Simple and easy method to isolate nucleic acids and protein from leaf sample

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Abstract

Here we report about the standardization of simultaneous extraction of nucleic acids and proteins from leaf samples. The protocol established a simple, user-friendly and affordable method to isolate DNA, RNA and protein from a single leaf sample suitable for genomic and proteomic studies. The result showed that the extracted biomolecules were qualitatively and quantitatively unaffected at the end of the extraction procedure. The protocol was successfully tested in thirty-three different leaf samples covering annual, biennial and perennial plants. It took three hours to complete the protocol. Nucleic acids and proteins were successfully used for downstream application.

The extracted DNA molecules were tested for restriction digestion with EcoRI and HindIII, gene cloning and molecular marker analysis with SSR marker. Complementary DNA synthesis, gene cloning and gene expression analysis were done by taking isolated total RNA molecules. Protein profiling was done by performing SDS-PAGE and Q-TOF MS analysis.

Keywords: DNA/ RNA/ protein extraction, genomics, proteomics.

Introduction

An organism's systemetics is an interdisciplinary field that stems from our understanding of molecular mechanisms within cells. Nowadays, omics research is gaining popularity[1]. The global study of proteins, transcripts, genes, metabolites and methylated DNA or changed histone proteins in chromosomes, respectively, is called proteomics, transcriptomics, genomics, metabolomics and epigenomics. Technological advancements in these analyses allow for high-throughput monitoring and thorough understanding of biomolecules[10]. Synergistic study of genomics and proteomics is necessary to decipher the entire molecular mechanism taking place inside any biological system at any given time and under any particular set of conditions. Therefore, it is necessary to develop a protocol for simultaneous extraction of DNA, RNA, proteins from a single biological sample.

There are a few reports on simultaneous extraction of DNA, RNA and protein[3-5,7-9,11,12]. Besides time consuming, most of the methods follow complex lengthy procedure with low yield and purity of DNA, RNA and protein isolated. A few kits are available in market but most of the quality kits are costly making it unaffordable for the common people specially the students, newly recruited scientists and teachers. The global market size for molecular biology enzymes, reagents and kits was valued at USD 15.48 billion in 2024 and is projected to reach USD 34.11 billion by 2033, growing at a CAGR of 9.99% from 2025 to 2033 (https://www.grandviewresearch.com). Therefore, there is an opportunity to develop a kit or molecular biology chemicals for performing such work.

The protocol described in our previous publication on tea plant [8; Indian patent No. 534593] was adopted for our present study.

Material and Methods

Thirty three plant samples (Supplementary 1) were used for the analysis. Both tender and mature leaf samples were used for experiments. The plants and plant components used in this study were collected from the local areas of Guwahati, Assam, India and were carefully inspected and verified before being collected. It should be mentioned that since the plants were gathered from local markets and locations with the verbal consent of a landowner, no license was needed. Our collection procedure was carried out in compliance with the applicable laws and policies controlling the purchase of plant materials. Following the relevant local (Indian) guidelines, plants were collected for this investigation. Since the plants were acquired either commercially or with the proper permission from the landowner and not directly from wild or protected regions, it is significant to underline that no particular national guidelines are needed for gathering plants from local areas.

Trizol reagent and Qiagen kits were used as gold standards. As demonstrated in our previous work[5], a leaf sample weighing 400 micrograms was collected in a mortar and grinded in liquid nitrogen. The mixture was then transferred to a centrifuge tube with 800 µL of extraction buffer and 800 µL of low-pH phenol, it was thoroughly mixed and placed on ice for 10 minutes and then spun for additional 10 minutes at 5500 rpm at 4ºC. Phase separation occurred as shown in supplementary 3 and fig. 1. The aqueous phase, or supernatant, was put in a fresh tube and equally divided for DNA and RNA extraction separately; the organic phase was used for protein extraction.

The extracted nucleic acids were resolved in 1% (w/v) agarose gel. The quality and concentration of DNA/ RNA

samples were analyzed in spectrophotometer. The extracted DNA samples were tested for its downstream application by conducting restriction digestion and PCR analysis whereas the extracted RNA samples were checked for complementary DNA preparation, PCR analysis with gene specific primers and SSR primers and real time PCR analysis. Downstream application of protein samples was done by performing SDS-PAGE and Q-TOF MS analysis.

Genomic DNA samples were digested with EcoRI and HindIII restriction enzymes using 1.5 U/µg of DNA. The reaction mixtures were incubated at 37°C for one hour. Digested DNAs were separated on 1% agarose gel. Gene specific primers were designed in Primer3 output and synthesized externally (Supplementary 2). SSR primers were used from the references given in supplementary 4. PCR amplification reaction was carried out in a 10 µL reaction mixture comprising of 1X PCR buffer, 0.5 unit of Taq DNA polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM primers and 20-50 ng of genomic DNA.

PCR profile was initial denaturation for 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s annealing temperature, 30 s at 72°C and a final extension of 7 min at 72°C. PCR product was resolved by electrophoresis on 1.5% agarose gel. DNA sequencing of PCR amplicons was done by outsourcing with RASci Tech Solutions, Guwahati. The DNA sequences after sequencing were checked for homology search by using the free online available BLASTN program at NCBI (https:// blast.ncbi.nlm.nih.gov/Blast.cgi) by following the default parameters against nucleotide collection (nr/nt) database.

Supplementary 1

Description about the biological samples considered for present analysis are listed in the table. Thirty three plant samples were collected from local area for conducting the analysis [#].

S.N.	Common Name (Scientific Name)	Organism Type	Tissue Considered
1	Marigold (*Tagetes erecta*)	Annual Plant	Leaf
2	Rice (*Oryza sativa*)	Annual Plant	Leaf
3	White Mustard (Brassica alba)	Annual Plant	Leaf
4	Mung (*Vigna radiata*)	Annual Plant	Leaf
5	Bottle gourd (*Lagenaria siceraria*)	Annual Plant	Leaf
6	Pumpkin (*Cucurbita maxima*)	Annual Plant	Leaf
7	Mustard (*Brassica juncea*)	Annual Plant	Leaf
8	Bean (*Phaseolus vulgaris*)	Annual Plant	Leaf
9	Long bean (Vigna unguiculata ssp. sesquipedalis)	Annual Plant	Leaf
10	Cabbage (*Brassica oleracea*)	Biennial Plant	Leaf
11	Carrot (*Daucus carota*)	Biennial Plant	Leaf
12	Beetroot (*Beta vulgaris*)	Biennial Plant	Leaf
13	Spinach (*Brasica rapa*)	Biennial Plant	Leaf
14	Onion (*Allium cepa*)	Biennial Plant	Leaf
15	Potato (*Solanum tuberosum*)	Perennial Plant	Leaf
16	Tea (*Camelia sinensis*)	Perennial Plant	Leaf
17	Kadi patta (*Murraya koengii*)	Perennial Plant	Leaf
18	Ghost chilli (*Capsicum chinense* cultivar Peach Savina)	Perennial Plant	Leaf
19	Bamboo (*Bambusa textilis*)	Perennial Plant	Leaf
20	Chilli (*Capsicum frutescens*)	Perennial Plant	Leaf
21	Tomato (*Solanum lycopersicum*)	Perennial Plant	Leaf
22	Lemon (*Citrus limon*)	Perennial Plant	Leaf
23	Brinjal (*Solanum melongena*)	Perennial Plant	Leaf
24	Tulsi (*Ocimum tenuiflorum*)	Perennial Plant	Leaf
25	Papaya (*Carica papaya*)	Perennial Plant	Leaf
26	Buffalo grass (*Stenotaphrum secundatum*)	Perennial Plant	Leaf
27	Capsicum (Capsicum annuum)	Perennial Plant	Leaf
28	Moss	Bryophate	Moss
29	Mango (*Mangifera indica*)	Perennial Plant	Leaf
30	Banana (Musa acuminata)	Perennial Plant	Leaf
31	Guava (*Psidium guajava*)	Perennial Plant	Leaf
32	Ladies finger (*Abelmoschus esculentus*)	Perennial Plant	Leaf
33	Sugarcane (Saccharum officinarum)	Perennial Plant	Leaf

#: Genomic DNA isolation done, downstream analysis done with restriction digestion and PCR with gene specific and SSR primers; total RNA isolation done followed by downstream processing like: complementary DNA synthesis, PCR with gene specific primers and real time PCR; protein extraction done and protein profiling was checked with, SDS-PAGE and Q-TOF MS analysis.

