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PROTOCOL NOTE

Applications in Plant Sciences

An efficient and effective RNA extraction protocol for ferns

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Abstract

Premise: The extraction of high-quality RNA is the critical first step for the analysis of gene expression and gene space. This remains particularly challenging in plants, and especially in ferns, where the disruption of the cell wall and separation of organic compounds from nucleic acids is not trivial.

Methods: We developed a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol that consistently performs well across a large phylogenetic breadth of ferns—a lineage of plants high in secondary compounds—and in an array of tissue types. Two alternative options (precipitation vs. clean-up without intermediate precipitation) are presented, both of which yield high-quality RNA extracts with optical density (OD) ratios of OD 260/280 = 1.9–2.1 and OD 260/230 > 1.6, and RNA integrity numbers >7.

Conclusions: This study presents an efficient protocol for the extraction of high-quality RNA from multiple tissues and across the fern phylogeny, a clade of plants that still lags behind other major lineages in the development of genomic resources. We hope that this method can be used to help facilitate the closing of this gap.

KEYWORDS

cetyltrimetylammonium bromide (CTAB), ferns, RNA extraction, secondary compounds

The isolation of nucleic acids from plants remains a challenging task, especially in lineages with high levels of secondary metabolites. During the lysis of plant cells, these compounds (e.g., polyphenols; Katterman and Shattuck, 1983) can contaminate and remain in the DNA/RNA extract, and have profound impacts on our ability to extract high-quality nucleic acids as they can irreversibly bind to DNA/RNA or inhibit downstream reactions involved in the preparation of sequencing libraries and other molecular biological methods (Fang et al., 1992; Pandey et al., 1996). Therefore, it is necessary to produce clean, contaminant-free extractions for these applications, particularly for high-throughput sequencing library preparation.

RNA, in particular, presents significant challenges as it degrades rapidly and is subject to the activity of ubiquitous and durable RNases. Taken together with the sturdy cell wall and high abundance of secondary compounds, RNA extractions in plants can be complicated and frustrating. While many commercial kits are available, they rely on lysis buffers that are often insufficient to lyse tissue from plants and simultaneously sequester unwanted organics. One common solution is to employ cetyltrimethylammonium bromide (CTAB), a strong detergent that works particularly well in difficult plant tissues (Doyle and Doyle, 1987) as it facilitates the separation of polysaccharides and nucleic acids in high-salt conditions (1.4 M NaCl) based on their different solubilities in the presence of the detergent (Heikrujam et al., 2020). Incorporating polyvinylpolypyrrolidone (PVPP) into the CTAB-based lysis buffer further works to sequester polyphenols and prevent them from binding with nucleic acids (Laborde et al., 2006). While traditional CTAB-based extractions rely on hazardous chemicals such as chloroform, the use of commercial kits generally does not consistently produce high-quality extracts usable for next-generation sequencing technologies.

Many ferns are rich in secondary compounds (Vetter, 2018; Castrejón-Varela et al., 2022) and have proven to be particularly difficult taxa for efficient nucleic acid extractions

(Dempster et al., 1999; Pelosi et al., personal observations and conversations with many researchers). Many of these secondary metabolites are either constitutively produced in plant tissues (e.g., flavonoids) or induced (e.g., polyphenols) as herbivore defenses (War et al., 2012), and they are ubiquitous across the fern phylogeny (Castrejón-Varela et al., 2022). Despite recent advances in the production of genomic resources for ferns (e.g., Li et al., 2018; Qi et al., 2018), tools and resource development for this clade still lag behind most other major plant lineages (Szövényi et al., 2021). We hope that this protocol will help researchers with the extraction of high-quality RNA from various tissue types across ferns and ultimately broaden our understanding of gene space and gene expression in this clade.

