Methods in Plant Molecular Biology

A LABORATORY COURSE MANUAL

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Front cover: A chimeric tobacco plant with transformed and wild-type plastid genome copies. In the yellow sectors the photosynthetic *psbA* gene was replaced with the bacterial *aadA* gene by targeted gene insertion. Plant growth is supported by the photosynthetically competent green sectors. (Photograph by Zora Svab and Pal Maliga [Waksman Institute, Rutgers University].)

Back cover: In situ hybridization was used to detect the activation of the pathogenesis-related PR-1 genes in a tobacco plant responding to an infection by tobacco mosaic virus. Darkfield microscopy reveals the presence of silver grains over the leaf section indicating the accumulation of PR-1 mRNA within the cells of this infected leaf. (Photograph by David C. Dixon [U.S. Department of Agriculture/ARS/SRRC] and Daniel F. Klessig [Waksman Institute, Rutgers University].)

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Methods in Plant Molecular Biology

Preface

This manual began as a collection of laboratory protocols handed out to participants of the three-week course Molecular and Developmental Biology of Plants, held every summer since 1981 at Cold Spring Harbor Laboratory. The editors of this book were instructors on the course with an overlapping tenure for a total of four years: Pal Maliga (1989–1992), Joseph Varner (1989–1991), Wilhelm Gruissem (1989–1990), Daniel Klessig (1991–1992), and Anthony Cashmore (1992). The "plant course" was established by Frederick Ausubel (1981–1982) and John Bedbrook (1981–1982), and subsequently organized by Ian Sussex (1983–1988), Russel Malmberg (1983), Joachim Messing (1984–1988), and Robert Horsch (1984, 1986, 1987). Since its inception, the course has been supported by the National Science Foundation.

The course program consisted of a series of daily lectures to present a comprehensive overview of the frontiers in plant sciences and hands-on teaching of molecular techniques in laboratory sessions. The laboratory sessions were designed to demonstrate a diverse set of principal methods in plant biology. Since the experiments were sometimes performed with participants who had limited experience in molecular biology, we tried to prepare as detailed and specific a protocol as feasible. The manual reflects the philosophy of the training course: it is a collection of detailed protocols, each designed to both demonstrate a principal method and maximize the probability of success.

Each chapter of the laboratory manual is authored by the instructor responsible for teaching the protocol at the course. As a consequence, we had to make difficult choices in selecting among similar protocols taught by different scientists during the years. As a principle, in the case of two essentially identical procedures, we chose the protocol of the instructor who was participating in the course at the time the decision was made to publish the course manual. This is why chapters by Ken Keegstra, Chris Sommerville, and Sue Gibson are missing, although they proposed and implemented laboratory exercises at the course that were later taught by others. We would like to take this opportunity to thank them and all the other invited instructors and speakers for their contributions to the course, as well as for their dedicated service in training a new generation of plant scientists. We are also indebted to our colleagues at Cold Spring Harbor Laboratory who have supported the course over the years, and who have often found their own research programs grind to a halt when course participants suddenly needed their equipment.

We also extend our gratitude to the staff of Cold Spring Harbor Labora-

tory Press, particularly Nancy Ford and John Inglis, who kept the project of assembling this laboratory manual alive, and Maryliz Dickerson, Dorothy Brown, Lee Martin, and Joan Ebert for their excellent editorial and technical assistance. A great part of the credit should go to Catriona Simpson, whose careful editing made this manual more accessible, and who executed her work with extreme patience and understanding.

The Editors

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• Section 1

Polyethylene Glycol-mediated Transformation of Tobacco Leaf Mesophyll Protoplasts: An Experiment in the Study of Cre-*lox* Recombination

This section describes a protocol for the transformation of plant leaf mesophyll protoplasts with nucleic acids and the transient expression of the encoded proteins. Although the use of stably transformed materials may be preferable in most instances, the transient introduction of macromolecules into plant protoplasts permits a more rapid analysis of the biological activity of the introduced material. The transient assay approach has facilitated, for example, the identification of *cis*-acting regulatory sequences of promoter regions (see, e.g., Ebert et al. 1987; Ellis et al. 1987; Ow et al. 1987), the comparison of transcriptional strength among different promoters (see, e.g., Fromm et al. 1985; Boston et al. 1987; Hauptmann et al. 1987), and the study of environmental factors that affect gene expression (see, e.g., Howard et al. 1987; Marcotte et al. 1988). In experiments in which cells are transformed with RNA molecules, notable progress has been made in defining the *cis* requirements for efficient maturation, stability, and translation of messenger and viral RNAs (see, e.g., Callis et al. 1987; Gallie et al. 1989). More recently, proteins have been introduced transiently into plant cells in the analysis of transcription factors (Katagiri and Chua 1992). In this section, the biological activity of a DNA molecule is examined in transgenic plant cells, exemplifying the combined use of stably transformed and transiently introduced gene constructs.

