm and 260 nm/230 nm) were extracted from several organs. The yield was the highest for axillary buds (5.72 μg 20 mg^{-1} fresh weight), and lower for fruit skin and fruit pulp (0.59 μg and 0.50 μg 20 mg^{-1} fresh weight, respectively). The absorbance ratios were comparatively low for calyces and fruit pulp, specifically 1.67 : 0.46 and 1.58 : 0.31 (260 nm/280 nm : 260 nm/230 nm) respectively, although the values were equivalent or greater than 1.8:1.0 for the other organs (Table 2). The standard curve was prepared from total RNA extracted from fruit skin using its amount and the Ct value, providing a coefficient of determination as high as 0.99 (Figure 2).

Additionally, to verify the effectiveness of this method, analysis was performed of the seasonal change of a photosynthesis-associated gene that was closely associated to the productivity of persimmon. Leaves of Maekawa Jiro were sampled from a tree located in Fukuoka Agricultural Research Center (Fukuoka, Japan) during April–October. Total RNA was extracted using the FastPure+Fruit-mate method to conduct real-time PCR using *DkrbcL* as a target. Results showed that the expression amount increased rapidly after May, when a certain amount of leaf development was secured, transited at a high level from July–September, and decreased in abscission after September (Figure 3).

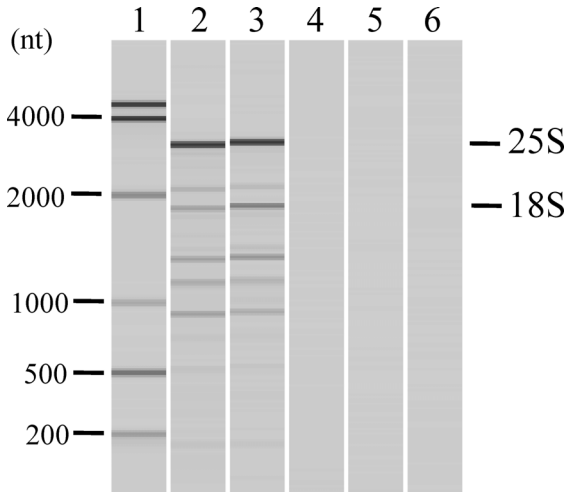

Figure 1. MultiNA image of RNA isolated from persimmon leaf tissues. Lane 1, RNA marker; Lane 2, SDS+PVP; Lane 3, FastPure RNA+Fruit-mate; Lane 4, FastPure RNA; Lane 5, RNeasy RLT Buf; Lane 6, Rneasy RLC Buf. 1.5 ul of RNA solution was set at each lane.

Table 1. Comparisons of the amount of total RNA from leaf samples of persimmon using different extraction methods.

Method	Amount of extracted total RNA (μg 40 mg^{-1} fresh weight)[Z]	Absorbance ratio (260 nm/280 nm)	Absorbance ratio (260 nm/230 nm)
SDS+PVP	2.01 ± 0.71	2.01	2.00
RNeasy (RLT buf.)	0.45 ± 0.28	1.68	0.18
RNeasy (RLT buf.)	0.38 ± 0.13	1.64	0.31
FastPure	0.34 ± 0.08	1.12	0.23
FastPure+Fruit-mate	2.38 ± 0.25	2.10	1.27

[Z] Data are means ± SE or means (n=3).

Table 2. Yields of total RNA from various persimmon organs.

Organ	Amount of extracted total RNA (μg 20 mg^{-1})	Absorbance ratio (260 nm/280 nm)	Absorbance ratio (260 nm/230 nm)
Leaf	2.52	2.15	1.44
Calyx	3.03	1.67	0.46
Fruit skin	0.59	1.83	1.36
Fruit pulp	0.50	1.58	0.31
Axillary bud	5.72	2.11	1.61
Root	3.32	2.18	1.58
Branch	1.04	2.15	0.98

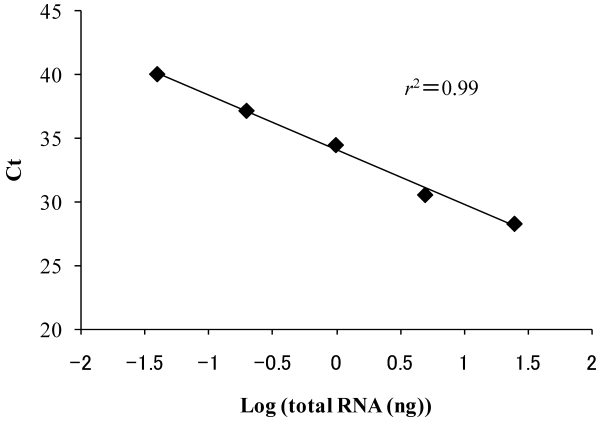

Figure 2. Q-PCR Standard curve generated from total RNA amount and Ct value targeting *Actin* gene in fruit skin. The PCR was performed on the real-time PCR system utilizing the SYBR method. The standard assay was performed on purified total RNA in a series of ten-fold dilutions over the range of 0.0025 ng to 25 ng. (*slope*=−4.31, r^2=0.99)

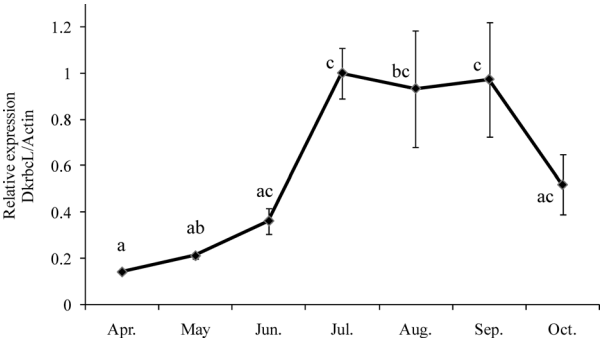

Figure 3. *DkrbcL* mRNA expression of persimmon leaves measured using Q-PCR during April to October (Apr.–Oct.) in 2008. Bars indicate SE (n=3). Means with different letters are significant at 1% level as determined by Tukey's multiple comparisons procedure for seven months experiments.

These characteristics of the time course of *DkrbcL* expression agreed with characteristics of the seasonal change of the photosynthesis rate obtained using a photosynthesis measurement instrument (Hino et al. 1974). *DkrbcL* is presumed to code a key enzyme that determines the rate of photosynthesis reaction in persimmon. Therefore, this identity suggests that total RNA from vegetative organs extracted by FastPure+Fruit-mate is reliable and can be applicable to Q-PCR analysis.

Results of this study demonstrated that the RNA extraction protocol used by FastPure+Fruit-mate using conditions established in this study is extremely useful for samples from which it had been difficult to extract RNA. We clarified the change in the photosynthetic activity in the tree body through actual analysis of *rbcL* gene expression in leaves using this method. This is the first report of the gene level analysis of physiology in the vegetative organs of persimmon. For future study, further advances in using this method are expected in the molecular genetics of plant physiology of the entire persimmon tree in addition to that of persimmon fruits.

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