METHODS AND RESULTS

RNA extraction and purification protocol

Fresh, young tissues are ideal for RNA extractions as these are the most RNA-rich and lowest in secondary metabolites (Moreira and Oliveira, 2011), although purification from older tissues is possible. All equipment (e.g., mortar, pestles, spatulas) was bleached for 24–48 h and subsequently autoclaved and baked at 200°C for >8 h to sterilize and denature exogenous RNases. Both field-collected and lab-grown materials were extracted using our protocol, and tissues were either preserved by flash-freezing in liquid nitrogen or stored in RNApreserve (BIONOVAS Biotechnology Co., Toronto, Ontario, Canada). A column-based clean-up is recommended if tissues are preserved in RNA-stabilizing solutions such as RNApreserve and RNA*later* (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For flash-frozen tissues, we either ground the tissues and immediately proceeded to extraction or stored whole tissue at −80°C until use. We found that pre-grinding material and keeping it frozen can lead to lower RNA quality as quinones, produced by the oxidation of polyphenols, can still bind to nucleic acids at low temperatures. Approximately 50–100 mg of frozen material was ground in a pre-cooled mortar to a fine powder, free of non-ground chunks or lumps. For smaller tissue amounts (<40 mg) such as gametophytes, we disrupted these tissues by placing them in a screw-cap tube with 1.5-mm high-impact zirconium molecular-biology-grade beads (Benchmark Scientific, Sayreville, New Jersey, USA), flash-freezing, and pulverizing with a Bead Blaster 24 (Benchmark Scientific) for 30 s, then resubmerging in liquid nitrogen. This step was repeated until the tissue was a fine powder (5–7 bead-blasting steps). Alternatively, gametophytes can be ground using a sterile blue pestle in a 1.5-mL RNase-free tube. The mechanical disruption of tissue is critical; under-grinding tissue leads to lower RNA quality and yield. The powder was then transferred using a spatula to a 2.0-mL RNase-free tube that had been pre-cooled in liquid nitrogen and left in liquid nitrogen until the lysis buffer was added.

The lysis buffer contains a high-concentration CTAB buffer prepared with diethyl pyrocarbonate (DEPC)-treated double-distilled H_2O (0.1 M Tris-Cl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 30 mg/mL CTAB), to which 15 mM beta-mercaptoethanol (5 µL/mL) and 4% PVPP (4 mg/mL) were added just before use and heated to 55°C. To each sample, 1 mL of the lysis buffer was added and vortexed to ensure all the powder was suspended. The sample was centrifuged at 8000 × *g* for 5 min at 4°C to remove tissue debris, and the liquid was transferred to a new tube without disturbing the pellet, followed by an incubation at 55°C for 10 min with intermittent inversions. Two half-volume chloroform extractions (0.5 mL 24:1 chloroform:isoamyl alcohol followed by 0.5 mL >99% chloroform) were used to separate organic compounds from the nucleic acids. For some taxa, using >99% chloroform for both extraction steps improved the quality of the extraction.

The aqueous phase was (A) precipitated with an equal volume of chilled isopropanol, redissolved, and cleaned with the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or treated with TURBO DNase (2 U/µL, Thermo Fisher Scientific) just prior to library construction or (B) immediately purified with either the Spectrum Total Plant RNA Kit (Millipore Sigma, Darmstadt, Germany; purification with this kit starts with binding the RNA in the aqueous solution to the column following the manufacturer's protocol, see Appendix 1) or the Zymo RNA Clean and Concentrator-25 kit (Zymo Research, Irvine, California, USA). Both options included a DNase treatment with either the Qiagen or Zymo on-column DNase sets or TURBO DNase. After the final wash in each of the column-based protocols, the collection tube was discarded and the column was applied to a new collection tube for an additional drying step by centrifuging for 1 min at 10,000 × *g*. We found this necessary to avoid the carryover of wash buffer components including ethanol in the elution of the RNA. In some cases (such as with gametophyte extractions), a second elution into a new 2.0-mL tube may yield improved RNA quality. To compare our method to the commercially available kits, we extracted RNA from four fern taxa using the most widely used kit, Qiagen's RNeasy Plant Mini Kit, following the manufacturer's protocol and our method.

Quality and quantity assessment

RNA quality is typically assessed using optical density (OD) ratios (at 260/280 nm and 260/230 nm), calculated using a spectrophotometer (e.g., NanoDrop [Thermo Fisher Scientific]), and from the results of denaturing gel electrophoresis. Generally, the optimal OD 260/280 is 1.9–2.1, the optimal OD 260/230 is greater than 2.0, and the RNA integrity numbers (RIN) as measured with automated gel electrophoresis (e.g., TapeStation [Agilent, Santa Clara, California, USA]) are greater than 7. Sequencing cores require high-quality RNA (e.g., OD 260/230 > 1.6, RIN > 7; University of Florida ICBR Gene Expression and Genotyping Core Facility, RRID:SCR_019145) for construction of high-throughput sequencing libraries. The quality of extracted RNA was

assessed using a NanoDrop, TapeStation 2100, and/or Agilent 5400 Fragment Analyzer. We calculated the quantity of each extraction using the Qubit 2.0 fluorometer (Thermo Fisher Scientific) with the broad-range RNA assay using 1 μL of each elution or by NanoDrop.