POLYETHYLENE GLYCOL-MEDIATED TRANSFORMATION

A large variety of methods have been developed to transfer DNA into plant cells (for review, see Potrykus 1991). Among these, polyethylene glycol (PEG)-mediated transformation has been used to establish both stable transformation and transient gene expression (Krens et al. 1982; Paszkowski et al. 1984; Shillito et al. 1985). PEG-mediated trans-

formation offers several attractive features. First, conditions that maximize transient gene expression are associated with high survival and division rates in the transformed cells (Negrutiu et al. 1990). Second, PEG-mediated transformations utilize common, inexpensive supplies and equipment. Finally, extensive studies have demonstrated that this simple and reliable method can be adapted to a wide range of plant species and tissue sources used for protoplast preparation. The protocol presented here was devised to study the site-specific recombination of a transgenic construct within the genome of tobacco (*Nicotiana tabacum*) leaf mesophyll protoplasts.

EXPERIMENTAL DESIGN

The objective of this experiment is to detect an inversion event in the tobacco genome mediated by transforming DNA. The site-specific recombinase is the 38.5-kD product of the bacteriophage P1 *cre* (control of *re*combination) gene, which catalyzes recombination between 34-bp sequences known as *lox* (*lo*cus of cross[*x*]over) sites. As has been shown previously, this site-specific recombination system can mediate the excision, inversion, and cointegration of DNA in plant cells (Dale and Ow 1990, 1991; Odell et al. 1990). The target of the recombinase in this experiment is a construct, pED32 (Dale and Ow 1990), that is stably incorporated into the *Nicotiana tabacum* genome by *Agrobacterium*-mediated transformation. This construct consists of a cauliflower mosaic virus *35S* RNA promoter (*35S*) transcriptionally fused to a fragment containing a firefly luciferase cDNA-nopaline synthase poly(A) region (*luc-nos3'*). However, as shown in Figure 1, the *luc-nos3'* fragment is in an inverted orientation relative to the *35S* promoter. This fragment is also flanked by a *lox* site on each end, but the two sites are of opposing orientations. Transcription of the *luc* cDNA would require inversion of the *luc-nos3'* fragment via recombination at the flanking *lox* sequences. The recombinase required for this inversion event is provided by the transient expression of *cre* from pMM23, a *35S-cre-nos3'* construct. This chimeric *cre* gene is similar to the previously described pED23 (Dale and Ow 1990) except that the prokaryotic ribosome binding site has been removed. The Cre recombinase produced by pMM23 must enter the plant nucleus to catalyze the site-specific inversion event, which can then be scored as luciferase activity (light production). The efficiency of DNA transformation can be assessed using a positive control construct such as pDO432, a *luc* expression plasmid (Ow et al. 1986). For a recent review of the *luc* marker gene, see Millar et al. (1992).

Table 1 illustrates the data from a typical experiment. Without the addition of a *cre* expression plasmid, leaf mesophyll protoplasts

