Vegetable Science (2012) 39 (2) : 118-122

A simple, rapid and cost-effective method of DNA extraction from different plant species for a wide range of PCR based applications

Maya Kumari, Vikas Y Patade, Zakwan Ahmed

Received : April, 2012 / Accepted : November, 2012

Abstract: Although there is no dearth of published protocols for DNA extraction in wide range of species, most of them are lengthy, time consuming and require more number of chemicals hence, not cost-effective. Also the amount of sample required for DNA extraction is large, which makes it difficult in extracting DNA from limited tissues. We have developed a simple, rapid and resource saving DNA extraction protocol for limited amount of plant tissues. This protocol work efficiently in wide range of plant species tried and hence, can be used in any species from which one wishes to extract DNA. This is also applicable for frozen preserved tissues. The extracted DNA was tested for PCR analysis using molecular markers like RAPD, EST-SSR primers and gene specific primers for evaluation of transgenic lines in capsicum and pea and also for hybridity confirmation of *in vitro* rescued wide hybrids in chickpea. The DNA extracted from this protocol was also found suitable for multiplexing using different primers of different product size but same melting temperature. This demonstrates the usefulness of this protocol since, DNA from more than 100 samples can be manually or mechanically extracted within a day, making it a promising alternative in wide range of PCR based applications.

Keywords: DNA isolation, Extraction buffer, PCR applications, Multiplexing

Introduction

Screening of mapping population for genotyping and of transgenic lines for gene integration confirmation requires large number of samples to be analyzed in a short time. This requires a good DNA extraction protocol, efficient for high throughput applications, should process large number of samples in a day, with minimum steps, bypassing the use of hazardous chemicals. The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols, however, the fundamentals of DNA extraction remains the same. There are three basic and one optional step in DNA extraction: cell disruption or lysis, to expose the DNA by grinding the sample, removing of membrane lipids by adding detergent, removing proteins by adding protease or sodium or ammonium acetate, or extract them with a phenol:chloroform:isoamyl alcohol (P:C:I) 25:24:1 or C:I (24:1) to dissolve non-nucleic acid biomolecules prior to DNA precipitation (optional), precipitating the DNA with an ice-cold ethanol or isopropanol and washing in 70% ethanol to remove salts. Addition of a chelating agent sequesters divalent cations such as Mg^{2+} and Ca^{2+} to stop DNAse enzymes from degrading the DNA. Sodium chloride helps to remove proteins that are bound to the DNA. It also helps to keep proteins dissolved in the aqueous layer so that they do not precipitate in alcohol along with the DNA. It should yield adequate intact DNA with good stability (at least 1 yr) and reasonable purity. DNA extraction from plant tissue can vary depending on the material used. After DNA extraction, purity is tested by measuring the intensity of absorbance of the DNA solution at wavelengths 260 nm and 280 nm. DNA absorbs UV light at 260 and 280 nm, while protein absorbs UV light at 280 nm; a pure sample of DNA has the 260/280 ratio at 1.8. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.8. The biochemical composition of plant tissues varies considerably across species therefore it is virtually difficult to apply one single extraction protocol for all plant species (Weishing *et al*. 1995; Matasyoh *et al*. 2008). Most of the available plant DNA isolation methods are multi-step as a result less number of samples can be processed in a day. Also most of them make use of hazardous chemicals. Generally, these methods are not suitable for high throughput applications. We modified the DNA extraction protocol from Edwards *et al*. (1991) for high-throughput DNA isolation from different plant species attempt to demonstrate its

Maya Kumari, Vikas Y. Patade, Zakwan Ahmed
Defence Institute of Bio-Energy Research (DIBER),
Goraparao, PO Arjunpur, Haldwani-263139,
Uttarakhand, India.
Email: madhulogin@yahoo.co.in

efficiency in various PCR-based applications and comparing it with different available protocols for quick DNA isolation.