Nearly all our extractions have OD 260/280 around 2.0, indicative of pure RNA purifications (mean OD 260/280 = 2.08 ± 0.11 [mean ± SD]; Tables 1–3). The OD 260/230 ratios were, on average, 1.66 ± 0.53, which is consistent with other methods (e.g., mean of 1.63 in Johnson et al., 2012) and consistently greater than the most widely used commercial kit (Table 1). This ratio varied with the protocol used and was lowest for option A using the Qiagen clean-up step (mean OD 260/230 = 1.33 ± 0.67), compared to option A using TURBO DNase (1.99 ± 0.42; measured prior to DNase treatment), option B using Sigma clean-up (mean OD 260/230 = 1.82 ± 0.50), or option B using Zymo clean-up (mean OD 260/230 = 1.58 ± 0.44). We found that the Qiagen columns yielded the least consistent results (OD 260/230 range = 0.14–2.22), with the lower ratios likely due to carry-over of guanidinium salts in the elution from the column. When we compared the same tissue type for taxa across each protocol, we found that the commercial kit consistently underperformed, with either negligible/low yield or non-pure RNA extracts (Table 1). Option A with a clean-up using the Qiagen columns resulted in higher-quality RNA extractions, although we found that the OD 260/230 ratios were highly variable (Table 1). Option B with clean-up protocols using the Sigma and Zymo kits had the highest OD 260/230 ratios and yields (Table 1). The ratios also varied greatly between tissue types: the mean OD 260/230 ratios were 1.73 for foliar tissue, 1.41 for mature fertile leaves, 2.03 for developing leaves, 0.98 for rhizomes, 1.06 for rachises, 1.76 for roots, and 1.38 for gametophytes. The variation in these values likely correlates to differences of metabolite constitutions of these organs.

The sample RINs varied with tissue type, largely due to differences in the occurrence of organellar rRNAs, which can lead to artificially lower RINs (Figure 1). The mean RIN for our extractions was 6.84 (±1.05). Mature leaf tissue (mean RIN = 6.73) and gametophytes (mean RIN = 5.53) had the lowest RINs and also displayed the largest peaks corresponding to organellar rRNAs (Figure 1). The highest RINs were in tissues lacking chloroplasts, such as roots (mean RIN = 8.56), and those that were still developing, such as fiddleheads (mean RIN = 8.70) and unfurling leaflets (mean RIN = 7.00).

The average RNA yield from the column-based clean-up protocols (option A with Qiagen clean-up and option B; 6.16 μg) is lower than the TURBO DNase treatment in option A (45.38 μg). These values, however, reflect different amounts of input material, developmental stages, and tissue types. Notably, the TURBO DNase treatment was performed after taking NanoDrop readings, which likely led to an overestimation of RNA yield for these samples. We also show that our method can be applied to small amounts of tissue (as few as five gametophytes, or <30 mg of starting material) with adequate yield for standard sequencing library preparation.

The quality of metrics from the extractions produced using this protocol exceeds the standards set by the 1000 Plant Transcriptomes Initiative (One Thousand Plant Transcriptomes Initiative, 2019), i.e., an RIN > 5 and total RNA > 30 μg. It should be noted that most library preparation protocols only require 100–400 ng of total RNA for standard next-generation sequencing library construction, and low-input libraries require as little as 10 pg of input material (University of Florida ICBR Gene Expression and Genotyping Core Facility). Our extracts also exceed the optical density metric standards from Johnson et al. (2012): OD 260/280 > 1.9, OD 260/230 > 1.5. We show that this protocol consistently performs well for a variety of tissues and across the fern phylogeny, generating high-quality, sequencing-ready RNA. Although we provide several options for the purification of RNA following tissue lysis in the CTAB buffer, we recommend using option B, which includes an on-column DNase step and washes without precipitation, and yields consistently high-quality, pure RNA. By bypassing RNA precipitation, the user avoids loss of product, which leads to lower yields; moreover, while the

TABLE 1 RNA quality and quantity metrics of sample extractions from sterile leaf tissue using a commercial kit (Qiagen RNeasy Plant Mini Kit) and our modified CTAB protocol (options A and B) with DNase treatment and clean-up.

