Springer-Science+Business Media, B.V.

Dear Reader

We would very much appreciate receiving your suggestions and criticisms for the *Plant Molecular Biology Manual, 2ⁿᵈ Edition*. They will prove to be most helpful during our preparations for future supplements.

Would you please answer the questions listed below, and send your comments with any further suggestions you may have, to *Jacco Flipsen* at the abovementioned address. Alternatively you may send a fax to +31 78 6392 334 or e-mail, BIOSCI@wkap.nl.

Thank you for your assistance!

Jacco Flipsen
Publisher

— —

PLANT MOLECULAR BIOLOGY MANUAL

1. What errors have you found? (list page numbers and describe mistakes)
2. What protocols do you find to be confusing or lacking in detail? (list chapter numbers and page numbers and describe problems)
3. What protocols do you feel should be replaced in future supplements with newer (better) methods?
4. What new topics or other material would you like to see included in future supplements?

Please print or type your answers in the space below, and continue overleaf.

Name: Date:

Address:

PLANT MOLECULAR BIOLOGY MANUAL

PLANT MOLECULAR BIOLOGY MANUAL

Second edition

Edited by

STANTON B. GELVIN
Purdue University, West Lafayette, Indiana, USA

ROBBERT A. SCHILPEROORT
Leiden University, Leiden, The Netherlands

Neither Kluwer Academic Publishers nor any person acting on its behalf is responsible for the use which might be made of the information contained herein.

Printed on acid-free paper

Plant Molecular Biology Manual, 2nd Edition

Supplement 4, 2000

INSTRUCTIONS FOR SUPPLEMENT 4

Preliminary pages
Pages I–XVIII should be replaced by new pages i–xxi.

Section E
Add after Chapter E2:

Chapter E3:   A.H. Meijer, J. Schouten, P.B.F. Ouwerkerk, J.H.C. Hoge/Yeast as versatile tool in transcription factor research

Section H
Add after Chapter H4:

Chapter H5:   S. Choi, R.A. Wing/The construction of bacterial artificial chromosome (BAC) libraries

Section N
Insert Chapters N1–N3:

Chapter N1:   L.A. Lyznik, D. Peterson, Z.-Y. Zhao, X. Guan, B. Bowen, B. Drummond, G. St. Clair, L. Tagliani, C. Baszczynski/Gene transfer mediated by site-specific recombination systems

Chapter N2:   C. Machida, H. Onouchi, E. Semiarti, T. Ishikawa, Y. Machida/Use of the R-RS site-specific recombination system in plants

Chapter N3:   H. Albert, D.W. Ow/Cre-lox directed integration of transgenes into the tobacco genome

Section O
Insert Chapter O1:   C.J. Staiger, B.C. Gibbon, H. Ren/Plant actin isolation and characterization

Index
Replace

Contents

*** Included in Supplement 3.

** Included in Supplement 2.
*** Included in Supplement 3.

SECTION F: BLOTTING AND GENE DETECTION SYSTEMS

SECTION G: *IN SITU* HYBRIDIZATION AND IMMUNODETECTION

---

* Included in Supplement 1.
** Included in Supplement 2.
**** Included in Supplement 4.

** Included in Supplement 2.
*** Included in Supplement 3.
**** Included in Supplement 4.

---

* Included in Supplement 1.
*** Included in Supplement 3.

*** Included in Supplement 3.
**** Included in Supplement 4.

List of Contributors

Author	Chapter	Address
Albert, H.	N3	Tropical Fruit, Vegetable, and Sugarcane Research Laboratory, USDA/ARS, 99-193 Aiea Heights Dr., Aiea, HI 96701, USA
Baszczynski, C.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA
Bendich, A.J.	D1	Botany Dept. KB-15, University of Washington, Seattle WA 98195, USA
Bennett, M.J.	K3	University of Warwick, Dept. of Biological Sciences, Coventry CV4 7AL, Great Britain
Bent, A.F.	B7	Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.
Bisaro, D.M.	L2	Ohio State University, Biotechnology Center, 1060 Carmack Road, Columbus OH 43210, U.S.A.
Bisseling, T.	D5	Dept. of Molecular Biology, Transitarium, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands
Boutry, M.A.	J2	Biochemie Physiology, University of Louvain, Croix du Sud 2-20, 1348 Louvain-La-Neuve, Belgium
Bowen, B.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA
Castle, L.A.	B5	Oklahoma State University, Botany LSE 104, Stillwater OK 74078, USA
Chapman, S.N.	L1	University of Florida, CREC, Lake Alfred, FL 33850, USA
Chee, P.P.	F4	Molecular Biology Unit 7242, The Upjohn Company, Kalamazoo, MI 49007, USA
Choi, S.	H5	Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843, USA; Departments of Agronomy & Biological Sciences, Clemson University Genome Center, Clemson University, Clemson, SC 29634, USA
Christou, P	A2	Agracetus, Inc., Research & Development, 8520 University Green, Middleton WI 53562, USA