Materials and methods:

Working protocol for DNA extraction

10-100 mg fresh leaf samples from 2-3 months old plants (Table 1) were collected and crushed in 1.5ml eppendorf tubes using micro pestle by adding liquid nitrogen (if available) directly in tubes or in buffer without any liquid nitrogen. 20 mg of 4-10 days old seedlings (jatropha, mung, chickpea, mustard, and methi) were also crushed directly in 1.5ml eppendorf tubes using micro pestle in DNA extraction buffer. All the samples were processed as mentioned below and worked in 3 replicates for all the extraction methods.

Table 1. Plant species from which DNA was extracted following different extraction protocols.

Sr. No.	Code used	Species	Common name	Family
1	Ca	*Capsicum annuum*	Sweet pepper	Solanaceae
2	Le	*Lycopersicon esculentum*	Tomato	
3	Bj	*Solanum melongena*	Brinjal	
4	N	*Nicotiana tabacum*	Tobacco	
5	Lp	*Lepidium latifolium*	Lepidium	Brassicaceae
6	A	*Arabidopsis thaliana*	Arabidopsis	
7	M	*Brassica juncea*	Mustard	
8	G	*Allium sativum*	Garlic	Alliaceae
9	O	*Allium cepa*	Onion	
10	Ps	*Pisum sativum*	Field Pea	Fabaceae
11	Tf	*Trigonella foenum-graecum*	Methi	
12	Mg	*Vigna mungo*	Mung bean	
13	Ci	*Cicer arietinum*	Chickpea	
14	J	*Jatropha curcus*	Jatropha	Euphorbiaceae

1. Add 500µl lysis buffer (Table 2) and invert tube 3-4 times for mixing. Then add C:I and vortex it. Spin tubes at 10,000 rpm for 5 min or 13,000 rpm for 1 min, collect aqueous phase and discard rest.
2. Add equal volume of isopropanol and keep for 30 min for precipitation at -20°C. Spin at 10,000 rpm for 5 min or 13,000 rpm for 1 min and discard supernatant.
3. Rinse pellet with 70% ethanol. Allow the pellet to dry and then resuspend in TE buffer or sterile distilled water. Store at 4°C for immediate use or at -20°C to be used latter.

Table 2. Different DNA extraction protocols tried to extract DNA from leaves and seedling samples from different plant species.

Protocol	Extraction buffer composition	Reference
A	200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS	Edwards et al, 1991
B	Buffer A+C:I	Modified
C	150 mM Tris-HCl (pH 7.5), 200 mM NaCl, 20 mM EDTA, and 0.5% SDS	Modified
D	Buffer C+C:I	Modified
E	1%SDS + 0.5M NaCl	Kotchani et al, 2009
F	CTAB extraction buffer	Doyle and Doyle, 1987
X	500mM NaCl, 100mM Tris HCl (pH 7.5), 50mM EDTA (pH 7.5), 20% SDS	Kang and Yang, 2004
Y	200mM Tris HCl (pH 7.5), 250mM NaCl, 25 mM EDTA, 0.5% SDS	Kasajima et al, 2004

DNA quality confirmation and quantification

Agarose gel electrophoresis of the DNA was done to check its quality, while spectrophotometer readings gave an indication of the concentration and cleanliness. Make a 0.8% agarose gel with 1x TAE and 0.1µl of ethidium bromide (10mg/ml) per 10ml solution. Load samples (3µl sample + 5µl SDW + 2µl 6x Loading Dye) in the wells, along with uncut Lamda DNA (100ng/µl of DNA) of known concentration, for quality. The gel was run for 30 min at 100 V. Expose the gel to UV light under gel documentation system. Presence of a highly resolved high molecular weight band indicates good quality DNA, while presence of a smeared band indicates DNA degradation.

Genomic DNA restriction digestion

To show that the extracted DNA can be digested for southern analysis, RNAse (10mg/ml stock) treatment was given to one set on DNA from all the species. The digestion mix consisted of 50ml reaction mix (5mg DNA, 6U restriction enzyme *EcoRI*, 10X buffer and sterile water) incubated for 2 hrs at 37°C in water bath.