	Qiagen RNeasy Plant Mini Kit			Option A with Qiagen clean-up			Option B with Sigma clean-up			Option B with Zymo clean-up		
Species	OD 260/280	OD 260/230	Quantity (μg)	OD 260/280	OD 260/230	Quantity (μg)	OD 260/280	OD 260/230	Quantity (μg)	OD 260/280	OD 260/230	Quantity (μg)
Lygodium microphyllum	2.29	0.52	3.12	2.04	1.95	2.35	2.15	1.94	2.16	2.14	2.19	31.2
Thelypteris palustris	1.38	0.64	<0.03	2.13	1.18	4.48	—	—	—	2.03	2.05	10.02
Dryopteris cristata	1.06	0.02	<0.03	2.13	1.99	4.15	2.12	2.01	5.33	2.09	1.54	2.03
Pleopeltis michauxiana	12.39	0.12	0.65	2.11	1.01	1.84	—	—	—	2.06	1.72	3.12

Note: OD = optical density ratios at 260/280 nm and 260/230 nm.

TABLE 2 RNA quality and quantity metrics for several fern species across the phylogeny. An asterisk (*) denotes that the quantity of RNA was determined prior to DNase treatment. Note that extractions had varying quantities of starting material; values can be found in Appendix S1.

Species	Order/Family	Protocol	Clean-up/DNase protocol	OD 260/280	OD 260/230	RIN	Quantity (μg)
Sceptridium formosanum	Ophioglossales/Ophioglossaceae	A	TURBO	2.12	2.19	—	9.49*
Sahashia stricta	Ophioglossales/Ophioglossaceae	B	Sigma	2.1	2.36	—	16.20
Ptisana robusta	Marattiales/Marattiaceae	B	Sigma	2.1	2.19	—	11.31
Didymoglossum tahitense	Hymenophyllales/ Hymenophyllaceae	A	TURBO	2.01	1.63	—	1.00*
Schizaea dichotoma	Schizaeales/Schizaeaceae	A	TURBO	2.08	2.03	—	4.85*
Lygodium microphyllum	Schizaeales/Lygodiaceae	B	Zymo	2.14	2.19	7.7	2.13
Lygodium japonicum	Schizaeales/Lygodiaceae	A	TURBO	2.04	2.26	—	38.55*
Adiantum caudatum	Polypodiales/Pteridaceae	A	TURBO	2.02	2.15	—	69.16*
Hymenasplenium murakami-hatanakae	Polypodiales/Aspleniaceae	A	TURBO	2.02	2.08	—	6.34*
Thelypteris palustris	Polypodiales/Thelypteridaceae	B	Zymo	2.03	2.05	—	10.02
Nephrolepis 'bostoniensis'	Polypodiales/Nephrolepidaceae	A	TURBO	1.96	2.31	—	24.12*
Lomariopsis boninensis	Polypodiales/Lomariopsidaceae	B	Sigma	2.13	2.14	—	4.94
Dryopteris ludoviciana	Polypodiales/Dryopteridaceae	B	Zymo	2.11	2.2	7.8	1.52
Dryopteris cristata	Polypodiales/Dryopteridaceae	A	Qiagen	2.12	2.01	7.6	5.33
Polybotrya sp.	Polypodiales/Dryopteridaceae	A	TURBO	2.03	1.81	7.8	24.79*
Tectaria devexa	Polypodiales/Tectariaceae	A	TURBO	2.07	1.79	—	8.67*
Goniophlebium formosanum	Polypodiales/Polypodiaceae	A	TURBO	2.01	2.28	—	55.01*
Microgramma sp.	Polypodiales/Polypodiaceae	A	TURBO	2.03	1.81	7.7	24.79*

Note: OD = optical density ratios at 260/280 nm and 260/230 nm; RIN = RNA integrity number.

TABLE 3 RNA quality, quantity, and sequencing metrics from several different tissue types of *Lygodium microphyllum*.

Tissue	OD 260/280	OD 260/230	RIN	Quantity (μg)	Number of read pairs	% Reads > Q30
Sterile pinna	2.14	2.19	7.7	2.13	26,149,022	94.10
Fertile pinna	2.07	1.67	5.7	5.60	23,643,650	93.31
Unfurling leaf	2.13	2.18	7.2	10.44	26,228,047	93.14
Fiddlehead	2.07	2.1	8.7	0.89	25,359,777	94.06
Rhizome	2.05	0.98	8.2	0.49	25,782,304	93.72
Rachis	2.02	1.4	7.2	1.69	29,852,850	92.32
Roots	2.07	1.73	8.6	2.02	24,100,378	93.98
Gametophyte	2.15	1.74	6.4	0.83	24,575,038	94.42

Note: OD = optical density ratios at 260/280 nm and 260/230 nm; RIN = RNA integrity number.

total amount of nucleic acid extracted was greatest with option A, these measurements were taken prior to DNase treatment. Furthermore, an on-column DNase treatment is preferred, as the DNase protein complex does not elute with the RNA, whereas in option A the inactivated DNase enzyme remains in the RNA solution and may impact downstream applications.