Citovsky, V. | J4 | U.C. Berkeley, Plant Biology, Berkeley CA 94720, USA

Clough, S.J. | B7 | Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, U.S.A.

Crosby, W.L. | M1 | Gene Expression Group, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon SK, S7N OW9, Canada

Dawson, W.O. | L1/L4 | University of Florida, CREC, Lake Alfred, FL 33850, USA

De Jong, H. | G5 | Department of Genetics, Dreijenlaan 2, Wageningen Agriculture University, Wageningen, The Netherlands

Dessaux, Y. | C3 | Institut des Sciences Végétales, Bâtiment 23 C.N.R.S., Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

Dilkes, B.P. | K3 | University of Arizona, Dept. of Plant Sciences, Tucson, Arizona 85721, USA

Dreyer, D.A. | G3 | Texas A & M University, Biology Dept., College Station, TX 77843-3258, USA

Drong, R.F. | F4 | Molecular Biology Unit 7242, The Upjohn Company, Kalamazoo, MI 49007, USA

Drummond, B. | N1 | Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA

Duck, N. | G1 | Monsanto Company, 700 Chesterfield Village Parkway, St. Louis MO 63198, USA

Feldmann, K.A. | K3 | University of Arizona, Dept. of Plant Sciences, Tucson, Arizona 85721, USA

Ferl, R.J. | I3 | Dept. of Botany, University of FLorida, Gainesville FL 32611, USA

Fischer, A. | F6 | c/o F. Hoffmann-LaRoche AG, PRPG 66/704, Grenzacherstrasse, CH-4070 Basel, Switzerland

Fransz, P. | G5 | Department of Molecular Biology, Dreijenlaan 3, Wageningen Agricultural University, Wageningen, The Netherlands

Gelvin, S.B. | B4 | Dept. of Biological Sciences, Purdue University, Lilly Hall of Life Sciences, West Lafayette IN 47907, USA

Gibbon, B.C. | O1 | Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

Gronenborn, B. | L3 | C.N.R.S., Institut des Sciences Végétales, 91198 Gif-sur-Yvette, France

Guan, X.   N1   Department of Crop Protection, Pioneer
                              Hi-Bred International, Inc. P.O. Box 1004,
                              Johnston, IA 50131, USA

Haughn, G.W.   M1   Department of Botany, University of British
                              Columbia, 3529-6270 University Boulevard,
                              Vancouver BC, V6T IZ4 Canada

Hille, J.   K2   Genetics, Free University, De Boelelaan
                              1007, 1001 HV Amsterdam, The Netherlands

Hinchee, M.   B6   Monsanto, Plant Protection Improvement,
                              700 Chesterfield VP, St. Louis MO 63198,
                              USA

Hoge, J.H.C.   E3   Institute of Molecular Plant Sciences, Leiden
                              University, Clusius Laboratory, P.O. Box
                              9505, 2300 RA Leiden, The Netherlands

Hooykaas, P.J.J. B3   Clusius Laboratory, Wassenaarseweg 64,
                              2333 AL Leiden, The Netherlands

Huijser, P.   K1   MPI für Züchtungsforschung, Carl von Linne
                              Weg 10, 5000 Köln 30, Germany

Ishikawa, T.   N2   Laboratory of Developmental Biology, Divi-
                              sion of Biological Science, Graduate School
                              of Science, Nagoya University, Chikusa-ku,
                              Nagoya 464-8602, Japan

Iwabuchi, M.   I1   Kyoto University, Faculty of Science/botany,
                              Ktrashirikawa, Kyoto 606-01, Japan