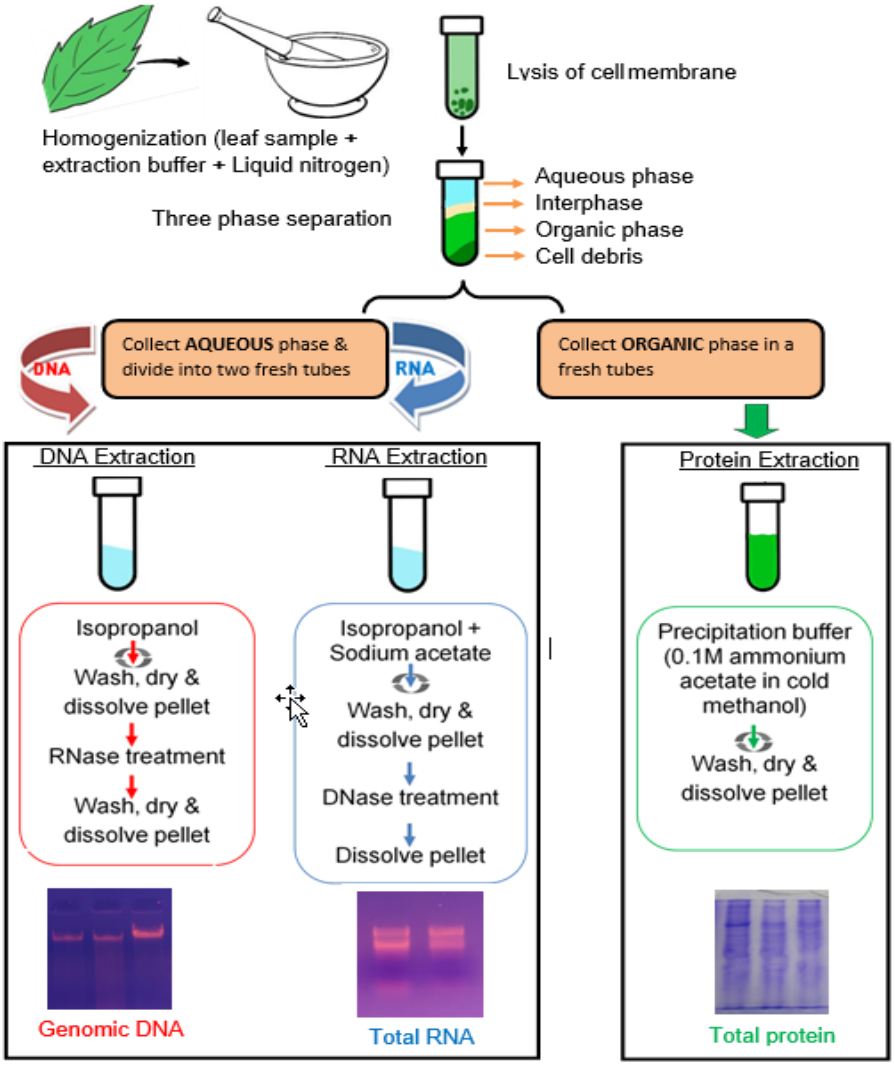
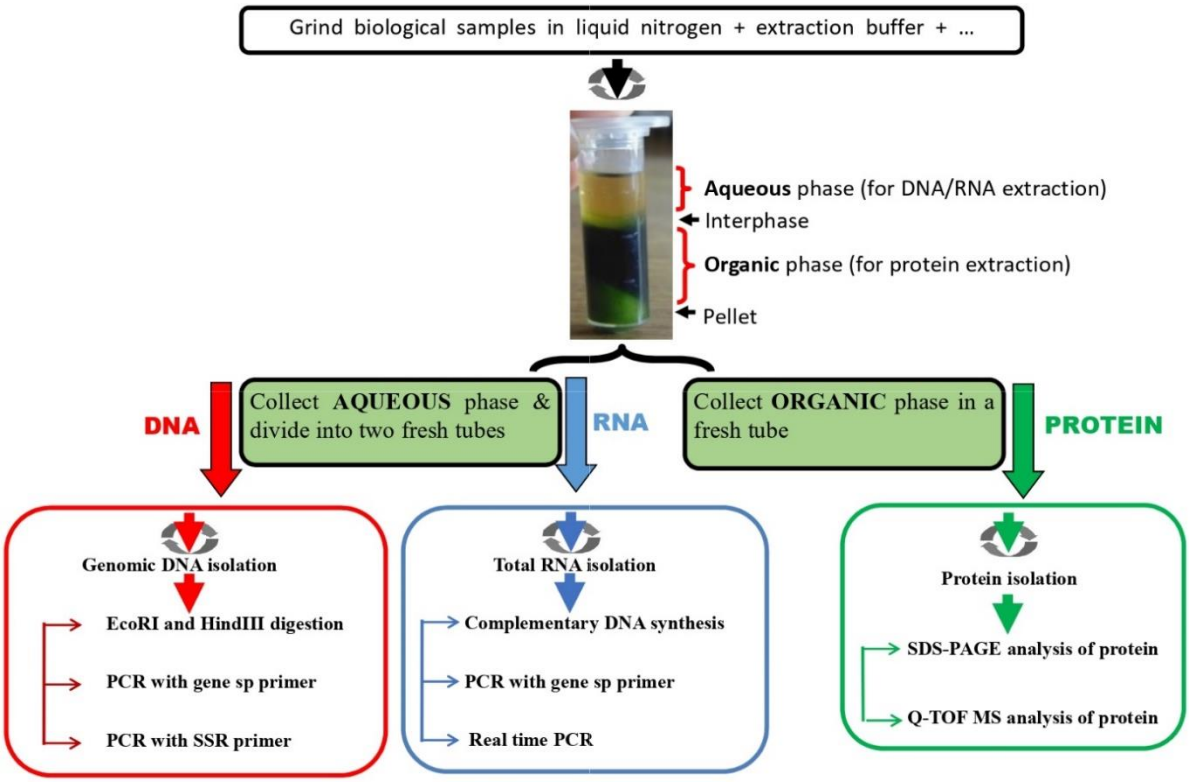


Fig. 1: Outline of the present protocol. Leaf samples were grinded with extraction buffer in liquid nitrogen which resulted in the separation of three phases: aqueous phase (transparent), interphase (brown) and organic phase (green). Aqueous phase was taken for DNA/ RNA extraction; the organic phase was used for protein extraction. The procedure shown here in the figure is in accordance to our previous report, Hazarika and Singh[5] Indian patent No. 534593 granted on April 24, 2024

Supplementary 2

Details of gene specific primers used to amplify a partial sequence of some target genes

S.N.	Primer sequence (5' to 3')	Tm (°C)	Gene Bank Id	Organism	Amplicon size (bp)
1	CAP F: GGCTCAGTCTGATTGCACAA CAP R: CAGCTACACCAGTCGAGGAA	52 54	AY804336	Ghost chilli	931
2	SOL_F: ATTGCTGCTTTCTTTGCCCA SOL_R: CCAAAAGGACTCTGGTTGCC	50 54	AF153195	Potato	778
3	LYC_F: GGTGGAAGAGTCAGTTGCAT LYC_R: ACTGCACTTGACGAACATTGT	52 50	D11112	Tomato	526
4	BAM_F: GGCGCTCAATAAGGAAGCAT BAM_R: GCGACCTTCTGAACGTCATC	52 54	GU062907	Bamboo	803
5	VIG_F: TCAGCTTCTTGTTCTTGCTCC VIG_R: CAACAGTTGACGAGGCAATAG	52 52	AY437639	Mung	308
6	ALL_F: CCAACCTCCACTCCATTCCT ALL_R: CTTTGGCGGTTCACAGAACA	54 52	EF192598	Onion	976



Supplementary 3: Flowchart of the work undertaken during the present study. Nucleic acids and proteins will be extracted from leaf sample; the extracted biomeolecules will be tested for their applicability for downstream processing. The extracted DNA molecules will be taken for restriction digestion with EcoRI and HindIII enzymes, PCR analysis with gene specific and SSR marker primers. The isolated RNA molecules will be used to perform complementary DNA preparation, PCR with gene specific primers and real time PCR. Protein samples will be taken for performing SDS-PAGE and Q-TOF MS analysis.

Supplementary 4
Details of SSR primers used to test the downstream applicability of the extracted DNAmolecules in molecular marker analysis

S.N.	Primer sequence (5' to 3')	Tm (°C)	Organism
1	CAPSR1_F: CTGAAGTCGGCTAGATGCCTA CAPSR1_R: TCAAAGCTATGGAGGAAAAGGA	54 52	Ghost chilli
2	SOLSR1_F: AATAGGTGTACTGACTCTCAATG SOLSR1_R:TTGAAGTAAAAGTCCTAGTATGTG	52 51	Potato
3	LYCSR1_F:GATGGACACCCTTCAATTTATGGT LYCSR1_R: TCCAAGTATCAGGCACACCAGC	54 57	Tomato
4	BAMSR1_F: AAACCTGTCGTGCCAGC BAMSR1_R: ATTACCGCCTTTGAGTGAG	49 49	Bamboo
5	VIGSR1_F: GGTGTTGTCGCTGTGGTTTT VIGSR1_R: CATCGCTGAATCTACGACCA	52 52	Mung
6	ALLSR1_F: GCCGGAAGAGGAGAAGAAGT ALLSR1_R: CATAATTCCCATGGCTTTGC	54 50	Onion

Reverse transcription and complementary DNA synthesis were done with the help of High-Capacity cDNA Reverse Transcription Kit (Invitrogen) following manufacturer's manual. The complementary DNAs were used as a template for PCR analysis. Real time PCR analyses were done by outsourcing with MrBiologist LLP, Guwahati. The organic phase was used for protein extraction. After processing, the pellets were air dried and dissolved in rehydration buffer. The extracted protein samples were tested for its downstream applicability by conducting SDS-PAGE analysis and Q-TOF MS analyses. Standard SDS-PAGE analysis procedure was used. Protein profiling by Q-TOF MS analysis was done by outsourcing with MrBiologist LLP, Guwahati. Comparison with gold standard chemicals (Trizol reagent and Qiagen kits) was done by following the manufacturer's protocol.

