PHYTOCHEMICAL BULLETIN, Vol. 19 (1): 11-15, 1987

A RAPID DNA ISOLATION PROCEDURE FOR SMALL QUANTITIES OF FRESH LEAF TISSUE

Jeff J. Doyle and Jane L. Doyle

L. H. Bailey Hortorium
466 Mann Library Building
Cornell University
Ithaca, NY 14853

Abstract - Of the several rapid and inexpensive DNA isolation procedures that have been described recently, one of the most popular is that of Saghai-Maroof et al. (1984), a procedure using hexadecyltrimethylammonium bromide (CTAB) and lyophilized tissue. We here describe a simple modification of this procedure that has been found to be efficient for nucleic acid isolations from fresh leaf tissue, and which we have also used for dried herbarium specimens.

Key Work Index - DNA isolation, hexadecyltrimethylammonium bromide, CTAB, molecular systematics

Introduction

Plant DNA isolation methodology has evolved rapidly in the last few years. From cumbersome, messy, and often inefficient large scale phenol procedures, DNA technology moved to more efficient methods involving ultracentrifugation in CsCl density gradients. Still, such procedures are slow and expensive, and not well suited for assaying large number of samples. More recently, a number of "miniprep" procedures have been developed that permit rapid isolation of DNA with a minimum of expense, using small amounts of tissue. There are a number of different protocols available for extraction of DNA from small samples, which is fortunate because it appears that different procedures work best for different plant groups, as might be expected considering the great diversity of plant secondary compounds that in many cases may interfere with a particular method of DNA isolation.

Here we report a method of isolation that has been found to work for a large number of different angiosperm groups, including

(Received March 31, 1987, Accepted May 3, 1987)

both monocots (Palmae, Orchidaceae, Cyperaceae) and dicots (Rosaceae, Leguminosae, Saxifragaceae, Juglandaceae, Fagaceae, Portulacaceae, Solanaceae). We have even had success with insect larvae! It is a very simple modification of a procedure originally described for barley by Saghai-Maroof et al. (1984), differing principally in that their procedure called for using lyophilized tissue, while we use fresh leaf material, and have compensated for the increased water content by increasing the concentration of the extraction buffer.

This procedure has been found to be effective on fresh tissue, and we have recently used it to extract usable DNA (high molecular weight, digestible with restriction endonucleases) from recently-dried herbarium specimens (Doyle and Dickson, 1987). It appears to be a very versatile procedure both in our hands and elsewhere in the molecular systematics community, where versions of the protocol have been rather widely circulated.

Experimental

1. Grind 0.5-1.5 g of leaf tissue in a mortar and pestle, using 7.5 ml 2x CTAB isolation buffer (2x CTAB isolation buffer; 100mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide ["CTAB"], 0.2% 2-mercaptoethanol). Pour grindate into a 30 ml Corex tube, rinse mortar with an addition 0.5 ml 2x CTAB buffer, and add to Corex tube. In addition:

A. we preheat our mortars and 2x buffer to 60C before the extraction.

B. for tough tissues, a pinch of sterile sand can be added.

C. it is also possible to use liquid nitrogen powdered tissue.

2. Incubate at 60C for 30 to 60 min with optional gentle swirling -- somewhat longer incubations, or in some cases shorter incubations (as little as 15 min) have been found by other workers to give better yields of high molecular weight DNA. We have recently been routinely using 30 min incubations with good success.

3. Extract once with chloroform-isoamyl alcohol (24:1), mixing gently but thoroughly.

4. Spin in clinical centrifuge to concentrate layers. We use setting 7 on our IEC for 10 min. Generally the aqueous phase will be clear (though often colored) by the time we get done with our spins, but this is not always the case.

5. Pull off aqueous phase with a wide-bore pipet, transer to a clear Corex tube, add 2/3 vol of cold isopropanol, and mix gently to precipitate nucleic acids.

In the best isolations, one gets a beautiful precipitation at this stage -- long strands of nucleic acid that can then be spooled out with a glass hook. Regrettably, this is not always the case. We have seen everything from no apparent precipitation (the aqueous phase actually got clearer!) to the formation of a cloudy precipitate, and in nearly all cases have still gotten good nucleic acids.

6. If possible, spool out nucleic acids with a glass hook, and transfer to 20-25 ml of wash buffer (76% EtOH, 10 mM ammonium acetate) for approximately 20 min.

If this is not possible, several approaches can be followed:

A. (best alternative) Spin in clinical centrifuge at low speed (e.g. setting 3 on IEC) for 1-2 min. Gently pour off as much of the supernatant as possible without losing nucleic acids, which will generally be a diffuse and very loose pellet at the bottom. Add wash buffer to the pellet and gently swirl to resuspend the pellet.

B. If you just don't see much of any precipitation, harder, more prolonged spins are unavoidable -- do whatever it takes, but the less the better as far as purity is concerned. After more than about 3 min at setting 3 on the IEC, the pellet is hard and very difficult to resuspend in wash buffer -- we generally resort to "tearing" it with a glass hook to try to wash it as much as possible. In other cases the precipitate at this point may look flaky, and that, too, can be disconcerting.