Jacobs, M.   G2   Plantengenetica VUB, University of Brus-
                              sels, Paardenstraat 65, St. Genesius Rode,
                              1640 Belgium

Jupin, I.   L3   Institut des Sciences Végétales, CNRS,
                              91198 Gif sur Yvette, Cedex, France

Karcher, S.J.   F3   Dept. of Biological Sciences, Purdue Univer-
                              sity, B-315 Lilly Hall, W. Lafayette IN
                              47907-13902, USA

Kay, S.A.   I2   Dept. of Biology, Gilmer Hall, University of
                              Virginia, Charlottesville WV 22901, USA

Kearney, C.M.   L1   University of FLorida, CREC, Lake Alfred
                              FL 33850, USA

Keegstra, K.   J1   University of Wisconsin, Dept. of Botany,
                              430 Lincoln Drive, Madison WI 53706, USA

Knight, H.   C4   University of Oxford, Dept. of Plant Sci-
                              ences, Oxford, OX1 3RB, UK

Knight, M.R.   C4   University of Oxford, Dept. of Plant Sci-
                              ences, Oxford, OX1 3RB, UK

Kohalmi, S.E.   M1   Department of Plant Science, University of
                              Western Ontario, 1151 Richmond Street N.,
                              London ON, N6A 5B7, Canada

Koncz, C.	B2	Max-Planck Institut für Züchtungsforschung, 5000 Köln 30, Germany
Lam, E.	I4	Rutgers University, AgroBiotech Center, Waksman University, Piscataway NJ 08854, USA
Laskowski, M.J.	F5	Dept. of Biology, Williams College, Williamstown, MA 01267, USA
Lee, Jeong Hee	C5	Universität Tübingen, Lehrstuhl für Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
Lindbo, J.A.	L4	CREC, University of Florida, Lake Alfred, FL 33850, USA
Lyznik, L.A.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA; Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47906, USA
Machida, C.	N2	Laboratory of Developmental Biology, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan; On leave from Kirin Brewery Co., Ltd. Central Laboratories for Key Technology, 1-13-5 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan
Machida, Y.	N2	Department of Plant Science, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan
MacKenzie, S.	D3	Dept. of Agronomy, Lilly Hall of Life Sciences, Purdue University, Lafayette IN 47907, USA
Martin, G.B.	H2	Purdue University, Dept. of Agronomy, 1150 Lilly Hall, West Lafayette IN 47907-1150, USA
Meijer, A.H.	E3	Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, P.O. Box 9505, 2300 RA Leiden, The Netherlands
Memelink, J.	F1	Clusius Lab./Molecular Plant Sciences Inst., Leiden University, Wassenaarsewag 64, 2333 AL Leiden, The Netherlands
Nickelsen, J.	D6	Lehrstuhl für Allgemeine Botanik, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany

Nowak, J.	M1	Gene Expression Group, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon SK, S7N OW9, Canada
Onouchi, H.	N2	Laboratory of Developmental Biology, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan
Ouwerkerk, P.B.F.	E3	Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, P.O. Box 9505, 2300 RA Leiden, The Netherlands
Ow, D.W.	N3	Plant Gene Expression Center, USDA/ARS, 800 Buchanan St., Albany, CA 94710, USA
Peterson, D.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA
Peytavis, R.	A3	Wageningen Agriculture University, Department of Molecular Biology, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
Pogue, G.P.	L4	CREC, University of Florida, Lake Alfred, FL 33850, USA
Potrykus, J.	A1	Institute of Plant Sciences, Eth Zentrum LFV-E20, 8092 Zürich, Switzerland
Price, C.A.	D4	Waksman Institute, Rutgers State University, Piscataway NJ 08855-0759, USA
Rafalski, A.	H4	DuPont Co. Agricultural Products & Biotechnology, P.O. Box 80402, Wilmington DE 19880-0402, USA
Raikhel, N.	J3	Michigan State University, Plant Research Laboratory, East Lansing MI 48824, USA
Reader, L.J.V.	M1	Gene Expression Group, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon SK, S7N OW9, Canada
Ren, H.	O1	Department of Biology, Beijing Normal University, Beijing 100875, China
Saedler, H.	F6	Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829, Köln, Germany
Samach, A.	M1	Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver BC, V6T IZ4 Canada
Schöffl, F.	C5	Universität Tübingen, Lehrstuhl für Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany