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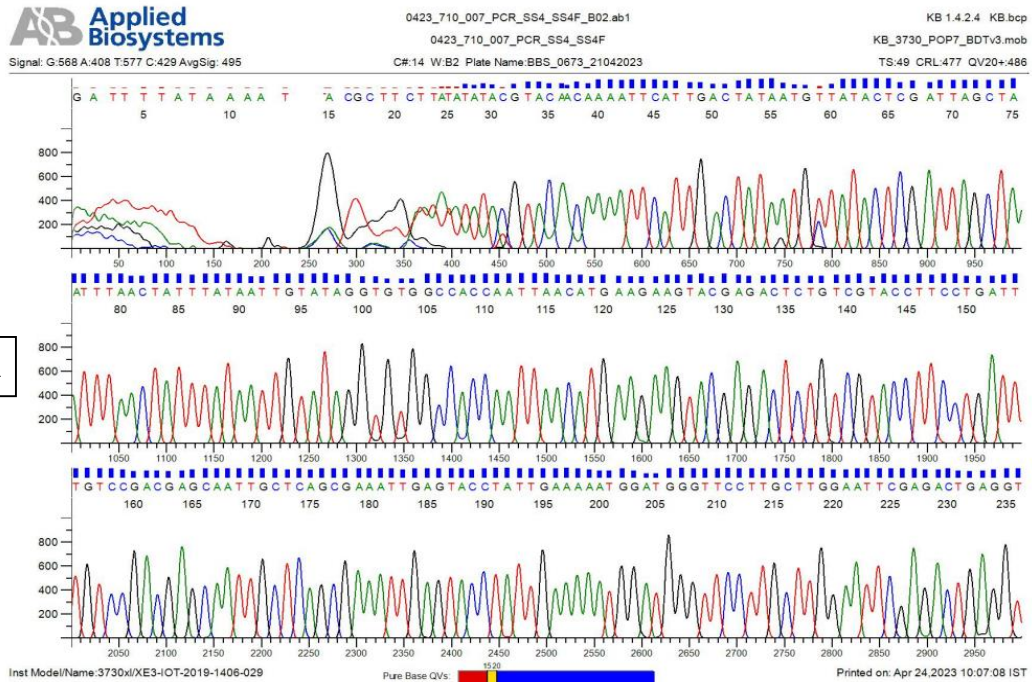

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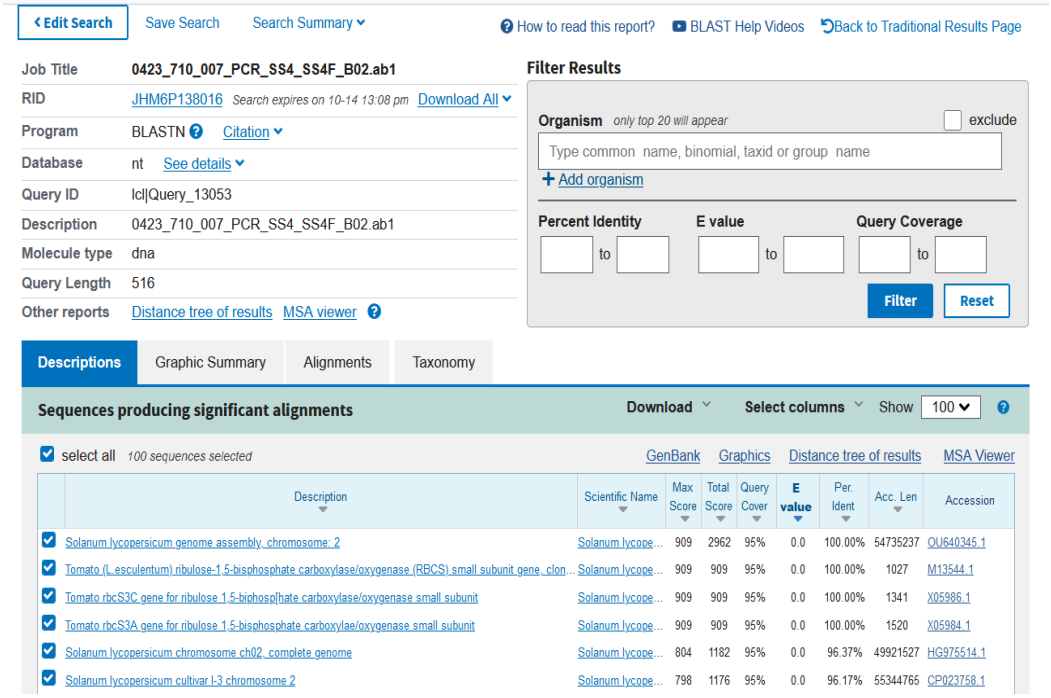

Supplementary 5: DNA sequence analysis of PCR amplicon. The PCR product using tomato gene specific primer as described in Table 2 was taken to perform sequencing. A) Electropherogram picture after the DNA sequencing was successful done. B) The DNA sequence determined after the completion of the sequencing. C) The DNA sequence was checked for sequence homology by BLASTN program at the National Centre of Biotechnology Information which showed identical with the gene of interest deposited in the GenBank of NCBI.

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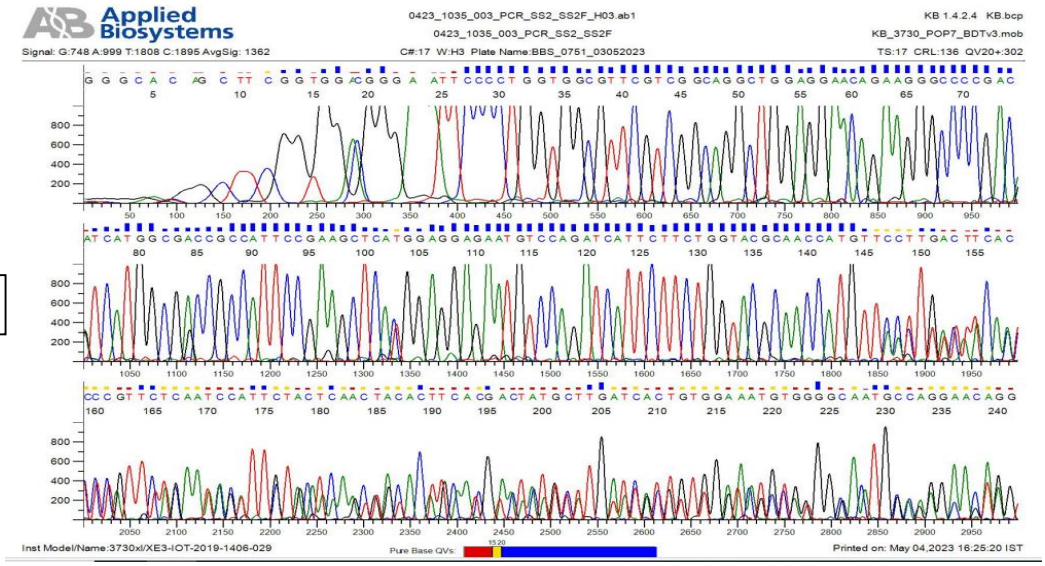

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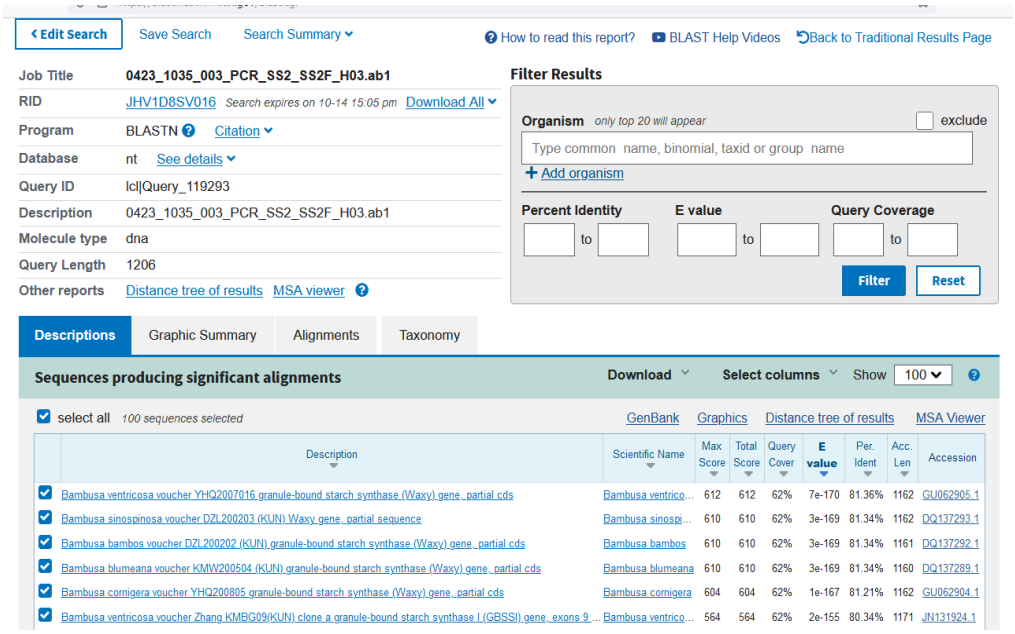

Supplementary 6: DNA sequencing and its analysis. The PCR amplicon as a result of taking bamboo gene specific primer as described in table 2 was taken for sequencing. A) Electropherogram picture showing successful DNA sequencing. B) The output result of DNA sequence determined by the sequencer. C) DNA sequence homology search done by BLASTN program at the National Centre of Biotechnology Information which showed about the homology with the DNA sequence of interest deposited in the GenBank of NCBI.