PCR amplification and DNA analysis

To show the efficacy of the extracted DNA for all types of PCR work, different types of primers were used. RAPD (OPJ 4 to 19; Quaigen synthesized), SSR, EST-SSR primers (TCGA synthesized) were used for

phylogenetic analysis in Capsicum. Gene specific primers for *cbf1, npt-II, rd29A, CaMv* and *NOS* along with actin gene were also used to evaluate capsicum transgenic lines. Multiplex PCR was carried out using cbf1, actin and npt-II gene specific primers. *Cry 1 Ac* gene primers were used for screening pea transgenic lines. In chickpea nuclear gene primer for ITS and plastid gene primer for Mat K was used for plastid inheritance study in wide hybrids rescued *in vitro* (Kumari et al, 2011). PCR amplification using single primer and multiplexing were performed on a MasterCycler Gradient (Bio-Rad), in 20µl reaction volume with 1µl of DNA template, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.5 µM primers, 0.2 mM dNTPs, and 1U Red Taq DNA polymerase (Banglore Genei), following programme: 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at Primer TM, 45 s at 72°C, and 7 min at 72°C. The PCR products were analyzed with 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Result and Discussion

More than 100 samples can be processed in a day. When using tissue lyser or grinder the grinding process takes less than a min. Here we compared different DNA extraction protocols published by different workers in different plant species. Buffer A, B, X and Y had same composition as that of Edwards et al (1991). Edwards's protocol is simple but DNA extracted from this protocol gave poor yield and was very impure. Impure DNA creates problem in PCR amplification and the result is sometimes not reproducible. Hence, this protocol was modified. In this study, we reduced the concentration of DNA extraction buffer and added C:I (24:1) in lysis step. The modified protocol gave best result, as DNA yield from both leaves and seedling samples from different species were highest in buffer D followed by buffer B (Table 3 and 4). DNA yield was lowest in brinjal and jatropha compared to other species. This may be because of high phenolics, secondary metabolites and other interfering compounds. DNA yield and purity was lowest in buffer E. DNA purity was highest in buffer F but yield was very low. DNA obtained from all theses buffers appeared turbid and faint yellow to green due to the presence of chlorophyll pigments. However, in case of modified protocol D, the DNA extracted was good in quality as there was no shearing and the quantity (Fig. 1) was higher compared to other published protocols (Table 3 and 4). The purity of extracted DNA was good in CTAB protocol but yield was lowest.

DNA obtained from all the samples with our modified protocol was of sufficient purity for almost all PCR

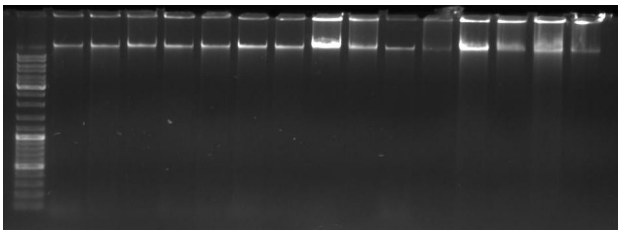


Figure 1. DNA integrity and quality of samples extracted from modified protocol (D)

Table 3. Quantification of DNA extracted using different buffers from leaf samples of different species, without any RNAse treatment.

Sample (100mg)	DNA (ng/µl)			
	Buffer A	Buffer B	Buffer C	Buffer D
Mustard	92.3	105.7	82.7	148.0
Lepidium	67.7	81.0	57.7	90.0
Jatropha	61.3	78.0	63.3	87.7
Capsicum	58.0	63.3	50.3	83.0
Brinjal	32.7	52.0	44.0	56.7
Chickpea	80.0	98.0	80.7	121.3
Tobacco	124.0	151.0	112.7	156.0
Pea	148.3	161.7	141.3	172.7
Tomato	79.7	89.7	71.3	94.0
SE (N=3)	5.2	3.6	4.7	5.5
5% LSD DF = 16	15.6	10.9	14.1	16.4

Table 4. Quantification of DNA extracted using different buffers from seedlings of different species, after RNAse treatment.