Schulz, B.	K3	University of Arizona, Dept. of Plant Sciences, Tucson, Arizona 85721, USA
Schouten, J.	E3	Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, P.O. Box 9505, 2300 RA Leiden, The Netherlands
Semiarti, E.	N2	Laboratory of Developmental Biology, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan
Shakya, R.	A3	Friedrich Miescher Institut, PO-box 2543, CH-4002 Basel, Switzerland
Sherrier, D.J.	G3	Texas A & M University, Biology Dept., College Station, TX 77843-3258, USA
Slightom, J.L.	F4	Molecular Biology Unit 7242, The Upjohn Co., Kalamazoo MI 49007-0199, USA
Snaed, M.	H1	Strategene Cloning Systems, La Jolla CA 92037, USA
Somssich, I.E.	E1	Max-Planck Institut, Biochemie, Carl von Linne Weg 10, 5000 Köln 30, Germany
Spiker, S.	D2	3530 Gardner Hall, North Carolina State University, Raleigh NC 27695, USA
Staiger, C.J.	O1	1392 Lilly Hall of Life Sciences, Dept. of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA
St. Clair, G.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA
Sunter, G.	L2	Ohio State University, Biotechnology Center, 1060 Carmack Road, COlumbus OH 43210, USA
Tagliani, L.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA
Teeri, T.	C2	Dept. of Genetics, University of Helsinki, Arkadiankatu 7, 00100 Helsinki, Finland
Theissen, G.	F6	Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829, Köln, Germany
Trewavas, A.J.	C4	University of Edingburgh, Institute of Cell and Molecular Biology, Edinburgh, EH9 3JH, UK
Turpen, T.H.	L1/L4	Biosource Technologies, Inc. 3333 Vaca Valley Pkwy, Vacaville, CA 95688, USA
Vandenbosch, K.	G3	Texas A & M University, Biology Dept., College Station TX 77843, USA

Van Kammen, A.	A3	Wageningen Agricultural University, Department of Molecular Biology, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
Van Montagu, M.	C1	Laboratorium Genetica, University Gent, Ledeganckstraat 35, 9000 Gent, België
Van Wordragen, M.	A3	ATO-DLO, PO-box 17, 6700 AA Wageningen, The Netherlands e-mail: m.f.vanwordragen@ato.dlo.nl
Velten, J.	B1	P.G.E.L., New Mexico State University, Box 3GL, Las Cruces NM 88003, USA
Verkerk, R.	A3	Wageningen Agricultural University, Department of Food Science, Bomenweg 2, 6703 HD Wageningen, The Netherlands
Werr, W.	F2	Institut für Genetik, Universität zu Köln, Germany
Wing, R.A.	H5	Departments of Agronomy & Biological Sciences, Clemson University Genome Center, Clemson University, Clemson, SC 29634, USA
Woo, Ho-Hyung	G4	University of Zrizona, Dept. of Plant Pathology and Molecular and Cellular Biology, Tucson, AZ 85721, USA
Yamazaki, K.	E2	Research Institute for Biochemical Regulation, School of Agricultural Sciences, Nagoya University, Nagoya, 46401, Japan
Zabel, P.	H3	Dept. of Molecular Biology, Agriculture University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands
Zabel, P.	A3	Corresponding author: e-mail: pim.zabel@mac.mb.wau.nl
Zabel, P.	G5	Department of Molecular Biology, Dreijenlaan 3, Wageningen Agriculture University, Wageningen, The Netherlands
Zhao, Z.-Y.	N1	Department of Crop Protection, Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA

The Construction of Bacterial Artificial Chromosome (BAC) Libraries

SANGDUN CHOI[1,2] and ROD A. WING[2]*

[1]*Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843, USA;*
[2]*Clemson University Genomics Institute, 100 Jordan Hall, Clemson, SC 29634, USA*
* Author for correspondence