Sequencing

RNA from *Dryopteris* spp. and *Lygodium microphyllum* (Cav.) R. Br. was used for library preparation and sequencing by Novogene (Sacramento, California, USA) on an Illumina NovaSeq 6000 to ca. 25 million 150 paired-end reads per sample, as part of ongoing studies on these systems. The read

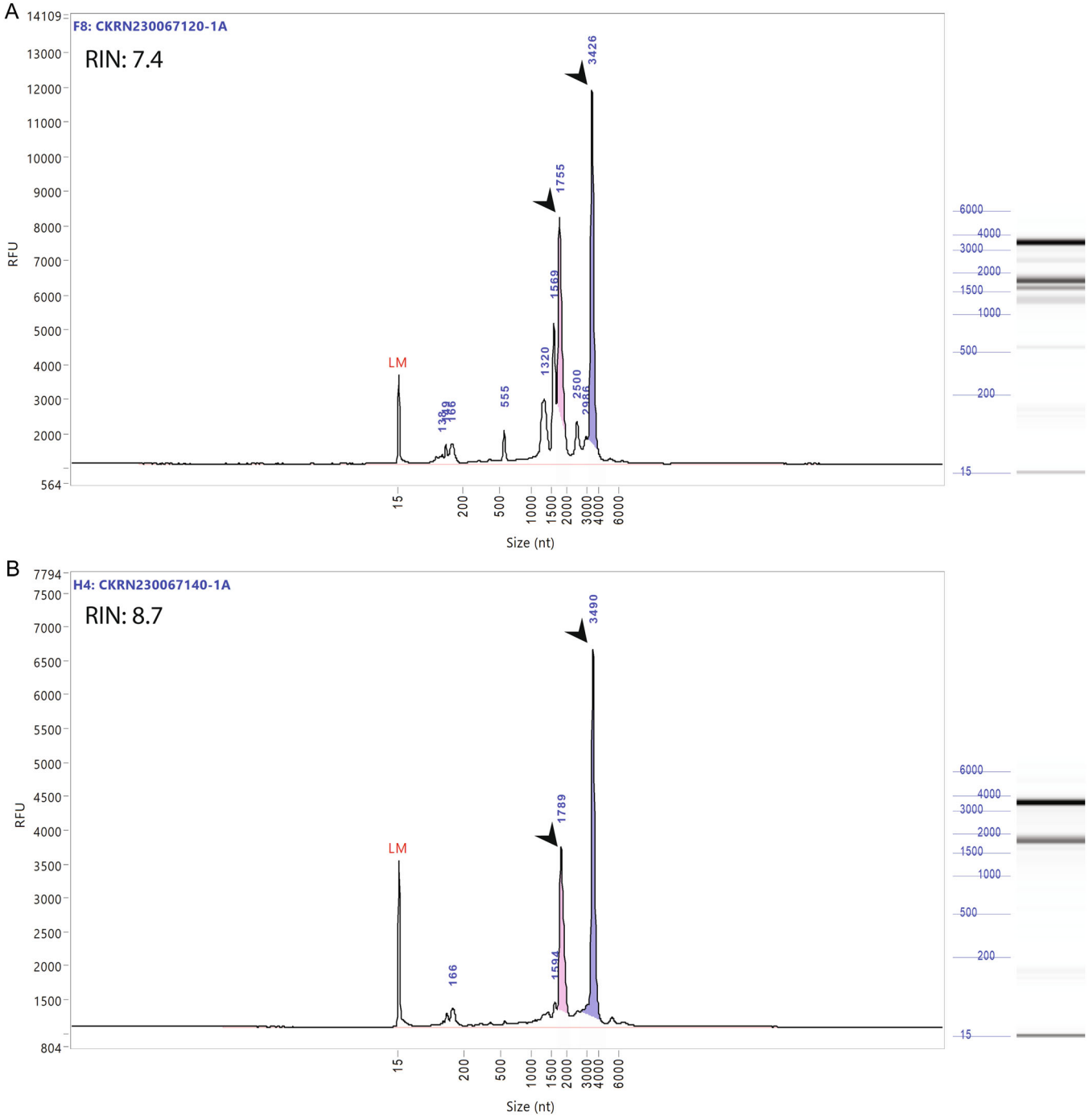

FIGURE 1 Integrity and size of RNA extracted from mature sterile leaflets (A) and roots (B) of *Lygodium microphyllum* on the Agilent 5400 Fragment Analyzer. rRNAs are largely intact in both samples, no degradation is shown in the lower fragment sizes, and no genomic DNA contamination is present. Major nuclear rRNA peaks (18S around 1.8 kbp and 25S around 3.4 kbp) are identified with black arrows; additional peaks in the samples are representative of organellar rRNAs, which can lead to artificially decreased RIN values. LM, lower marker; nt, nucleotides; RFU, relative fluorescence; RIN, RNA integrity number.