Sample	DNA (ng/µl) after RNAse treatment					
Code	Buffer A	Buffer B	Buffer C	Buffer D	Buffer E	Buffer F
Leaf (50mg)						
Tomato	122	173	124	204	104	114
Pea	39	48	44	61	34	39
Jatropha	75	156	78	204	68	75
Chickpea	97	159	204	212	77	86
Lepidium	37	61	38	73	39	42
Capsicum	38	45	37	58	34	38
Mustard	56	71	62	93	39	51
Onion	52	81	72	91	49	66
Tobacco	48	55	37	68	30	31
Garlic	54	67	59	87	48	61
Seedling (10mg)						
Fenugreek	23	24	23	26	22	21
Mustard	37	38	37	39	24	29
Jatropha	25	36	34	50	23	33
Arabidopsis	22	35	26	49	18	27
Chickpea	30	53	34	53	30	53
Capsicum	30	36	31	40	29	32
Mung	47	44	35	52	20	31
Purity	low	avg.	low	avg.	low	high
Yield	avg.	good	avg.	v. good	low	low

applications and also found amicable to digestion with restriction enzymes (Fig. 2). PCR amplification was observed in all the samples for different types of primers. RAPD, SSR, EST-SSR primers were used for phylogenetic studies in Capsicum and Jatropha (Fig 3). Gene specific primers for actin, *cbf1, npt-II, rd29A, CaMv* and *NOS* were used for screening capsicum transgenic lines (Fig. 4). Multiplexing could be achieved by using CBF-I, actin and NPT-II primers in single PCR mix with different product size but same melting temperature (Fig. 5). In chick pea nuclear gene primer ITS and plastid gene primer MatK was used for plastid inheritance study in wide hybrids (Kumari *et al*., 2011). The DNA extracted here was also used to screen pea transgenic lines for *Cry1Ac* gene integration.

Most of the available protocols have been optimized on a particular plant species (Alaey *et al*., 2005; Kasajiama *et al*., 2004; Wang *et al*., 2009; Mishra *et al*., 2008). Edwards (1991) gave a simple protocol for DNA

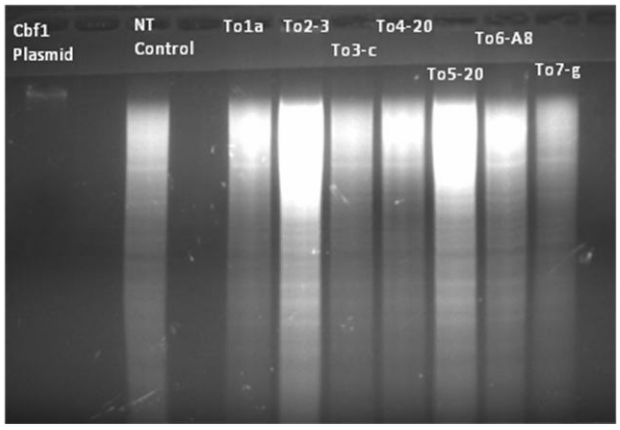
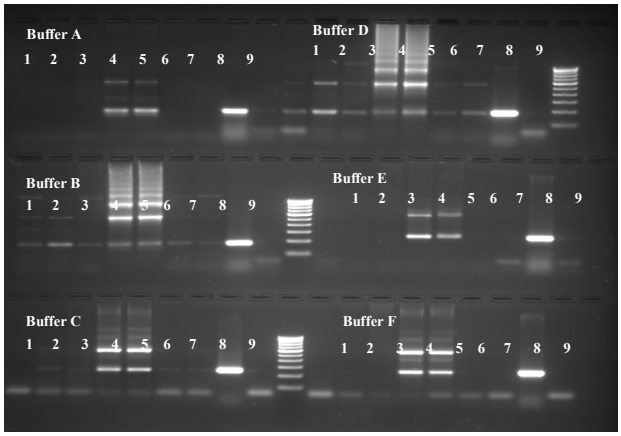
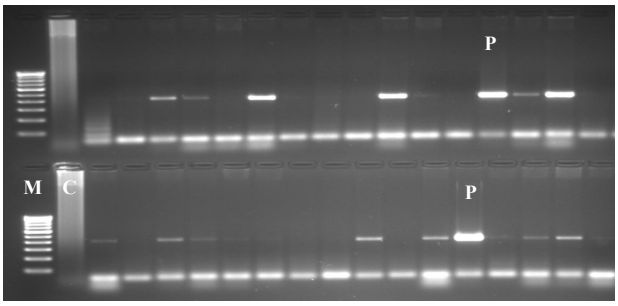