Results

The λ260 /λ280 ratios were in the range of 1.68 to 1.98 and the λ260 /λ230 ratios were between 1.71 and 2.21, indicating the extraction of comparative pure DNA samples. DNA yield was found to be in the range of 210 to 590 µg per gram of fresh tissue as shown in table 1. DNA concentration was found to be in between 0.105 to 0.295 µg per µL, sufficient to start any molecular biology work. The present protocol yielded high molecular weight and good quality DNA as shown in fig. 2A.

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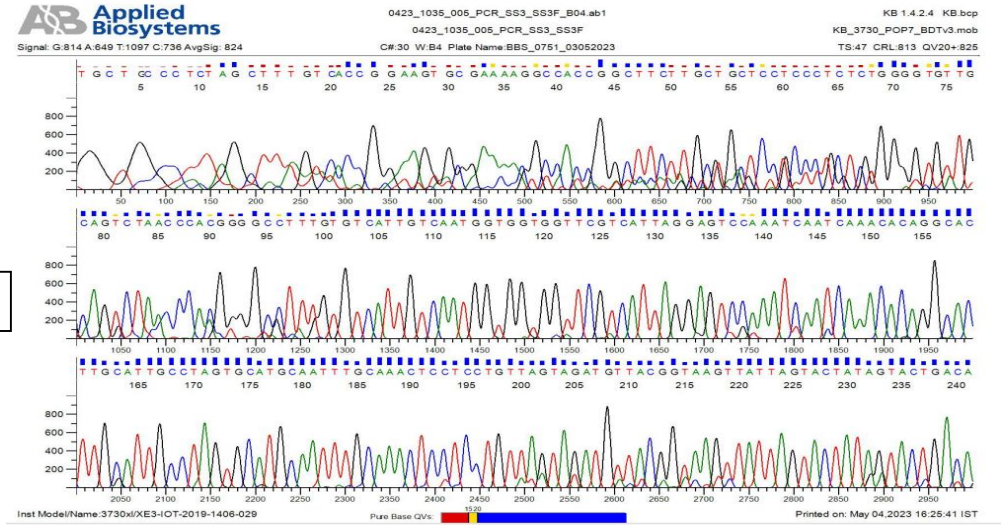

B

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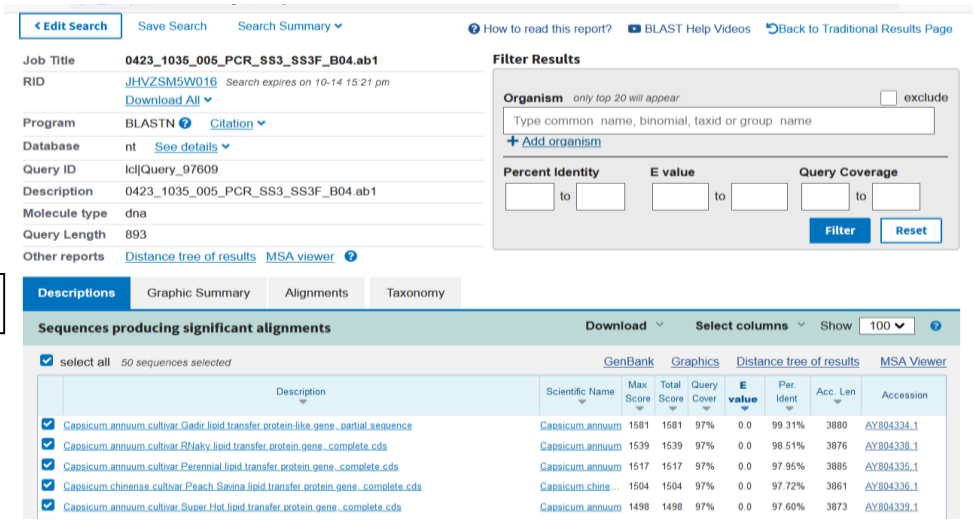
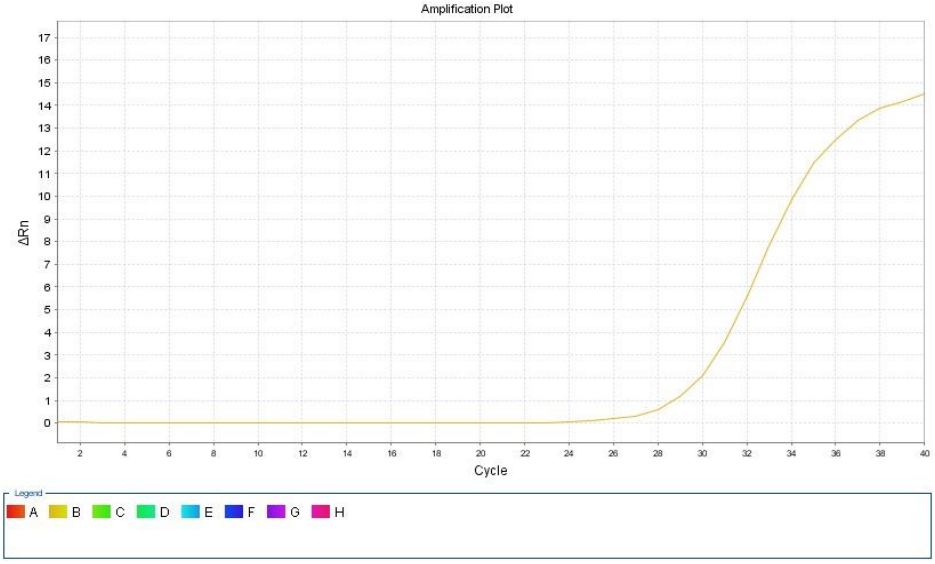


Supplementary 7: DNA sequencing of PCR product and its analysis. The product after performing PCR with ghost chilli gene specific primer as described in table 2 was taken to perform DNA sequencing.
A) Electropherogram picture showed the successful DNA sequencing. B) The DNA sequence shown as determined by the sequencer. C) The DNA sequence homology search was done by BLASTN program at the National Centre of Biotechnology Information which showed matching with the gene of interest deposited in the GenBank of NCBI.

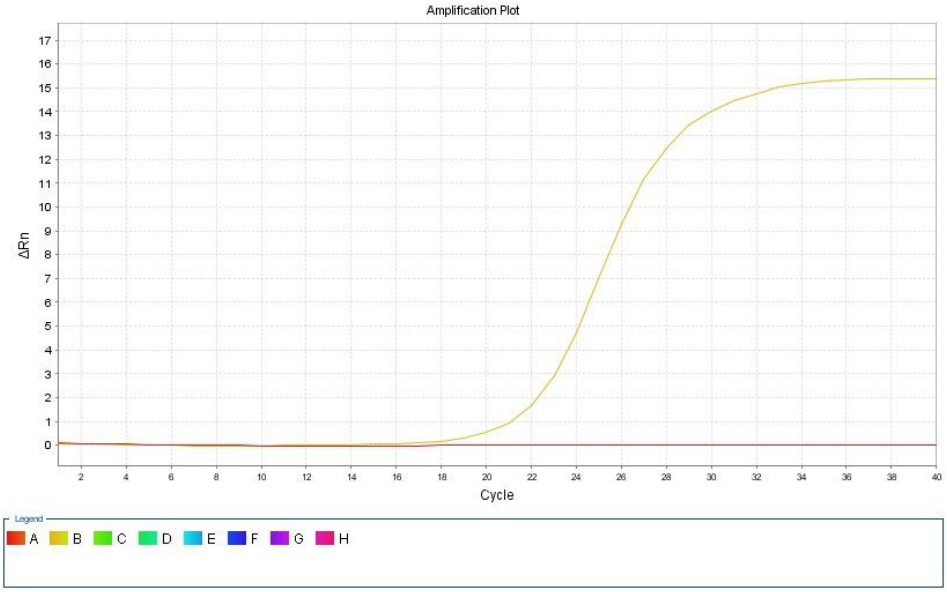
The DNA preparations were successfully used for restriction analysis with EcoRI and HindIII restriction endonucleases (Fig. 2B) clearly showing that the DNA samples can be used for southern hybridization technique. The extracted DNAs were also used as a template in PCR for partial amplification of specific genes. Fig. 2C showed the presence of desired single band size amplification representing that the extracted DNA can be used for gene cloning studies. Fig.2D showed about successful PCR amplification with SSR primers making the genomic DNA isolated eligible for molecular marker studies. The nucleotide sequences were found to be identical to the specific genes sequences deposited in GenBank of NCBI as shown in supplementary 5 (Tomato), supplementary 6 (Bamboo) and supplementary 7 (Ghost chilli).