data are highly accurate, with more than 90% of reads passing the Q30 filter for all samples sequenced (range: 93.71–94.52%; Table 3). Sequences from these libraries will be published with their respective studies. For the *L. microphylllum* data, we assessed the quality of reads from all tissue types using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), removed adapter and low-quality sequences with Trimmomatic v0.39 (Bolger et al., 2014), and assembled a preliminary transcriptome with Trinity v2.15.1 (Grabherr et al., 2011). We used BUSCO v5.3.0 (Manni et al., 2021) with the viridiplantae_odb10 database to determine the completeness of gene space in this assembly. Using this pipeline, we identified

99.1% complete benchmarking universal single-copy orthologs (BUSCO) sequences in the assembly, suggesting that we were able to capture a highly complete transcriptome from RNA extracted using this protocol.

CONCLUSIONS

We present a straightforward, easily adaptable, and consistent method for the extraction of high-quality RNA from fern tissues. Using this modified CTAB protocol, we were able to successfully extract RNA from species across the fern phylogeny (five orders and 12 families) and from several tissue types and preservation methods including sterile, fertile, and developing fronds; rachises; rhizomes; roots; and gametophytes. RNA extracted using our protocol has been used successfully for high-throughput sequencing on an Illumina platform, with all libraries passing quality control checks resulting in high-accuracy sequence data. We hope that the ease and accessibility of this protocol will facilitate further transcriptomic studies in ferns and close the gap in plant genomics.

AUTHOR CONTRIBUTIONS

L.-Y.K. developed the original protocol. J.A.P., R.D., and W.B.B. modified and updated the protocol. J.A.P., R.D., and L.-Y.K. performed the extractions. J.A.P., E.B.S., and L.-Y.K. acquired funding. J.A.P. wrote the manuscript with input from all authors. All authors revised and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

All data used in this study are found within this article and supplementary materials.

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REFERENCES

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15): 2114–2120.

Castrejón-Varela, A., B. Pérez-García, J. A. Guerrero-Analco, and K. Mehltreter. 2022. A brief review of phytochemical defenses of ferns against herbivores. *American Fern Journal* 112(4): 233–250.

Dempster, E. L., K. V. Pryor, D. Francis, J. E. Young, and H. J. Rogers. 1999. Rapid DNA extraction from ferns for PCR-based analyses. *Biotechniques* 27: 66–68.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Fang, G., S. Hammar, and R. Grumet. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *Biotechniques* 13: 52–54, 56.

Grabherr, M. G., B. J. Hass, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, et al. 2011. Trinity: Reconstructing a full-length transcriptome without a genome for RNA-Seq data. *Nature Biotechnology* 29(7): 644–652.

Heikrujam, J., R. Kishor, and P. B. Mazumder. 2020. The chemistry behind plant DNA isolation protocols. *In* O.-M. Boldura, C. Balta, and N. S. Awwad [eds.], Biochemical analysis tools: Methods for bio-molecules studies, 131–142. IntechOpen, London, United Kingdom.

Johnson, M. T. J., E. J. Carpenter, Z. Tian, R. Bruskiewich, J. N. Burris, C. T. Carrigan, M. W. Chase, et al. 2012. Evaluating methods for isolating total RNA and predicting the success of sequencing phylogenetically diverse plant transcriptomes. *PLoS ONE* 7: e50226.

Katterman, F. R., and V. I. Shattuck. 1983. An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. *Preparative Biochemistry* 13: 347–359.

Laborde, B., V. Moine-Ledoux, T. Richard, C. Saucier, D. Dubourdieu, and J.-P. Monti. 2006. PVPP-polyphenol complexes: A molecular approach. *Journal of Agricultural and Food Chemistry* 54: 4383–4389.

Li, F.-W., P. Brouwer, L. Carretero-Paulet, S. Cheng, J. de Vries, P.-M. Delaux, A. Eily, et al. 2018. Fern genomes elucidate land plant evolution and cyanobacterial symbioses. *Nature Plants* 4: 460–472.