Introduction

Cloning of exogenous DNA into bacterial artificial chromosomes (BACs) provides a new approach to the analysis of the genomes of higher organisms [1]. BAC libraries containing large genomic DNA inserts are important tools for positional cloning, physical mapping and genome sequencing. A number of human and plant BAC libraries have been constructed (e.g., human: [2], *Arabidopsis*: [3], rice: [4], sorghum: [5]). Bacterial artificial chromosome vectors utilize the *E. coli* single-copy fertility plasmid and can maintain genomic DNA fragments up to 350 kb. Very little or no rearrangement of the inserts or chimerism have been observed [1, 5–7]. Other systems for the cloning of large DNA fragments have been developed. The development of yeast artificial chromosome vectors (YAC: [8]) permits cloning of fragments of greater than 500 kb. However, some disadvantages of the YAC system include a high degree of chimerism and insert rearrangement that limit its usefulness [9–11]. Systems based on the bacterial F-factor (BAC: [1]) and bacteriophage P1 (PAC: [12]) have much higher cloning efficiencies, improved fidelity, and greater ease of handling as compared to the YAC system. Table 1 shows a comparison between the two most common large DNA fragment cloning systems, YACs and BACs. Because of BAC clone stability and ease of use, the BAC cloning system has emerged as the system of choice for the construction of large insert genomic DNA libraries.

Table 1. Comparison between YAC and BAC cloning systems.

Features	YAC	BAC
Configuration	Linear	Circular
Host	Yeast	Bacteria
Copy number/cell	1	1–2
Cloning capacity	Unlimited	up to 350 kb
Transformation	Spheroplast ($\leq 10^7$ T/μg)	Electroporation ($\leq 10^{10}$ T/μg)
Chimerism	up to 40%	None to low
DNA isolation	Pulsed-field-gel-electrophoresis – Gel isolation	Standard plasmid miniprep
Insert stability	Unstable	Stable

BAC vectors

The basic structure of BAC vectors is derived from the bacterial F factor (Figure 1). This backbone contains four essential regions that function in plasmid
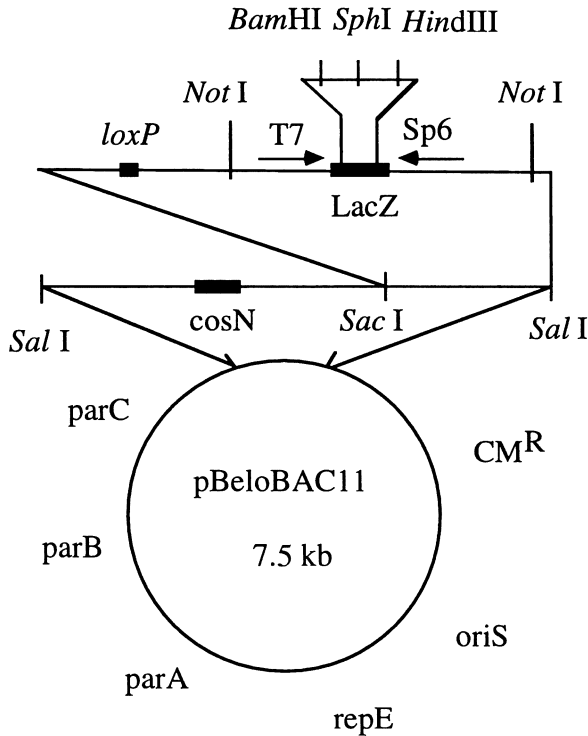
Fig. 1. Diagram of pBeloBAC11 vector. The plasmid is based on a mini-F plamid, pMBO131 [16]. CM^R = chloramphenicol resistance.

stability and copy number [13]. *parA, parB,* and *parC* are required for ParFIA partitioning. *parB* and *parC* are also required for incompatibility with other F factors. *oriS* is the origin of RepFIA replication and is unidirectional. *repE* encodes RepFIA protein E that is autoregulatory and essential for replication from *oriS*. A chloramphenicol resistance gene was incorporated for antibiotic selection of transformants. The most widely used BAC vector, pBeloBAC11, has the three unique cloning sites *Hind*III, *Bam*HI and *Sph*I within the *lacZ* gene and thus allows identification of recombinants by colony color through α complementation [2].

   Additional BAC vectors have been derived from pBeloBAC11 and are listed in Table 2. pECSBAC4 [14] has an unique *Eco*RI cloning site and pBACwich (Choi *et al.,* in preparation) has a promoterless hygromycin gene for transformation into plants. BIBAC2 is capable of replicating in both *E. coli* and *A.*

Table 2. Existing BAC vectors.