The range of acceptable pure RNA samples includes the λ260 /λ280 and λ260 /λ230 ratios, which were found to be between 1.73 and 2.09 and 1.77 and 2.84 respectively. Table 2 illustrates the range of 202 to 574 µg of RNA production per gram of fresh tissue. RNA concentration was found to be in the ranged of 0.101 to 0.287 µg per µL, making it appropriate to begin any molecular biology study. As seen in fig. 3A, electrophoresis of RNA samples revealed high-quality RNA molecules.

The complementary DNA preparations were successfully done as shown in fig. 3B and further used as a template for PCR analysis. Fig. 3C clearly demonstrated about the successful use of the isolated RNA molecules for gene cloning with gene specific primes. Real time PCR analysis was also possible as shown in fig. 3D and supplementary 8, 9, 10 and 11. Primers described in supplementary 12 were used. Gel pictures from fig. 4A clearly showed the isolated protein can be used for proteomic studies with one dimensional gel analysis called SDS-PAGE analysis. Protein profiling was done by Q-TOF MS for bamboo and ghost chilli as shown in fig. 4B.



Supplementary 8: Real time PCR Amplification plot for Potato cDNA with gene specific primer.



Supplementary 9: Real time PCR Amplification plot for Onion cDNA with gene specific primer.

Table 1
Spectrophotometric analysis of DNA yield obtained from different biological samples. The DNA concentration for each biological sample was calculated and expressed in µg/µl. The DNA yield was determined there after and expressed in µg/g tissue. The quality of the extracted DNA sample was checked by calculating λ260/ λ280 and λ260/ λ230 for each sample

S.N.	Sample Name	λ260/λ280	λ260/λ230	DNA Conc (µg/µl)	DNA Yield (µg/gtissue)
1	Marigold (*Tagetes erecta*)	1.88	1.93	0.175	350
2	Rice (*Oryza sativa*)	1.69	1.77	0.231	462
3	White Mustard (Brassica alba)	1.98	2.16	0.105	210
4	Mung (*Vigna radiata*)	1.84	1.96	0.265	530
5	Bottle gourd (*Lagenaria siceraria*)	1.77	2.07	0.195	390
6	Pumpkin (*Cucurbita maxima*)	1.92	2.17	0.133	266
7	Mustard (*Brassica juncea*)	1.94	1.85	0.205	410
8	Bean (*Phaseolus vulgaris*)	1.91	2.18	0.255	510
9	Long bean (Vigna unguiculata ssp. sesquipedalis)	1.77	1.90	0.191	382
10	Cabbage (*Brassica oleracea*)	1.80	1.99	0.285	570
11	Carrot (*Daucus carota*)	1.78	2.18	0.295	590
12	Beetroot (*Beta vulgaris*)	1.85	1.97	0.295	590
13	Spinach (*Brasica rapa*)	1.69	1.85	0.145	290
14	Onion (*Allium cepa*)	1.89	1.99	0.186	372
15	Potato (*Solanum tuberosum*)	1.81	2.11	0.185	370
16	Tea (*Camelia sinensis*)	1.76	1.94	0.287	574
17	Kadi patta (*Murraya koengii*)	1.78	2.13	0.105	210
18	Ghost chilli (*Capsicum chinense*)	1.90	2.17	0.291	582
19	Bamboo (*Bambusa textilis*)	1.82	1.74	0.177	354
20	Chilli (*Capsicum frutescens*)	1.71	1.82	0.292	584
21	Tomato (*Solanum lycopersicum*)	1.94	2.07	0.274	548
22	Lemon (*Citrus limon*)	1.77	1.90	0.205	410
23	Brinjal (*Solanum melongena*)	1.91	2.14	0.255	510
24	Tulsi (*Ocimum tenuiflorum*)	1.97	1.91	0.149	298
25	Papaya (*Carica papaya*)	1.68	1.71	0.211	422
26	Buffalo grass (*Stenotaphrum secundatum*)	1.87	1.97	0.126	252
27	Capsicum (Capsicum annuum)	1.91	2.21	0.179	358
28	Moss	1.68	1.86	0.286	572
29	Mango (*Mangifera indica*)	1.87	1.86	0.254	508
30	Banana (Musa acuminata)	1.90	1.99	0.200	400
31	Guava (*Psidium guajava*)	1.89	2.09	0.153	306
32	Ladies finger (*Abelmoschus esculentus*)	1.84	1.91	0.201	402
33	Sugarcane (Saccharum officinarum)	1.92	2.07	0.233	466

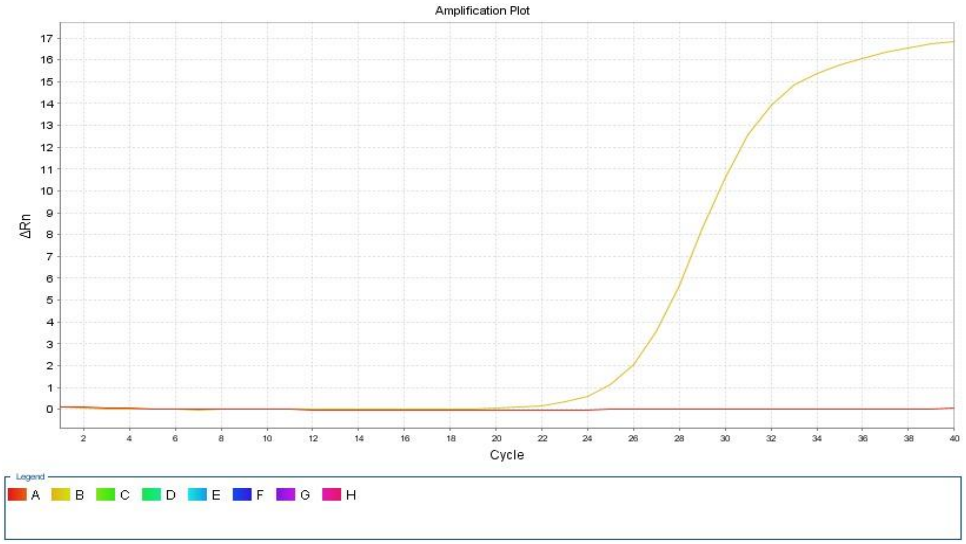
As seen in tables 3, 4 and 5, a gold standard comparative analysis using Qiagen (column-based) kits and Trizol (chemical-based) reagent produced results that were fairly close to our technique.

Discussion

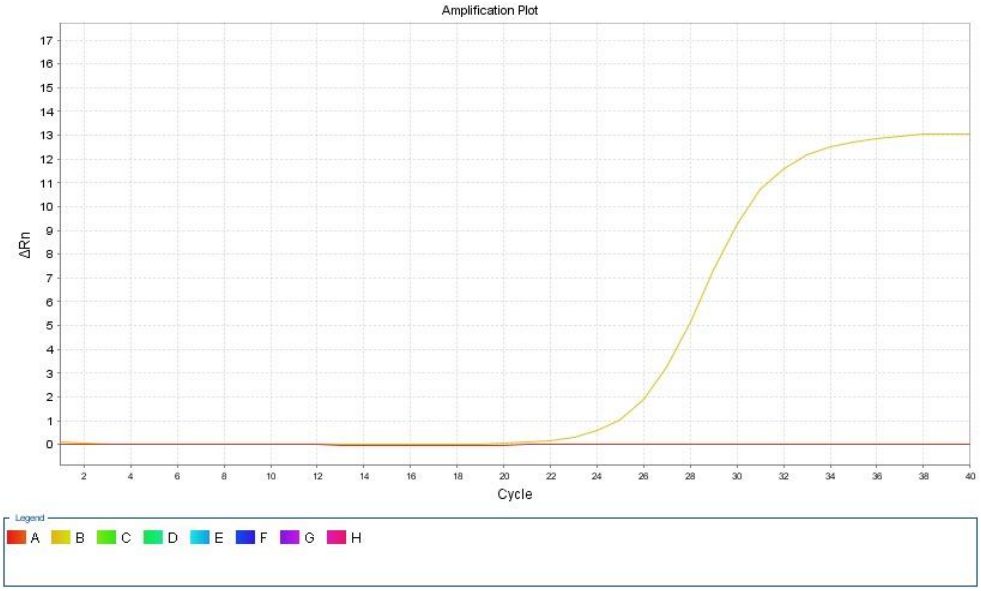
The problem we addressed was related to genomic and proteomic research. In coming years, molecular biology research is predicted to increase. For doing such work, we need to isolate DNA, RNA and protein from biological samples like plants, animals and microbes. Some reagents and kits are available in market for doing such work but the kits are costly in price and are often contaminated with DNA or RNA. Therefore, there is a need for an affordable and dependable kit for simultaneous extraction of DNA, RNA and protein from biological samples. As a solution for the problem addressed, we have developed a protocol for simultaneous isolation of DNA, RNA, protein from plant sample. The idea is supported by our previous publication on tea plant[5] and our Indian patent no 534593. The uniqueness of our protocol is: economical, rapid protocol (3 hours) and user-friendly

