PLANT MOLECULAR BIOLOGY MANUAL

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Kluwer Academic Publishers

Dordrecht / Boston / London

Distributors

for the United States and Canada: Kluwer Academic Publishers, P.O. Box 358, Accord Station, Hingham, MA 02018-0358, USA
for the UK and Ireland: Kluwer Academic Publishers, MTP Press Limited, Falcon House, Queen Square, Lancaster LA1 1RN, UK
for all other countries: Kluwer Academic Publishers Group, Distribution Center, P.O. Box 322, 3300 AH Dordrecht, The Netherlands

ISBN 0-7923-0240-0

Printed on acid-free paper

PRINTED IN BELGIUM

Preface

During the past ten years, great advances have been made in the area of plant molecular biology. Such formerly esoteric techniques as gene transfer and plant regeneration are now routinely performed, making the dissection of regulatory elements of genes a common practice in many laboratories. Along with this new technology has come an almost bewildering array of rapidly changing techniques, often making it difficult for the novice to select and perform the technique most appropriate for answering a given biological question. In 1986, some of us felt that many of these techniques had become routine enough to warrant the publication of a laboratory manual. The manual is designed both for advanced college level laboratory courses and as a 'bench guide' for use in the scientific laboratory. Recognizing the rapidly changing nature of plant molecular biology technology, the editors have designed a laboratory manual that is both easy to use in the laboratory and which will be updated as the techniques change and new technologies are devised. Additional chapters that can replace or be added to this first edition will be published periodically.

The editors recognize that many of the techniques described in this manual depend upon specialized plant genetic material, microbial strains, or recombinant plasmids. Those people desiring such material should contact the relevant authors directly. A list of the various contributors to this manual, including their addresses, is included.

The editors would like to thank the authors for the speed with which they contributed their chapters. Special thanks go to Ms. Wilma Foust (Purdue University) for her secretarial assistance.

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Direct DNA transfer to protoplasts with and without electroporation

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Introduction

The introduction of 'naked' DNA into plant protoplasts, in comparison to transformation methods used for bacterial, yeast and animal cells, has been a relatively recent development. The first conclusive demonstrations of uptake and integration of DNA into plant protoplasts were those of Davey *et al.* [1], Draper *et al.* [2] and Krens *et al.* [3] in which isolated Ti plasmid from *Agrobacterium tumefaciens* was applied to plant protoplasts in the presence of poly-l-ornithine or polyethylene glycol/Ca^{2+}. The presence of the Ti DNA in the plant genome was demonstrated both by the phenotype of hormone auxotrophic growth, production of the expected opine and by Southern blot analysis of DNA from the transformants. The DNA integrated into the genome appeared to be a random assortment of DNA derived from the Ti plasmid. The subsequent development of markers allowing positive selection in plant cells (i.e. antibiotic resistance markers) led to the development of a much simplified protoplast transformation system. Pazskowski *et al.* [4] constructed a simple plasmid based on pUC8 containing a selectable marker, the kanamycin resistance gene from the transposon Tn5 with expression signals from gene VI of the dsDNA virus cauliflower mosaic virus (CaMV). Using this plasmid (pABD1) and an uptake method derived from that of Krens *et al.* [3], they were able to demonstrate the uptake, integration and expression of the resistance marker in protoplasts of *Nicotiana tabacum*. The integrated DNA was shown to be retained through regeneration to plants and inherited through further generations. This methodology has been further developed by various laboratories using different 'chemical' [5, 6, 17] uptake methods or 'electrical' [7, 8] methods. Although the original methods were restricted to protoplasts from relatively amenable Solanaceous species, the methods function for a wide range of different plant or culture types [9–12]. In addition, different selectable markers have now been used successfully [6].