Name	Cloning sites	Recombinant selection	Features	Reference
pBAC108L (6.7 kb)	*Hind*III, *Bam*HI	none		[1]
pBeloBAC11 (7.5 kb)	*Hind*III, *Bam*HI, *Sph*I	*lac*Z		[2]
pECSBAC4 (9.3 kb)	*Eco*RI, *Hind*III, *Bam*HI	*lac*Z		[14]
BIBAC2 (23.5 kb)	*Bam*HI	*sac*BII	Plant transformation via *Agrobacterium*	[15]
pBAC wich (11 kb)	*Hind*III, *Bam*HI, *Sph*I	*lac*Z	Plant transformation via site-specific recombination	Choi *et al.,* unpublished
pBACe3.6 (11.5 kb)	*Bam*HI, *Sac*I, *Sac*II, *Mlu*I, *Eco*RI, *Ava*III	*sac*BII	High copy number is available	de Jong *et al.,* unpublished
pClasper (9.7 kb)	homologous recombination in yeast	*LEU2*	Yeast and bacteria shuttle vector	[17]

tumefaciens and has the characteristics of the Ti plasmid DNA [15]. pBACe3.6 allows positive selection for insert-containing BAC clones through inclusion of the sacBII gene. Additionally, pBACe3.6 contains a 'pUC-link' that increases the copy number of the vector for plasmid purification prior to BAC library construction (de Jong *et al.,* 1997: http:// 128.205.167.214/ cgi-bin/ vector-query? Vector= pBACe3.6 &Choice=I).

Plant transformation BAC vectors

DNA transfer into plants has been accomplished by several methods including *Agrobacterium*-mediated transformation, electroporation, biolistic bombardment, and microinjection. BAC vectors have been engineered for transformation of large DNA inserts into plant genomes. BIBAC (binary bacterial artificial chromosome) has been designed to replicate in both *E. coli* and *A. tumefaciens* and has all of the features required for transferring large inserts of DNA into plant chromosomes [15]. BIBAC test constructs containing 150 kb human DNA were introduced into several *A. tumefaciens* strains. Of these strains, those containing additional copies of *virG* or *virG/virE* produced transgenic tobacco plants in which the entire 150 kb human DNA fragment integrated into the tobacco genome randomly. Recently, a pBACwich system has been developed to achieve site-directed integration of DNA into the genome (Choi *et al.,* unpublished work). A 150 kb cotton BAC DNA was transferred into a specific *lox* site in tobacco by biolistic bombardment and Cre-*lox* site specific recombination. These results open up a number of new possibilities for plant molecular biology and the genetic engineering of plants. These systems will streamline positional cloning and the transfer of desirable traits into plants.

Bacteria-yeast shuttle vectors

The bacteria-yeast shuttle vector, pClasper, combines the bacterial origin of replication from BAC vectors with the *CEN6/ARS*4 yeast replication machinery and includes a yeast *LEU*2 gene and the bacterial chloramphenicol resistance gene for selection in yeast or bacteria, respectively. Bradshaw *et al.* [17] also cloned homologous sequences flanking the region to be targeted into the polylinker of pClasper to isolate regions from within a YAC. After transformation of linearized plasmids into the *LEU*⁻ strains carrying a specific YAC, yeast colonies were selected for the acquisition of the *LEU*⁺ phenotype. The recombinant was then shuttled to bacteria for preparation of plasmid DNA. Transformation-associated recombination (TAR: [18, 19]) cloning can be applied to the generation of circular YACs by using a single centromere vector containing various plant repeats. If the TAR vector contains an *E. coli* mini-F factor origin of replication and chloramphenicol resistance gene from the BAC vectors, circular YACs can be moved into bacterial cells and easily separated from yeast chromosomes. YACs are important tools for cloning large genome regions. However, it is laborious to isolate enough YAC DNA from yeast. The bacteria-yeast shuttle vectors are useful to clone specific regions from YACs, from hybrid cells, or directly from the genome, and advantageous for isolating DNA.

Megabase-size DNA isolation from plants

There are two general methods for preparing megabase-size DNA from plants. The protoplast method yields megabase-size DNA of high quality with minimal breakage, but the process is costly and labor-intensive. For example, to prepare protoplasts from tomato, young leaves are manually feathered with a razor blade before being incubated for four to five hours with cell-wall-degrading enzymes [20]. Furthermore, because each plant species requires a different set of conditions to generate protoplasts, the method will only work if a high-yielding protoplast method has been optimized for a given plant species.