Table 2
Spectrophotometric analysis of RNA molecules extracted from different plant samples. The concentration of RNA samples was calculated and expressed in µg/µl. The RNA yield was finally calculated in µg/g leaf sample. The RNA purity was determined from λ260/ λ280 and λ260/ λ230 ratios for each sample

S.N.	Sample Name	λ260/ λ280	λ260/ λ230	RNA Conc (µg/µl)	RNA Yield (µg/g tissue)
1	Marigold (*Tagetes erecta*)	1.91	2.28	0.145	290
2	Rice (*Oryza sativa*)	1.97	1.80	0.194	388
3	White Mustard (Brassica alba)	1.83	1.99	0.185	370
4	Mung (*Vigna radiata*)	1.73	2.18	0.195	390
5	Bottle gourd (*Lagenaria siceraria*)	1.75	1.87	0.195	390
6	Pumpkin (*Cucurbita maxima*)	1.88	1.77	0.155	310
7	Mustard (*Brassica juncea*)	2.09	1.99	0.281	562
8	Bean (*Phaseolus vulgaris*)	1.88	1.83	0.175	350
9	Long bean (Vigna unguiculata ssp. sesquipedalis)	1.79	1.87	0.133	266
10	Cabbage (*Brassica oleracea*)	1.98	2.16	0.105	210
11	Carrot (*Daucus carota*)	1.94	1.93	0.195	390
12	Beetroot (*Beta vulgaris*)	1.77	2.07	0.195	390
13	Spinach (*Brasica rapa*)	1.92	2.17	0.231	462
14	Onion (*Allium cepa*)	1.94	1.85	0.105	210
15	Potato (*Solanum tuberosum*)	1.81	2.11	0.285	570
16	Tea (*Camelia sinensis*)	1.96	1.94	0.287	574
17	Kadi patta (*Murraya koengii*)	1.97	1.91	0.149	298
18	Ghost chilli (*Capsicum chinense* cultivar Peach Savina)	2.04	1.81	0.211	422
19	Bamboo (*Bambusa textilis*)	1.77	1.77	0.126	252
20	Chilli (*Capsicum frutescens*)	1.91	2.21	0.179	358
21	Tomato (*Solanum lycopersicum*)	1.91	2.84	0.122	244
22	Lemon (*Citrus limon*)	1.81	2.13	0.101	202
23	Brinjal (*Solanum melongena*)	1.86	1.96	0.173	346
24	Tulsi (*Ocimum tenuiflorum*)	1.88	2.33	0.105	210
25	Papaya (*Carica papaya*)	1.90	2.77	0.200	400
26	Buffalo grass (*Stenotaphrum secundatum*)	1.82	2.04	0.170	340
27	Capsicum (Capsicum annuum)	1.81	2.22	0.222	444
28	Moss	1.85	2.20	0.111	222
29	Mango (*Mangifera indica*)	1.81	2.17	0.163	326
30	Banana (Musa acuminata)	1.86	2.72	0.113	226
31	Guava (*Psidium guajava*)	1.90	2.09	0.104	208
32	Ladies finger (*Abelmoschus esculentus*)	1.83	1.87	0.152	304
33	Sugarcane (Saccharum officinarum)	1.88	2.21	0.117	234



Supplementary 10: Real time PCR Amplification plot for Marigold cDNA with gene specific primer.



Supplementary 11: Real time PCR Amplification plot for Tomato cDNA with gene specific primer

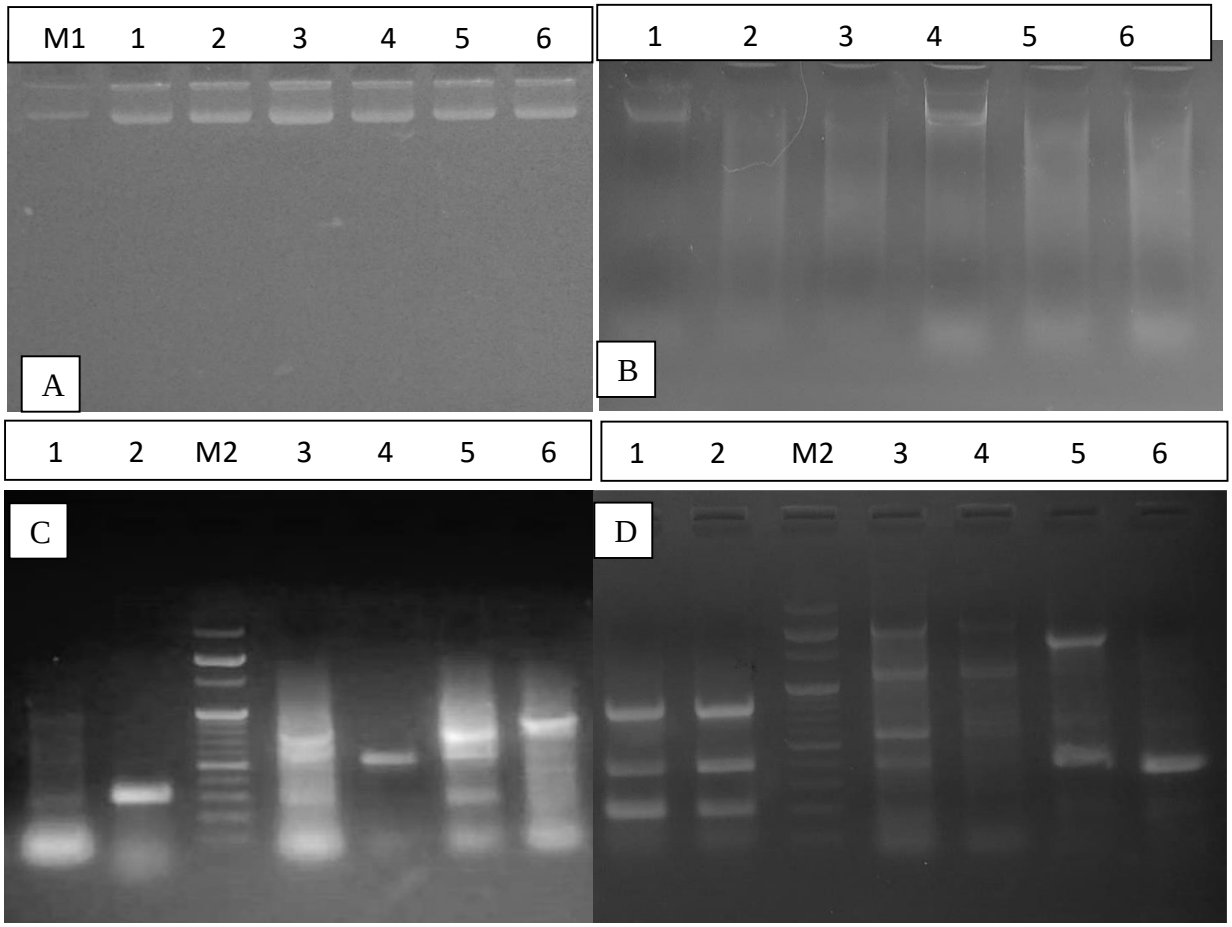


Fig. 2: Extraction of genomic DNA from plants and its downstream analyses. A) Analysis of genomic DNA isolated from leaf samples in 1% agarose gel electrophoresis. B) Restriction digestion analysis of extracted DNA molecules in 1% agarose gel electrophoresis. Genomic DNA molecules isolated from leaf samples were digested with EcoRI. C) PCR analysis of extracted DNA molecules with gene specific primers (described in Supplementary 3) in 1.5% agarose gel electrophoresis. Lane 1: Onion (976bp), Lane 2: Mung (308bp), Lane 3: Ladder (100bp), Lane 4: Bamboo (803bp), Lane 5: Tomato (526bp), Lane 6: Potato (778bp), Lane 7: Ghost chilli (931bp). D) Molecular marker analysis of extracted DNA molecules with SSR primers (described in Supplementary 4) in 1.5% agarose gel electrophoresis. [1: Onion, 2: Mung, 3: Bamboo, 4: Tomato, 5: Potato, 6: Ghost chilli, M1: Uncut λDNA (100 ng, approx 48 Kb), M2: Ladder (100bp)].