Manni, M., M. R. Berkeley, M. Seppey, F. A. Simão, and E. M. Zdobnov. 2021. BUSCO update: Novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of eukaryotic, prokaryotic, and viral genomes. *Molecular Biology and Evolution* 38: 4647–4654.

Moreira, P. A., and D. A. Oliveira. 2011. Leaf age affects the quality of DNA extracted from *Dimorphandra mollis* (Fabaceae), a tropical tree species from the Cerrado region of Brazil. *Genetics and Molecular Research* 10: 353–358.

One Thousand Plant Transcriptomes Initiative. 2019. One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574: 679–685.

Pandey, R. N., R. P. Adams, and L. E. Flournoy. 1996. Inhibition of random amplified polymorphic DNAs (RAPDs) by plant polysaccharides. *Plant Molecular Biology Reporter* 14: 17–22.

Qi, X., L.-Y. Kuo, C. Guo, H. Li, Z. Li, J. Qi, L. Wang, et al. 2018. A well-resolved fern nuclear phylogeny reveals the evolution history of numerous transcription factor families. *Molecular Phylogenetics and Evolution* 127: 961–977.

Szövényi, P., A. Gunadi, and F.-W. Li. 2021. Charting the genomic landscape of seed-free plants. *Nature Plants* 7: 554–565.

Vetter, J. 2018. Secondary metabolites of ferns. *In* H. Fernández [ed.], Current advances in fern research, 305–327. Springer International Publishing, Cham, Switzerland.

War, A. R., M. G. Paulraj, T. Ahmad, A. A. Buhroo, B. Hussain, S. Ignacimuthu, and H. C. Sharma. 2012. Mechanisms of plant defense against insect herbivores. *Plant Signaling & Behavior* 7: 1306–1320.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Quality assessment data for RNA extractions using the protocols presented in this study.

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Appendix 1. Protocol for high-quality RNA extraction from ferns.

Equipment and materials

- Dewar to hold liquid nitrogen
- Nuclease-free micropipette filter tips
- Micropipettes
- Ice
- Microcentrifuge capable of cooling to 4°C
- Water bath capable of heating to 55°C
- Nuclease-free tubes (2.0 mL)

Reagents

- Lysis buffer: The lysis buffer should be prepared for each extraction immediately before use. It is composed of 3× CTAB buffer (0.1 M Tris-Cl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 30 mg/mL CTAB), 15 mM beta-mercaptoethanol (5 µL/mL), and 4% PVPP (4 mg/mL). Heat the buffer in the water bath at 55°C for about 10–15 min and vortex immediately prior to use.
- 24:1 Chloroform:isoamyl alcohol
- >99% Chloroform
- Nuclease-free water

Option A:
- Chilled isopropanol (−20°C)
- Qiagen RNeasy Plant Mini Kit (including on-column DNase) (Qiagen, Hilden, Germany) or TURBO DNase (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Option B:
- Spectrum Plant Total RNA Kit (Millipore Sigma, Darmstadt, Germany) or Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, California, USA)
- 200-proof (100%) molecular-grade ethanol

Preparation

1. Precool centrifuge to 4°C and preheat water bath to 55°C.
2. Fill the Dewar with liquid nitrogen.
3. Gather required samples (kept at −80°C or in liquid nitrogen until ground), equipment, and reagents.
4. Clean workspace of clutter and possible contaminants. RNase Away or RNase Zap may be used to eliminate exogenous RNases in the workspace.

Protocol

1. Grind 25–100 mg of fresh tissue in a mortar with liquid nitrogen. Ensure that the tissue is ground to a fine powder. In a 1.7-mL Eppendorf tube, this corresponds to approximately 0.2 to 0.3 mL, although the weight of the sample should be determined directly from the sample. Transfer the ground tissue to a 2.0-mL tube and immediately add 1 mL of the pre-heated lysis buffer; do not let the sample thaw. Vortex to suspend all tissue in the buffer and place on ice.