Generally, nucleic acids will become much more white when rinsed in the wash buffer.

7. Spin down (or spool out) nucleic acids after around 20 min of leaving in wash buffer (the time of "washing" is not apparently critical).

8. Allow to air dry briefly, then add resuspension buffer (usually 1 ml, but this will depend on the amount of precipitate seen). (Resuspension buffer: 10 mM ammonium acetate, 0.25 mM EDTA; we have also been known to use TE [10 mM Tris-Cl pH 7.4, 1 mM EDTA, pH 8.0]). tris-EDTA

ethylenediaminetetraacetic acid

A. If you check this preparation on a minigel, in many cases there is not only good quality DNA, but also visible bands of ribosomal RNAs.

B. Saghai-Maroof et al. (1984), from whom this protocol was modified, use DNA at this stage for restriction digests. We have found some instances where DNA at this stage was resistant to endonuclease digestion, so we do some further cleaning.

9. Add RNAse A to a final concentration of 10 ug/ml, incubate 30 min at 37C.

10. Ethanol precipitation of the DNA: dilute sample with 2 volumes of distilled water, add ammonium acetate (7.5 M stock) to a final concentration of 2.5 M, mix, add 2.5 volumes of cold ethanol, and gently mix to precipitate DNA. Spin down DNA at high speed (10,000 xg for 10 min in refrigerated centrifuge, or setting 7 in clinical for 10 min).

A. By this precipitation, the DNA usually looks great, even in preps that didn't look so good initially.

B. In the past, we precipitated directly from the 1 ml volume, and found that the DNA formed a gelatinous complex with the alcohol. Even a hard spin leaves a lot of alcohol in prep, and if you resuspend too soon you still get a great deal of alcohol. Increasing the volume by diluting with water helps a great deal, but it is still necessary to dry the pellet, either under vacuum or, as we often do, by air drying on the benchtop overnight.

11. Air dry and resuspend in TE (or spool out, dab dry on a kimwipe, and resuspend directly).

Results and Discussion

DNA isolated by this method has been of uniformly good quality in terms of size, with the majority in excess of 30 kilobases in length. A260/A280 ratios are a bit low, however--generally anywhere from 1.4-1.7, so there obviously is some impurity. Yields are generally quite high -- apparently up to nearly 1 mg/g fresh wt, which is a bit deceptive because whenever possible we use young, unexpanded leaves that are packed with DNA. The DNA is digestible with virtually all restriction enzymes (we have not found any examples of an enzyme that consistently will not digest DNA prepared this way, though for any particular plant taxon there may be some enzymes that will not work, just as is true for DNA prepared in other ways). It is, of course, possible to clean the DNA further -- additional precipitations, for example or, even phenol extractions or passing the DNA over CsCl gradients. It is also possible to add reagents to the extraction buffer; for example, we have added 1% polyvinylpyrollidine (PVP 40) in one or two cases where we anticipated large amounts of phenolic compounds to be present.

We routinely use DNA prepared in this way for all studies in our laboratory as it is a total DNA preparation that includes both nuclear and organellar components. Using specific recombinant probes, it is therefore possible to detect chloroplast DNA restriction fragments as well as sequences from any nuclear gene by using conventional transfer and hybridization procedures.

Literature Cited

Doyle, J. J. and E. L. Dickson (1987) Taxon (in press).

Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen and R. W. Allard (1984) Proc. Natl. Acad. Sci. USA 81: 8014-8019.

Acknowledgements

This work was supported in part by grants from the National Science Foundation (BSR-8307106 and BSR-8516630).

[1] DNA Extraction method (CTAB)

. CTAB? → CetylTrimethylAmmonium Bromide ($C_{19}H_{42}BrN$)

= hexadecyltrimethylammonium bromide

Working Solution Preparation

1) 2X CTAB DNA extraction buffer:

100mM	Tris-HCl	pH 8.0	→ 6.06g	adjusted 500ml by H_2O
1.4M	NaCl		→ 40.91g	
20mM	EDTA		→ 3.72g	
2%	CTAB		→ 10g	녹인후 pH 8.0으로
0.2%	2-mercaptoethanol			

* 2-mercaptoethanol 은 extraction 직전에 넣어 사용한다.

ex) 10 sample의 extraction時 → stock 100ml을 취하여 200㎕의 2-mercaptoethanol넣어 섞은후 각 sample당 10ml씩 넣어 사용.

2) Chloroform: isoamyl alcohol solution(24 : 1 mixture) ← 500㎕ 정도 만들어 stock으로 사용

3) Isopropanol

4) 70% ethanol ← -20℃ 에 보관.

5) 95% ethanol ← normal quality.

6) 7.5M ammonium acetate(M.W.=77.08)

289.05g → 500ml D.W.

7) 2.5M sodium acetate(M.W.=136.08)

170.1g → 500ml D.W.

8) TE (Tris-EDTA) pH 8.0

Tris-Cl과 EDTA를 각각 10X stock으로 만들어 보관.

200ml 정도씩 1X로 만들어 사용.