Figure 2. Restriction digestion of genomic DNA of transgenic Capsicum extracted in buffer D for Southern hybridization. (Low melting agarose gel 1.0%; Electrophoresed at 60V for 4h)



1. Lepidium, 2. Onion, 3. Garlic, 4. Tomato, 5. Tobacco, 6. Pea, 7. Chickpea, 8. Capsicum, 9. Jatropha

Figure 3. RAPD analysis using DNA template from samples extracted in different extraction buffers.



(M:1kb ladder, C-non transgenic control, P-plasmid (830bp), Rest are regenerants)

Figure 4. Screening of putative transgenic capsicum regenerant lines (T0) for *Cbf1* gene integration.

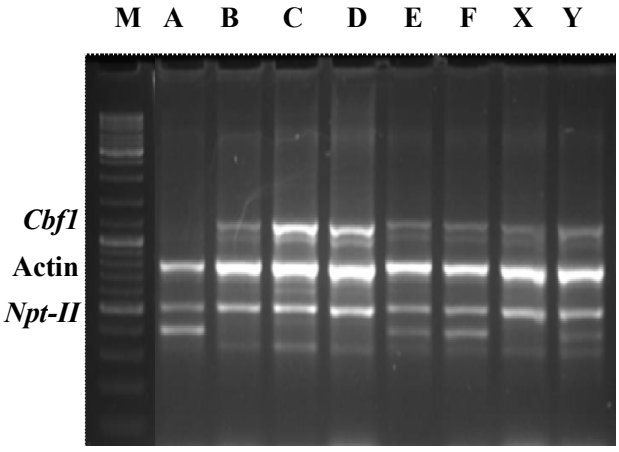


Figure 5. Multiplex analysis (*Cbf1*, Actin *and Npt-II* genes specific primers), using DNA template from transgenic capsicum samples extracted in different buffers.

extraction but the DNA extracted by this method was very impure, viscous with more pigments and was less stable. The DNA solution stability reported was one month for Kasajma et al (2004) modified protocol from Edwards *et al*. (1991) for *Arabidopsis* DNA extraction in TE buffer. For phylogenetic assay more numbers of primers are to be studied, hence relatively, large amount of DNA is needed with stability for at least one year. For large-scale application Kasajima's protocol may not be suitable. Kotchoni and Gachomo (2009) optimized their protocol and tested in single species *Arabidopsis*.

In the present study, none of the published protocols were found suitable for extracting DNA from Jatropha and Solanaceous crops like brinjal, tomato and tobacco. Most of the DNA extraction protocols available are not applicable to wide range of species and for wide range of PCR based applications. Therefore we modified Edwards (1991) protocol and reduced the salt concentration in buffer and added C:I along with the lysis buffer in one single step. This gave clear extract; DNA was intact, stable for more than a year. Our modified protocol is efficient for both small and large

quantity of samples. The DNA extracted by this method is completely digestible with restriction enzymes and is also amicable to RNAse treatment for purified DNA. This is more resource saving and we demonstrated its ability for multiplexing. DNA extraction and purification from Solanaceous and Euphorbiaceae crop species is hampered due to the presence of contaminants such as polyphenols and polysaccharides. The present protocol worked in wide range of species and for wider applications like RAPD, SSR, EST-SSR, GSP for transgenic screening, genotyping, phylogenetic studies and MAS. There are wide ranges of PCR applications and hence, require pure DNA. Some applications like screening of large population require very less amount of DNA and that too of average purity. For such applications simple protocol (1 or 2 step) is good enough but for some applications the requiring high purity and in higher quantity of DNA, ethanol washing of pellet and RNase treatment is essential. For species with high phenolics, PVP and Beta-mercaptoethanol is required to remove these compounds. For genotyping and phylogenetic studies, though average purity DNA works but the quantity of DNA needed is high since, large numbers of primers (molecular markers) have to be tested on each sample. Therefore a single extraction procedure is not applicable for all types of PCR applications.