Zhang *et al.* [21] developed a universal nuclei method that works well for several divergent plant taxa. Fresh or frozen tissue was homogenized with a blender or mortar and pestle, respectively. Nuclei were then isolated and embedded as described in the Materials and Methods section. DNA isolated from nuclei was often more concentrated and was shown to contain lower amounts of chloroplast DNA. Although the nuclei method was universal, it produced a relatively high degree of sheared DNA. The primary advantage of the method is that it is economical and not as labor-intensive as the protoplast method.

Once protoplasts or nuclei are produced they are embedded in an agarose matrix as plugs or microbeads. The agarose provides a support matrix to prevent shearing of the DNA while allowing diffusion of enzymes and buffers to the DNA. Thus the DNA is purified and manipulated in the agarose and is stable for over a year at 4 °C.

Generation and size selection of large DNA fragments for BAC cloning

Once megabase-size DNA has been prepared, it must somehow be fragmented and DNA in the desired size range isolated. In general, DNA fragmentation utilizes two general approaches: (1) physical shearing and (2) partial digestion with a restriction enzyme that cuts relatively frequently within the genome. Because physical shearing is not dependent upon the frequency and distribution of particular restriction enzyme sites, this method should yield the most random distribution of DNA fragments. However, the ends of the sheared DNA fragments must be repaired and cloned directly or restriction enzyme sites added by the addition of synthetic linkers. These subsequent steps may damage the megabase-size DNA and result in lower yields of clonable DNA. Because of the subsequent steps required to clone DNA fragmented by shearing, most protocols fragment DNA by partial restriction enzyme digestion. The advantage of partial restriction enzyme digestion is that no further enzymatic modifications of the ends of the restriction fragments are necessary. Four common techniques that can be used to achieve reproducible partial digestion of megabase-size DNA are: (1) varying the concentration of the restriction enzyme, (2) varying the time of incubation with the restriction enzyme, (3) varying the concentration of an enzyme cofactor (e.g., Mg^{++}), and (4) varying the ratio of endonuclease to methylase.

There are three cloning sites (*Hin*dIII, *Bam*HI, and *Sph*I) in pBeloBAC11, but only *Hin*dIII and *Bam*HI produce 5′ overhangs for easy vector dephosphorylation. These two enzymes are primarily used to construct BAC libraries. The optimal partial digestion conditions for megabase-size DNA are determined by wide and narrow window digestions. We recommend optimizing partial digestions to produce the majority of DNA fragments in the 300 to 500 kb range. This size range will usually results in BAC inserts between 50 to 300 kb due to trapping of smaller DNA fragments in the gel. Figures 2 and 3 show wide and narrow optimization gels used to construct a *japonica* rice BAC library [4]. For the wide window optimization (Figure 2) 1, 2, 5, 10, and 50 units of *Hin*dIII were separately added to 50 µl aliquots of rice DNA in agarose microbeads and incubated at 37 °C for 20 min. Figure 2 shows that the optimal digestion conditions were between 5 and 10 units. It also shows that the megabase-size DNA could be totally digested with 50 units of *Hin*dIII in 20 min. To determine a more narrow optimization window, 6, 8, and 10 units of *Hin*dIII were separately added to 50 µl aliquots of microbeads and incubated as above (Figure 3). Six units of enzyme gave the best results in which the size of most of the DNA ranged between 300 to 500 kb as shown in Figure 3.

Once the optimal partial digestion conditions are determined a large amount of megabase-size DNA is partially digested using the exact conditions. The digestion is subjected to electrophoresis through a preparative pulsed-field gel and DNA in the size range of 300 to 500 kb is excised from the gel. This DNA is ligated to the BAC vector or subjected to a second size selection on a pulsed-field gel under different running conditions. Studies have previously shown that

Fig. 2. Wide window: Partially digested rice, *Oryza sativa* L. (Lemont *japonica*), megabase-size DNA: rice DNA in 50 μl of agarose microbeads. (M): *Saccharomyces cerevisiae* (BIO-RAD), (C): rice DNA without enzyme, Lanes 3–7: rice DNA partially digested with *Hin*dIII (1) 1 U (2) 2 U (5) 5 U (10) 10 U (50) 50 U. The genomic DNA was subjected to CHEF through a 1% agarose gel in 0.5X TBE using a switch time of 90 s at 6 v/cm and 12 °C for 16 h.

two rounds of size selection can eliminate small DNA fragments comigrating with the selected range in the first pulsed-field fractionation [4, 5]. Such a strategy resulted in an increase in insert sizes and a more uniform insert size distribution.