1M Tris-Cl(pH 8.0) stock: 12.11g → 100ml D.W.

100mM EDTA(pH 8.0) stock: 3.722g → 100ml D.W.

2ml + 2ml → 200ml D.W. = 1X EDTA

Reference: Doyle J.J. and J.L.Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin, 19(1): 11-15., Ken Systma's Lab. protocol

Preparation

1. water bath를 60℃에 맞추어 놓는다.

Procedure

1. 채취한 Sample은 엽맥을 제거하고 liquid nitrogen으로 동결시켜 막자사발로 갈아서 -70℃ deep freezer에 보관한다.*1 또는 채취한 생체를 silica gel과 함께 vinyl pack에 보관하여 완전히 건조 시킨다(건조후 그대로 보관).
2. Sample 1g을 50ml polypropylene tube(Sastard or Falcon)에 2X CTAB buffer 10ml를 첨가하여 60℃ water bath넣고 30분간 Incubation 시킨다.*2
3. Incubation 후 Chloroform: Isoamyl alcohol solution(24 : 1 mixture)을 동량(10ml)넣고 뚜껑을 닫은 후 Invert시켜 두세번 섞어준다.
4. 상온에서 3500 rpm, 15분간 centrifugation 한다(Vision, VS 6000CF).*3
5. 상등액만을 다른 tube로 옮긴다.*4
6. 2/3volume(7ml)의 Isopropanol을 첨가하여 뚜껑을 닫고 두세번 invert시킨다(침전을 눈으로 확인할 수 있음).
7. -20℃에서 2시간 이상 침전 시킨다.*5
8. 4℃, 3500 rpm, 15분간 centrifugation. DNA pellet확인.
9. pellet을 제외한 상등액을 모두 제거한 후 10분 정도 방치*6.
10. TE 5ml을 넣어 pellet을 완전히 녹인다*7.
11. 1/2 volume (2.5ml)의 7.5M ammonium acetate를 첨가후 2 volume(15ml)의 100% ethanol*8를 넣은 후 2-3회 Invert 시켜 precipitation되는지 살핀다.
12. -20℃에서 2시간 이상 침전 시킨다.
13. 4℃, 3500 rpm, 15분간 centrifugation. DNA pellet확인.
14. pellet을 제외한 상등액을 모두 제거한 후 10분 정도 방치*6.
15. TE 5ml을 넣어 pellet을 완전히 녹인다.
16. 1/10 volume(500㎕)의 2.5M sodium acetate를 첨가 후 2 volume(11ml)의 100% ehthanol을 넣은 후 2-3회 invert 시킴.
17. -20℃에서 2시간 이상 침전 시킨다.
18. 4℃, 3500 rpm, 15분간 centrifugation. DNA pellet확인.
19. 상등액을 모두 제거한 후 -20℃에 보관시킨 70% ethanol 5ml을 넣어*9 5분간 방치후 tube를 Invert 시켜 ethanol을 모두 제거하고 벽에 묻어있는 방울도 제거시킨 후(kimwipe)시킨 후 10분간 방치시킴.
20. 400 ml의 TE로 완전히 녹임.
21. agarose gel로서 extraction상태를 확인한다. (→ [2] agarose gel loading 참조)

Reference: Doyle J.J. and J.L.Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin, 19(1): 11-15., Ken Bystma's Lab. protocol

*1. 생체를 liquid nitrogen에 갈아서 바로 실험에 들어가는 것이 가장 yield가 높다.

*2. incubation시에 tube의 뚜껑을 꽉 닫으면 안됨. 살짝 열어 놓는다. 10분 간격으로 tube를 흔들어 준다.

*3. CTAB이 있을 시에는 cooling 시키면 안됨.

*4. micropipet을 사용하되 하부의 침전물이 같이 빨려 올라왔을 경우에는 다시 centrifugation시켜 상등액 만을 취한다.

*5. 모든 alcohol precipitation은 충분한 시간을 두어 overnight 시키는 것이 가장 좋다. 급한 경우에는 -70℃ deep freezer에서 15분 정도 침전으로도 가능하다.

*6. tube를 거꾸로하여 pellet은 tube 끝에 붙어 있고, 나머지 상등액은 모두 흘러내리게 한 후 벽에 묻어있는 것도 kimwipe로 닦아낸다.

*7. DNA(and RNA) pellet이 양이 많아 TE가 포화되어 녹지 않을 경우 더 많은 양의 TE를 넣는다. 또는 45℃정도의 water bath에 중탕 하기도 한다. 그러나 1g의 sample을 extraction할 경우 대부분 5ml의 TE에 충분히 녹는다. 다만 pellet에 많은 불순물이 있는 경우 잘 녹지 않는데, 이때는 녹일 수 있는데 까지 녹이고 녹지 않은 pellet 덩어리 들은 centrifuse 시킨 후 버린다.

*8. 95%의 normal grade의 ethanol을 써도 무방하다.

*9. precipitation을 유도한 salt들을 제거하는 역할을 한다.

Reference: Doyle J.J. and J.L.Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin, 19(1): 11-15., Ken Bystma's Lab. protocol