सारांश

यद्यपि पौधों की बहुतायत जातियों का डीएनए निकालने की प्रकाशित पद्धतियों का अभाव नहीं हैं, लेकिन उनमें से अधिकतम बोझिल, अधिक समय और अधिक रसायनों की संख्या का प्रयोग करते हैं, इसलिए लागत–प्रभावकारी नहीं है। इनमें डीएनए निकालने के लिए अधिक मात्रा में नमूने की आवश्यकता पड़ती है। जो कि सीमित ऊतकों से डीएनए निकालने को कठिन बनाती है। हमने सीमित मात्रा के पादप ऊत्तकों से डीएनए निकालने की एक सहज, तेज और साधन की बचत करने की पद्धति विकसित की है। ये पद्धति आजमाये गये विस्तृत पादप जातियों में कुशलता से कार्य करती है इसलिए किसी भी जाति का डीएनए निकालने के लिए प्रयोग की जा सकती है। ये जमा के सुरक्षित किए गए ऊत्तकों के लिए भी उपयुक्त है। निकाले गये डीएनए का परीक्षण पीसीआर विश्लेषण के लिए जीवाणु संकेतकों जैसे–आर.ए.पी.डी., ई.एस.टी.–एस.एस.आर. प्रवेशिकाओं और जीन विशिष्ट प्रवेशिकाओं का प्रयोग करके मिर्च और मटर के पराजीनिक पौधों और चने के संकरीकरण के मूल्यांकन और पुष्टि के लिए किया गया। निकाले गये डीएनए को विभिन्न उत्पाद माप लेकिन समान गलनांक ताप वाले विभिन्न प्रवेशिकाओं का प्रयोग करके बहुविधिकरण के लिए भी उपयुक्त पाया गया। चूंकि इस पद्धति से 100 से अधिक नमूनों का डीएनए शारीरिक या यांत्रिक तरीकों से 1 दिन में निकाला जा सकता है, जो इसको बहुतायत पीसीआर आधारित उपयोगों के लिए एक आशाजनक विकल्प बनाता है और ये इस पद्धति की उपयोगिता को प्रदर्शित करता है।

References

Alaey M, Naderi R, Vezvaei A, Khalighi A and Salami A (2005) Comparing study between four different methods of genomic DNA extraction from *Cyclamen persicum* mill. Int J Agri Biol 7(6): 882-884.

Edwards K, Johnstone C and Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucl Acid Res 19: 1349.

Kasajima I, Ide Y, Ohtsu N O, Hayashi H, Yoneyama T and Fujiwara T (2004) A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis. Plant Mol Biol Rep 22: 49–52.

Kotchoni SO and Gachomo EW (2009) A rapid and hazardous reagent free protocol for genomic DNA extraction suitable for genetic studies in plants. Mol Biol Rep 36(6): 1633-11636.

Kumari M, Clarke HJ, Small CCF, Small I, Khan TN and Siddique KHM (2011) Albinism does not correlate with biparental inheritance of plastid DNA in interspecific hybrids in *Cicer* species. Plant Sci 180(4): 628-633.

Matasyoh LG, Wachira FN, Kinyua MG, Muigai AWT and Mukiama TK (2008) Leaf storage conditions and genomic DNA isolation efficiency in *Ocimum gratissimum* L. from Kenya. Afr J Biotechnol 7(5): 557-564.

Mishra MK, Rani NS, Ram AS, Sreenath HL and Jayarama (2008) A simple method of DNA extraction from coffee seeds suitable for PCR analysis. Afr J Biotechnol 7(4): 409-413.

Wang CT, Wang XZ, Tang YY, Zhang JC, Yu SL, Xu JZ and Bao ZM (2009) A rapid and cheap protocol for preparation of PCR templates in peanut. Electronic J Biotech 12 (2): 1-6.

Weishing K, Nybom H, Wolff K and Meyer W (1995) DNA isolation and purification. *In:* DNA fingerprinting in plants and fungi, pp44-59. CRC Press, Boca Raton, Florida.