During the course of these developments the transformation frequency which

could be obtained has been increased from approximately 1×10^{-5} per developing microcallus to $1-5 \times 10^{-2}$ [7]. These frequencies and the developments of the techniques now mean that the method can be applied to many different problems and that it can be considered as a real alternative to other methods for the introduction of DNA into plant cells (such as *Agrobacterium tumefaciens*). The main advantage of the method is that the form of the DNA applied to the protoplasts is controlled entirely by the experimenter and not by an intermediate biological vector. Therefore, methods such as co-transformation [13], transformation with λ clones or whole genomic DNA and various other manipulations of the DNA before application to the plant cells are easily carried out. The main disadvantage is that the system requires protoplasts and a functioning system for regeneration of these protoplasts to calli or whole plants. It is therefore not applicable to many plant systems which some laboratories may be using. In addition, the relatively random way in which DNA is integrated into the genome with these techniques means that, for the introduction of non-selectable genes, a thorough characterization of the transformants by Southern blot analysis is necessary to confirm the nature of the integration event. In practice, however, this does not involve much more work than does the characterization of transformation events with all other techniques.

As mentioned above the main application of the technique, apart from analysing the transformation process itself, is in introducing foreign genes to plant cells. This can either be accomplished by constructing a molecule containing a selectable marker and the gene of interest, or more easily by simply mixing DNA of the gene of interest with the selectable marker plasmid in a molar ratio of approximately 3:1 to 10:1, transforming, selecting for the marker and analysing transformants for the presence of the second gene [13]. The method can also be applied with DNA from many different sources. For example, total genomic DNA can be used followed by selection for a marker on the transforming genome. Although as yet this method has only been applied in a model system (using tobacco DNA from a transformant containing a single kanamycin resistance marker), it could be a technique of interest for transfer of uncharacterized genes to new genomes.

The transformation protocol presented here is restricted to mesophyll protoplasts from shoot cultures of *Nicotiana tabacum* cv. Petit Havana SR1 [14] and the plasmid pABD1 [4] that provides resistance to kanamycin. However, the protocol can be modified for application to other protoplast types. Mesophyll protoplasts from several different *Nicotiana* species [4, 6, 17], other Solanaceous [15] species, *Brassica* species [11] and cell culture protoplasts from Solanaceous and Graminaceous [8–10, 12] species have all been successfully transformed. The major factors affecting the efficiency of the method are the concentration of PEG or other uptake agent used and the strength and type of electric pulse applied. These factors should be varied accordingly for the different protoplasts and the optimum method derived empirically. The electroporation method

described is based on that of Neumann *et al.* [16] for animal cells, and uses a relatively high initial field strength (1–1.5 kV) with a low capacitance and, therefore, a short decay time. Other methods have been described with a low initial field strength and long delay time [8]. These methods may have different effects on protoplasts and can certainly lead to good transformation rates. An apparatus for the second method can be relatively easily constructed in the laboratory and is accordingly inexpensive. The method presented here uses a machine produced by Dia-Log GmbH (D-4000 Düsseldorf, West Germany). Although the present method uses kanamycin resistance as a selectable marker, several other markers can be used, perhaps the most effective being hygromycin resistance [6]. The second method presented here does not involve the use of electroporation. We have found that careful optimization of the conditions for washing protoplasts and the conditions during uptake can lead, in some cases, to transformation frequencies comparable to those with electroporation, i.e. approximately 1×10^{-2} [17]. In particular, the magnesium ion concentration in the uptake medium and the PEG concentration appear to be important. This method has been successfully applied to *Nicotiana plumbaginifolia*, albeit with ten-fold lower frequencies. With changes in some steps it could probably also be applied to other species.

Both transformation methods presented here and the method of bead culture of protoplasts are the subject of patent applications. The plasmid pABD1 can be obtained on application to Dr J. Paszkowski, Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland.

Procedures

Preparation, culture and plant regeneration from protoplasts from a sterile shoot culture of N. tabacum

The example given is for protoplasts from shoot cultures of the widely used genotype of *N. tabacum* cv. Petit Havana, SR1 [14]. This material is grown as sterile axenic shoot cultures. The protocol for protoplast isolation is modified from that of Nagy and Maliga [18].