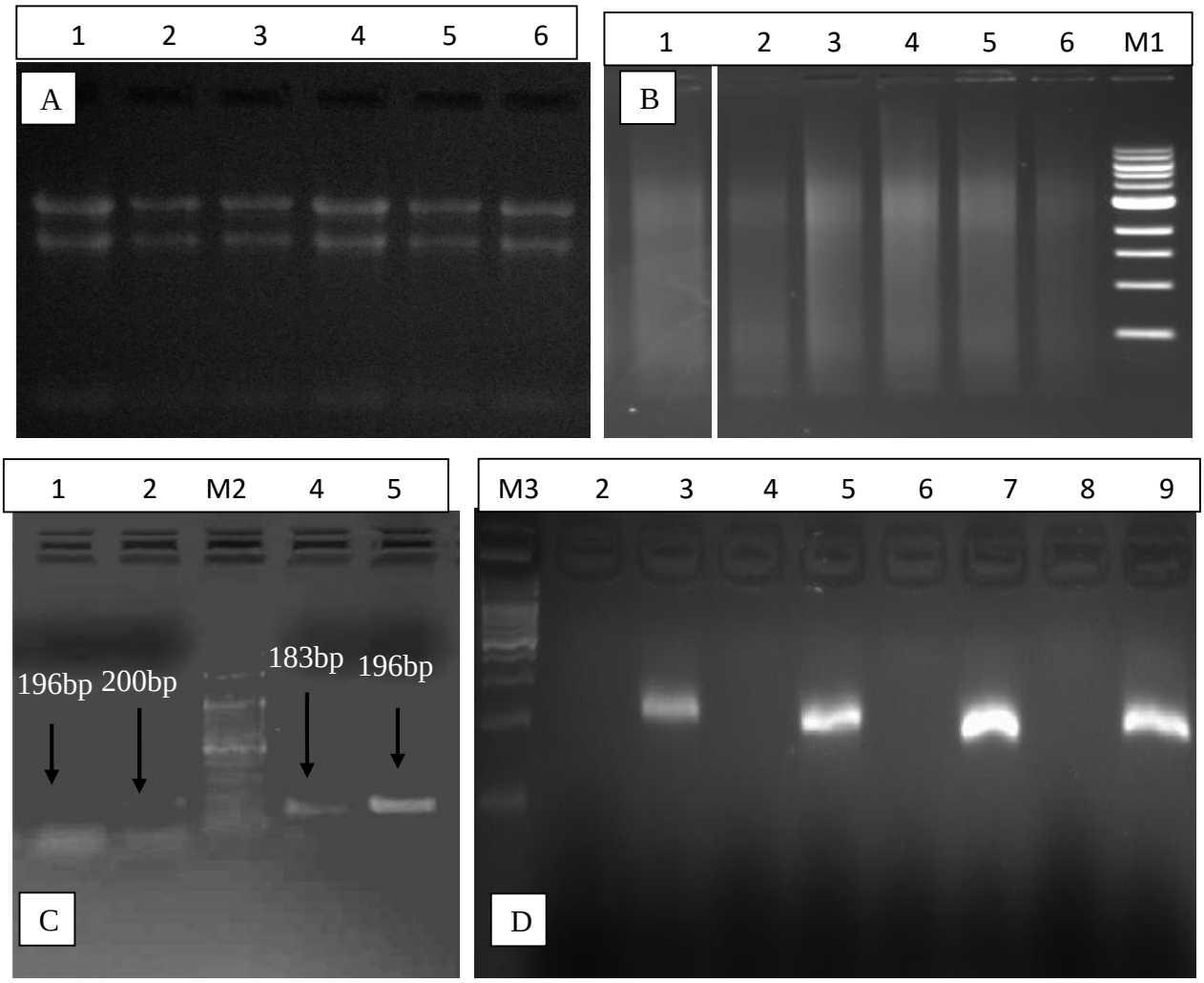


Fig. 3: Total RNA extraction and its analysis for downstream applicability. A) Total RNA isolated from leaf samples [Lane 1: Onion, Lane 2: Mung, Lane 3: Bamboo, Lane 4: Tomato, Lane 5: Potato, Lane 6: Ghost chilli]. B) Reverse transcription of isolated RNA from leaf samples [Lane 1: Onion, Lane 2: Mung, Lane 3: Bamboo, Lane 4: Tomato, Lane 5: Potato, Lane 6: Ghost chilli, Lane 7: M1, Ladder (100bp)]. C) PCR analysis of complementary DNA with primers (described in Supplementary 12) to be used for real time PCR in 1.5% agarose gel electrophoresis. PCR amplifications with the real time primers were done to standardize the annealing temperatures of primer pairs. [Lane 1: Onion cDNA (58 ºC annealing temp, 196bp) Lane 2: Potato cDNA (58ºC annealing temp, 200bp), Lane 3: M2, Ladder (100bp), Lane 4: Marigold cDNA (55ºC annealing temp, 183bp), Lane 5: Tomato cDNA (55ºC annealing temp, 196bp)]. D) Real time PCR analysis of complementary DNA with primers (described in Supplementary 12) in 2% agarose gel electrophoresis [Lane 1: M3, Ladder (100bp), Lane 3: Potato (200bp) Lane 5: Onion (196bp), Lane 7: Marigold (183bp), Lane 9: Tomato (196bp), Lane 2,4,6,8: No template].

Supplementary 12
Details of gene specific primers used for real time PCR analysis

S.N.	Primer sequence (5' to 3')	Tm (°C)	Gene Bank Id	Organism	Amplicon size (bp)
1	ALL_RT_F: ACCACCAGCTCCAAGAGTAGAG ALL_RT_R: GGCTGCTTTGGTCACTTTCTC	60 61	EF192598	Onion	196
2	SOL_RT_F: GTCCTTCTCCCAAACCACCTAC SOL_RT_R: GGTATCACCAGTAGTGCCAAAG	61 59	AF153195	Potato	200
3	TAG_RT_F: AGCCGAAGTTGAAGAACACC TAG_RT_R GGACGAGCAACGAGTGATGT	59 61	KX792459	Marigold	183
4	LYC_RT_F: ACCGTGAGAACCATAAGTCACC LYC_RT_R: GCGATGAAACTGATGCACTG	60 60	D11112	Tomato	196

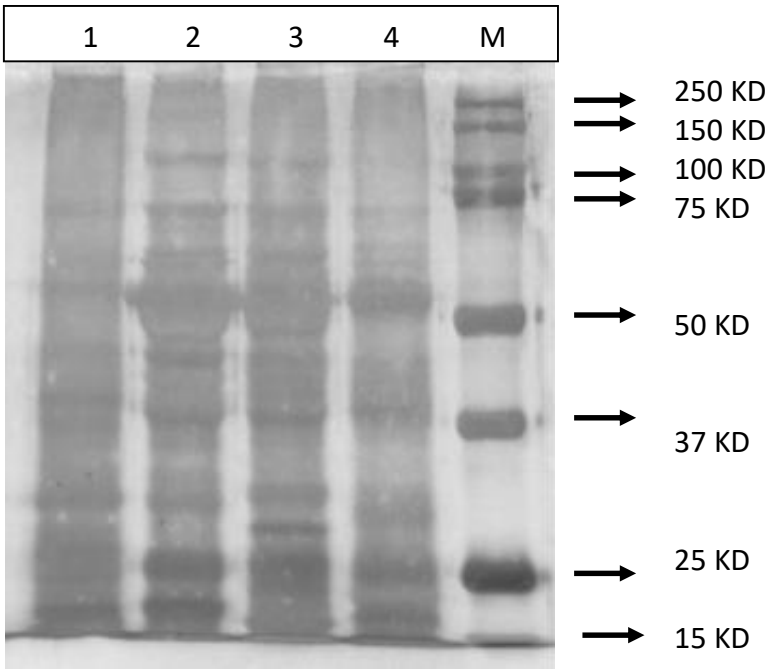


Fig. 4A: 10% SDS-PAGE analysis of protein isolated from leaf samples [Lane 1: Tomato, Lane 2: Marigold, Lane 3: Ghost Chilli, Lane 4: Bamboo, Lane 5: Protein Ladder].