   Note: If working with small amounts of tissue (e.g., gametophytes), the tissue can be mechanically disrupted by placing the tissue in a screw-cap tube with nuclease-free beads (e.g., 1.5-mm high-impact zirconium molecular-biology-grade beads), flash-freezing, pulverizing in a bead blaster for 30 s, and then resubmerging the tubes in liquid nitrogen. Repeat this step until the tissue is a fine powder (up to eight bead blastings). Ensure that the tissue remains frozen and does not thaw.
2. Centrifuge at 8000 × *g* at 4°C for 5 min to remove debris. Without disturbing the pelleted material, transfer the liquid to a new 2.0-mL tube.
3. Incubate at 55°C for 10 min. Invert/shake every 3 min.
4. Add 0.5 mL of 24:1 chloroform:isoamyl alcohol to each 2.0-mL tube and vortex to produce a homogenous solution. On a small cooler, invert/shake for 5 min.
5. Centrifuge at 13,000 × *g* at 4°C for 10 min. Transfer the aqueous phase to a new 2.0-mL tube.
6. Add 0.5 mL of >99% chloroform to each 2.0-mL tube and vortex to produce a homogenous solution. On a small cooler, invert/shake for 5 min.
7. Centrifuge at 13,000 × *g* at 4°C for 10 min. Proceed to either option A or option B.

Option A:

A1. Transfer the aqueous phase to a new 2.0-mL tube.

A2. Add an equal volume of chilled isopropanol to the tube and invert/mix a few times.

A3. Centrifuge the samples at 10,000 × *g* or higher at 4°C for 10 min.

A4. Remove the isopropanol by pouring, being careful not to lose the pellet, which may be difficult to see. Spin down samples for 1 min at 4°C and remove any remaining isopropanol with a micropipette.

A5. Dry the pellet at room temperature for 3 min.

A6. Dissolve the pellet with 15 µL or less of nuclease-free water. Flip the tubes to confirm that the pellets are well-dissolved. Note: Stopping at A6 will result in a solution containing both RNA and DNA. Proceed to A7 for DNase treatment and clean-up.

A7. Bring the volume of the extraction up to 100 µL with RNase-free water. Then follow the RNA Clean Up

protocol provided in the RNeasy Mini Handbook (Protocol "RNA Cleanup" in the RNeasy Mini Handbook 04/2023) including the DNase treatment, and then follow steps A8–A11 or proceed with the DNase treatment with TURBO DNase following the manufacturer's protocol.

A8. After the final wash, discard the collection tube and apply the column to a new collection tube. Dry the column by centrifuging for 1 min at 10,000 × *g*.

A9. Discard the collection tube and apply the column to a new RNase-free 2.0-mL tube.

A10. Add at least 25 μL of nuclease-free water directly onto the membrane of the column. Let the column sit for 1 min at room temperature.

A11. Centrifuge at 10,000 × *g* for 1 min.

Option B (Recommended):

B1. Transfer the aqueous phase to a new 2.0-mL tube. The following steps can be performed at room temperature unless otherwise noted. The aqueous phase is your sample that will be bound to the column.

B2a. If purifying using the Spectrum Plant Total RNA Kit, proceed to Step 4: Bind RNA to Column, Protocol A, which is recommended for difficult tissues. At this step, add 3–5× volume of binding buffer provided in the kit and allow the tube to sit at room temperature for 1 min prior to applying the solution to the column. If there is any precipitate that forms after adding the binding buffer, mix and transfer the solution with the precipitate to the column, then proceed with the washes and on-column DNase treatment. Complete steps 4 through 8 of the Spectrum Plant Total RNA Kit and then proceed to step B3 of this protocol.

B2b. If purifying with the Zymo RNA Clean and Concentrator Kit, proceed directly to Step 1 of the Total RNA Clean-up protocol (manual version 3.1.0) and add 2× volume of binding buffer provided in the kit. Complete steps 1 through 6 of the Zymo Total RNA Clean-up protocol and then procced to step B3 of this protocol.

B3. After the final wash, discard the collection tube and apply the column to a new collection tube. Dry the column by centrifuging for 1 min at 10,000 × *g*.

B4. Discard the collection tube and apply the column to a new RNase-free 2.0-mL tube.

B5. Add at least 15–25 μL of nuclease-free water (minimum 15 μL for Sigma, 25 μL for Zymo) directly onto the membrane of the column. Let the column sit for 1 min at room temperature.

B6. Centrifuge at 10,000 × *g* for 1 min and save the elute. This is your purified RNA extraction.

B7. Optionally, repeat steps B5 and B6 for a second elution. This may be recommended for tissues that are RNA-rich. Discard the column.

Quality control

Aliquot 4 μL of RNA elution into a new 2.0-mL RNase-free tube for quality control by TapeStation/Bioanalyzer, NanoDrop, and Qubit. The remaining sample should be kept at −80°C and freeze–thaw cycles should be avoided to preserve RNA integrity. Optimal RNA extractions should have OD 260/280 of 2.0, OD 260/230 > 2.0, and RINs above 7.0, without signs of degradation.