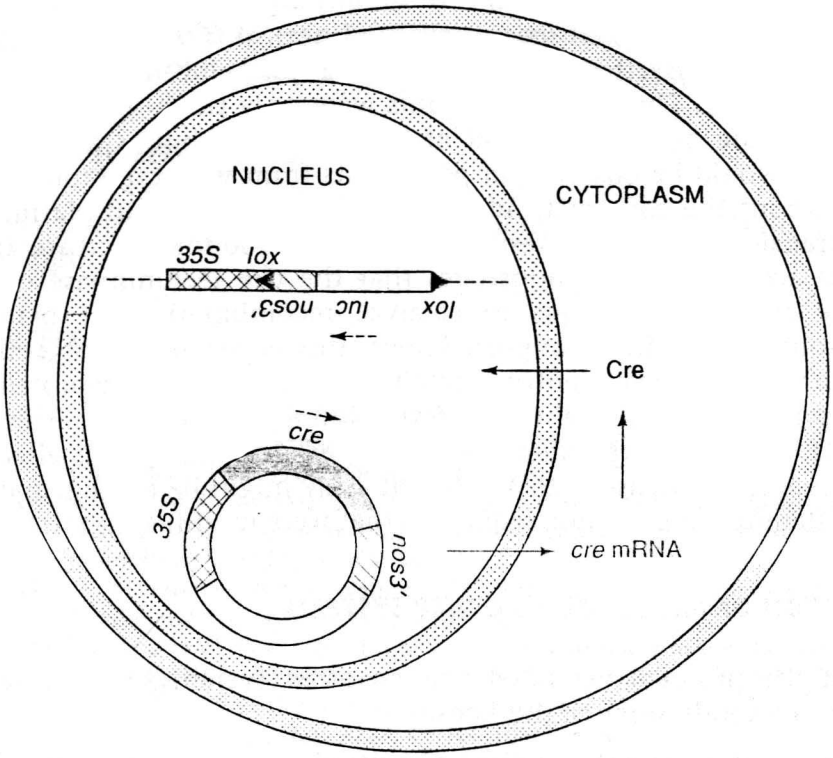

Figure 1
Cre-mediated site-specific inversion of DNA in the plant genome. The Cre recombinase, expressed from the transforming DNA, flips the coding region of the *luc* transgene which results in luciferase activity.

Table 1 **Cre-induced Inversion of Plant Chromosomal DNA**

Transgenic *luc* Plants	Transforming DNA[1]	Light Units[2]	Relative Activity
nt35.9	carrier only	820	1
nt35.9	carrier + 10 μg of pMM23	1.14×10^5	139
nt35.10	carrier only	1190	1
nt35.10	carrier + 10 μg of pMM23	1.57×10^5	132

[1]Transformation of approximately 2×10^6 protoplasts with 100 μg of calf thymus carrier DNA and 10 μg of pMM23, where indicated.
[2]Extracts were prepared 26 hours after transformation. Each extract was adjusted to 1 ml total volume with luciferase extraction buffer. 100 μl of extract were combined with 100 μl of luciferase reaction buffer followed by injection of 100 μl of 0.5 mM D-luciferin. Light emission was counted for 30 seconds in integrated mode. An average of three readings was taken. The baseline count of a blank (extraction buffer only) was 279 light units.

from two independent transgenic tobacco lines (nt35.9 and nt35.10) that harbor the inverted luciferase construct produced very little luciferase activity. These baseline counts were only three- to fourfold higher than the background level of the extraction buffer alone or values that would be observed in protoplasts from wild-type tobacco plants without the construct. Addition of the *cre* expression plasmid to the transformation mix resulted in over a 100-fold increase in luciferase activity. This demonstrates that the Cre recombinase can penetrate the plant nucleus to catalyze recombination of plant chromosomal DNA. In its original intent, this experiment was conducted to test the nuclear entry capability of this prokaryotic protein prior to proceeding with stable transformation experiments (Dale and Ow 1991). Since this experiment demonstrated that the assay is effective, a possible future use of this transient assay might be for the analysis of different Cre-encoding sequences that were modified in vitro.

ADAPTATION OF PROTOCOL TO OTHER SYSTEMS

To adapt the protocol described here to other systems, it may be necessary to modify some of the steps.

- The tissue source and the method of protoplast preparation may affect the transformation process (Negrutiu et al. 1987, 1990). It has been suggested that the results of transformation experiments are superior if the protoplasts are isolated by methods in which the cell wall is digested under mild conditions for a long period of time (e.g., overnight) (Negrutiu et al. 1990). For advice on alternative methods of protoplast isolation, see Potrykus and Shillito (1986).
- Researchers have noted that heat shock increases levels of transient expression in some systems (Oliveiria et al. 1991) but decreases levels of transient expression in others (Negrutiu et al. 1990). It may be useful to test the transformation with and without the brief heat treatment.
- If DNA is being used in the transformation process, the topology of the plasmid template (circular versus linear) may affect expression levels (Ballas et al. 1988). If the plasmid is linearized, cleavage at a site distant from the 3′ end of the gene may enhance levels of gene expression (Negrutiu et al. 1990).
- The divalent cation in the PEG solution is another variable worth testing. Negrutiu et al. (1990) have suggested that the use of Ca^{++} instead of Mg^{++} results in higher transient expression levels and that a divalent cation concentration of 5–15 mM results in a good balance between cell survival and DNA uptake.
- Finally, different PEG concentrations and treatment times should be tested. In addition, the source and molecular weight of the PEG