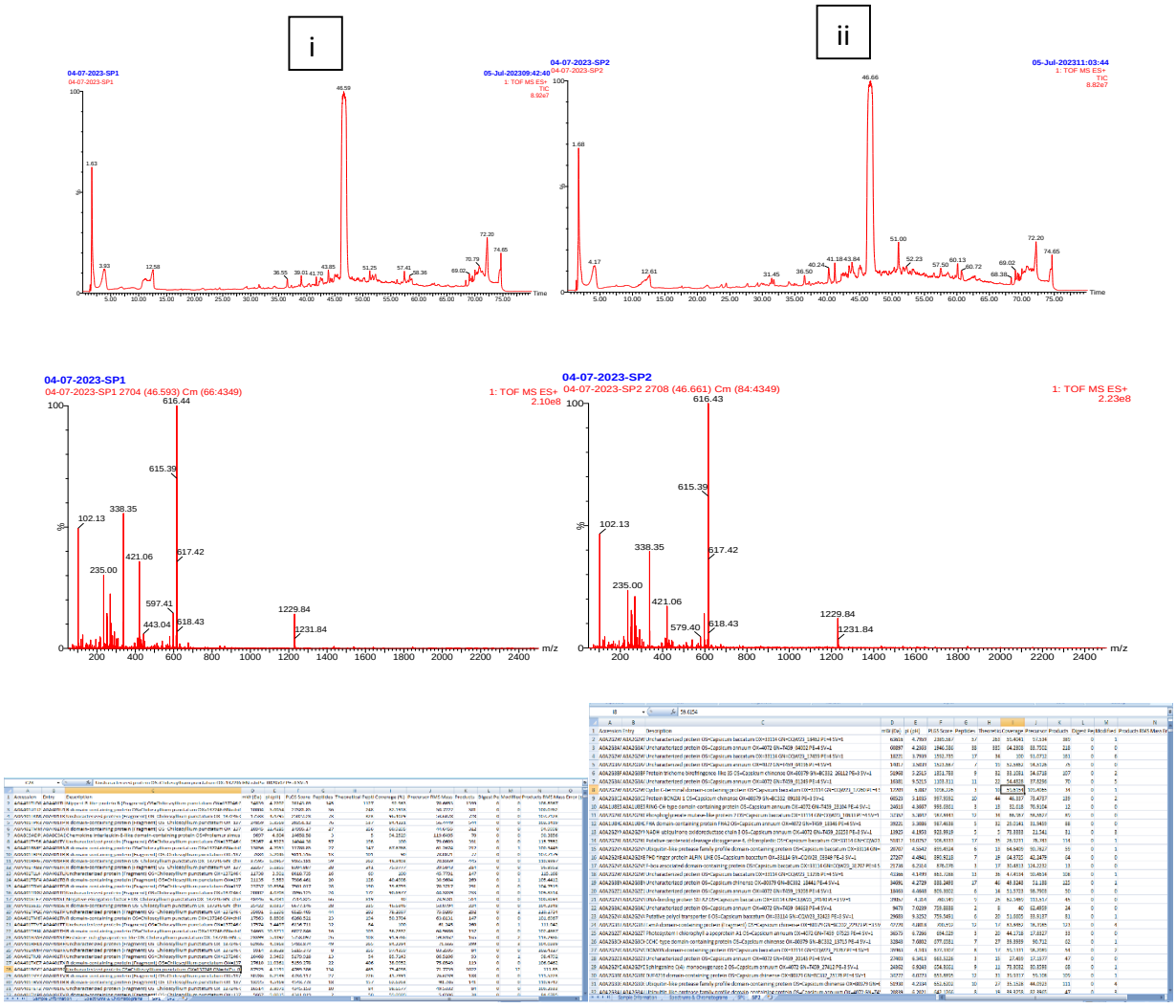


Fig. 4B: Profiling of protein sample (bamboo plant) with the help of Q-TOF Mass Spectrometry analysis along with identification by MASCOT. i) Bamboo plant ii) Ghost chilli plant

Table 3
Gold standard comparative analysis of DNA yield and purity obtained from tomato leaf

Protocol	$\lambda_{260}/\lambda_{280}$ ± SE	$\lambda_{260}/\lambda_{230}$ ± SE	DNA concentration, µg/µL ± SE	DNA yield, µg/g tissue ± SE
Our Protocol	1.94 ±0.05	2.07 ±0.08	0.274 ±0.03	548 ±23
Trizol (T)	1.82 ±0.05	2.65 ±0.06	0.228 ±0.015	456 ±21
Qiagen (Q)	1.83 ±0.03	2.33 ±0.03	0.216 ±0.02	432 ±28

Table 4
Gold standard comparative analysis of RNA yield and purity obtained from tomato leaf

Protocol	$\lambda_{260}/\lambda_{280}$ ± SE	$\lambda_{260}/\lambda_{230}$ ± SE	RNA concentration, µg/µL ± SE	RNA yield, µg/g tissue ± SE
Our Protocol	1.91 ±0.08	2.84 ±0.07	0.122 ±.013	244 ±22
Trizol (R)	2.01 ±0.03	2.18 ±0.07	0.187 ±0.017	374 ±25
Qiagen (Q)	1.89 ±0.05	2.67 ±0.08	0.110 ±0.011	220 ±23

Table 5
Gold standard comparative analysis of protein yield obtained from tomato leaf

Protocol	Protein concentration,µg/µL ± SE	Protein yield, µg/g tissue ± SE
Our Protocol	0. 527±0.07	1054 ±43
Trizol (R)	0.449 ±0.06	898 ±48
Qiagen (Q)	0.428 ±0.04	876 ±37

TRIzol (Invitrogen)/ TRI (Sigma) reagents are generally used for simultaneous extraction work. People also use column based kits like Qiagen etc. available in market which are costly in price. Similar work highlighted in Medicago plant by Xiong et al[12] by using commercially available TRIzol reagent and not any self-developed extraction buffer or solution. TRIzol reagent composes of guanidinium thiocyanate and phenol where as our extraction buffer compose of Tris HCL, SDS, EDTA and glycerol.

For doing extraction from limited samples like recalcitrant tissue culture leaf sample (For example transgenic tea) is time taking since it takes months for the growth of tissue culture plants, the sample for isolation work. Therefore, if we can do all three biomolecules (DNA, RNA or protein) isolation from a single tissue culture leaf sample, then it will save our time of waiting for the growth of plant to a sufficient leaf tissue. This can be elaborated by the instance that if we need 100 mg of leaf sample for the isolation of DNA or RNA or protein, then we will need 300 mg for completing all three isolations (DNA, RNA and Protein) works. Suppose a tissue, culture plant takes six months to become a size with body mass of 100 mg, then to reach to 300 mg body mass tissue culture tea plant will take approximately eighteen months. But if we can do all the isolation from only 100 mg, then we can save approximately one year of waiting period.

So this was one reason for finding for an alternative extraction buffer for simultaneous isolation of DNA, RNA and protein. The second reason was that regents like TRIzol/ TRI reagents (About 20,000 INR) and kits like Qiagen (About 66,000 INR) are available in market which is costly. So we thought of developing a new type of reagent/ kit for simultaneous isolation of DNA, RNA and protein from plant which will be economical and dependable one. Finally, we successfully developed a kit for this type of work which will cost 5,000 INR tentatively for 50 reactions.

Simultaneously extraction of all three biomolecules (DNA, RNA and protein) with high molecular weight and good quality indicated by $\lambda_{260}/\lambda_{280}$ and $\lambda_{260}/\lambda_{230}$ ratios[6] is quite tricky and difficult to do. Initially we took Tris HCL, SDS and EDTA for preparing the extraction buffer. From literature it is known that Tris HCl- stabilizes the pH of solution, SDS- disrupts cell membrane and promotes nucleic acid extraction, EDTA acts as a chelating agent thereby inhibiting nuclease activity (Nuclease is responsible for DNA and RNA degradation). Taking these three points, we prepared the extraction buffer.

From the aqueous liquid portion during phase separation after the first centrifugation we got DNA and RNA but from the organic phase, we were unable to extract protein. May be due to SDS, after cell membrane disruption, protein got precipitated to cell debris as pellet at the bottom of the tube below the organic phase.

All three recipes Tris HCL, SDS, EDTA are very frequently used by many workers in the preparation of extraction buffer for individual extraction of DNA or RNA or protein along with other chemicals like guanidinium thiocyanate, CTAB, KCl, NaCl, PVP, PVPP, DTT, acetone, TCA, urea, acrylamide, bis-acrylamide, methanol, phenol etc.[2] Glycerol

is generally used in protein precipitation since it is known for protein stabilization. It also acts partially as a carrier for nucleic acids. Indeed, it is used in gel loading buffers. It helps to delivery nucleic acids at the bottom of the gel loading well. This information made us to try for a new type of extraction buffer with glycerol which can be used for simultaneous isolation of all three biomolecules (DNA, RNA and protein).

Initially we started from minimal concentration and slowly we reached our claimed concentration where we got prominent protein band in SDS PAGE and downstream analysis by Q-TOF Mass Spectrometry analysis. Next hurdle we faced was when after successful extraction of nucleic acids, we were unable to use them for downstream processing because a standard SDS concentration is needed to get high yield and purity of nucleic acid otherwise the interaction with nucleic acid inhibits subsequent amplification assay. After keeping on adjusting the concentration of SDS, Tris HCl and EDTA, we were able to get the pure form of nucleic acids which were able to use for downstream processing like restriction digestion, PCR analysis, DNA sequencing, complementary synthesis and real time PCR.

Therefore, two important factors are responsible for the surprising effect of our present protocol: i) Addition of glycerol in the extraction buffer, ii) Isolation of protein from the organic phase after first step of centrifugation.

The main player of our protocol which is doing a major role in the simultaneous isolation is the extraction buffer. The other solutions/ chemicals like isopropanol, phenol, precipitation buffer with ammonium acetate etc. have follow-on secondary functions. This can be elaborated by the fact that a biomolecule supposes say DNA or RNA or protein can be precipitated during the course of protocol only if it is at first isolated/ released successfully from the grinded leaf sample with extraction buffer.

Conclusion

To sum up, the process for isolating DNA, RNA and proteins from plant samples has been well standardized. The isolated DNA, RNA and protein molecules were found to be compatible for genomic studies like restriction digestion analysis, gene cloning, markers analysis, gene expression and proteomic analysis by SDS-PAGE and Q-TOF MS. The outcomes gave great encouragement for the development of a kit for simultaneous isolation of DNA, RNA and protein. A gold standard comparative analysis using Qiagen (a column-based kit) and Trizol (a chemical-based kit) produced results that were equivalent. However, our protocol's most notable feature is its cost-effectiveness and user friendliness.

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