Steps in the procedure

1. Take three fully expanded leaves of the shoot cultures under sterile conditions, place them in a 14-cm petri dish and wet them thoroughly with enzyme solution. Remove the mid-ribs, cut the leaves into squares of 1–2 cm and wet both sides with enzyme solution.
2. Float the pieces, bottom side down, on enzyme solution in a petri dish (10 ml enzyme solution in a 9-cm petri dish). Seal the dishes with Parafilm and incubate overnight at 26 °C in the dark.
3. Gently agitate the mixture and incubate for a further half four to complete digestion. Filter the solution through a 100-μm stainless steel mesh sieve and wash it through with one half volume of the 0.6 M sucrose solution.
4. Mix the protoplast suspension gently and distribute into two capped centrifuge tubes. Centrifuge for 10 min at 600 rpm in a clinical centrifuge. The protoplasts collect at the upper surface of the medium.
5. Remove the medium and pellet from under the protoplasts with a sterile cannula (A.R. Howell Ltd., Kilburn High Rd., London NW6, UK) attached to a 20-ml disposable plastic syringe. This must be done slowly so as to avoid disturbing the layer of protoplasts excessively.
6. Resuspend the protoplasts in K3 medium containing 0.4 M sucrose, and repeat the centrifugation and resuspension steps in fresh medium two times. Take a 0.1-ml sample for counting before the last centrifugation, and dilute it in 0.9 ml of 0.17 M $CaCl_2$ solution.
7. Resuspend the protoplasts following the last flotation step in 0.4 M mannitol, containing 6 mM $MgCl_2$ to stabilize the protoplasts, at a cell density of 1.6×10^6 per ml.

Notes

1. The sterile shoot cultures used for this isolation are established from seed sterilized with sodium hypochlorite (5 min, 1.4% w/v containing 0.05% w/v Tween 80). The shoots are

maintained by subculture every 6 weeks as cuttings on T medium (Nitsch and Nitsch [19], Table 1) solidified with 0.8% w/v cleaned agar at 26 °C in 16 h per day light (1000–2000 lux) in a growth chamber. Take fully expanded, undamaged leaves for the protoplast isolation.

2. One such dish should produce about 3×10^6 protoplasts.
3. Protoplasts can be released from leaf debris by sucking through a pipette with a broken off tip.
5. The protoplasts from two tubes can be combined into one tube for washing.
6. The protoplasts float in this medium. Protoplasts can also be washed by sedimentation in 0.17 M $CaCl_2$, but care should be taken to carry over as little as possible wash solution into the transformation mix.

Solutions (sterile)

In general all solutions and media are sterilized by filtration through 22-μm filters. Some simple salt solutions can be sterilized by autoclaving.

Table 1. The composition of the media used. All of the media shown here are derivatives of media in the indicated publications.

Table 1A. Inorganic salts.

	Medium				
	T[19]	LS[22]	K3[18]	H[23]	A[24]
Macro elements (mg/ml final concentration)					
KNO_3	950	1900	2500	1900	1010
KH_2PO_4	68	170		170	136
NH_4NO_3	720	1650	250	600	800
$NaH_2PO_4 \cdot H_2O$			150		
$CaCl_2 \cdot 2H_2O$	220	440	900	600	440
$MgSO_4 \cdot 7H_2O$	185	370	250	300	740
$(NH_4)_2SO_4$			134		
$NH_4 \cdot$ Succinate					50
Micro elements[a] *(mg/l final concentration)*					
Na_2EDTA	74.6	74.6	74.6	74.6	74.6
$FeCl_3 \cdot 6H_2O$	27.0	27.0	27.0	27.0	27.0
H_3BO_3	10.0	6.2	3.0	3.0	3.0
KI		0.83	0.75	0.75	0.75
$MnSO_4 \cdot H_2O$	17.25	16.9	10.0	10.0	10.0
$ZnSO_4 \cdot 7H_2O$	10.0	8.6	2.0	2.0	2.0
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25
$CoCl_2 \cdot 6H_2O$			0.025	0.025	0.025
$CoSO_4 \cdot 7H_2O$		*0.03*			

[a] Macroelements are usually made up as 10×, and microelements as 1000× concentrated stock solutions. Na_2EDTA and $FeCl_3$ are kept as separate stock solutions 200× concentrated.