A practical approach to performing size selections is to test first for the number of clones/μl of ligation and insert size from the first size selected material. If the numbers are good (500 to 2,000 white colonies/μl of ligation) and the size range is also good (50 to 300 kb), then a second size selection is practical. When performing a second size selection a 80 to 95% decrease in the number of recombinant clones per transformation is expected.

Ligation

Twenty to two hundred nanograms of the size-selected DNA is ligated to dephosphorylated BAC vector (molar ratio of 10 to 1 in BAC vector excess). Table 3 shows a summary of BAC library ligation conditions for a number of BAC/ PAC libraries. Most of the BAC or PAC libraries used a molar ratio of 5 to 15:1 (size-selected DNA: BAC vector).
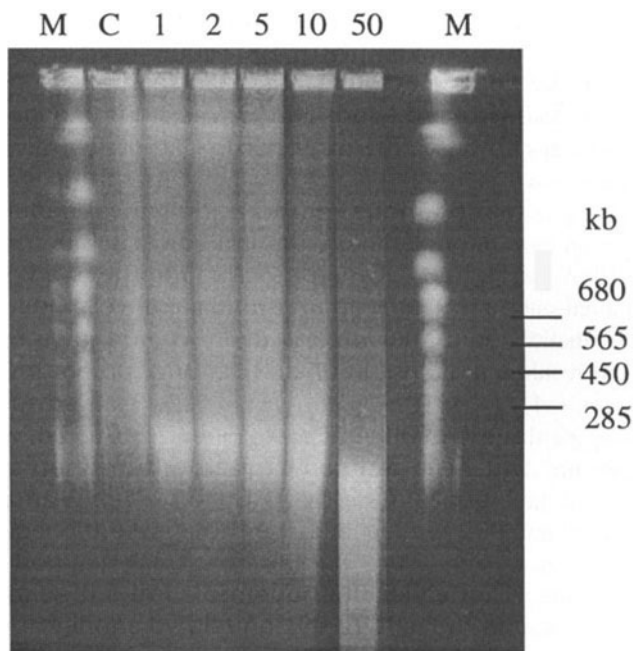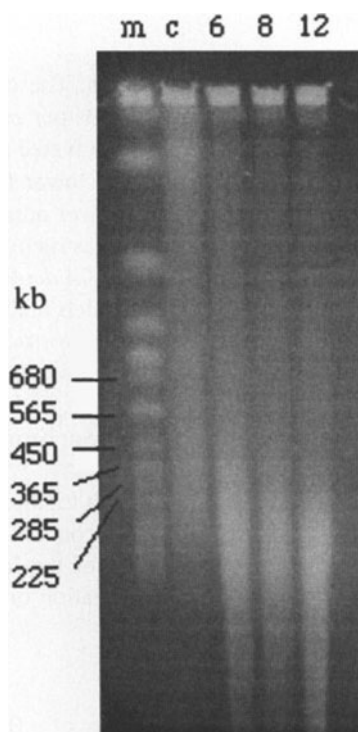
Fig. 3. Narrow window: Partially digested rice, *Oryza sativa* L. (Lemont *japonica*), megabase-size DNA: rice DNA in 50 μl of agarose microbeads. (m): *Saccharomyces cerevisiae* (BIO-RAD), (c): rice DNA without enzyme, Lanes 3–5: rice DNA partially digested with *Hin*dIII (6) 6 U (8) 8 U (12) 12 U. The genomic DNA was subjected to CHEF through a 1% agarose gel in 0.5X TBE using a switch time of 90 s at 6 v/cm and 12 °C for 16 h.

Table 3. BAC libraries ligation conditions.

Constructor	Organism	Insert DNA (average size in kb)	Ratio (insert: vector)	# of clone	# of Ligations (size select)	Clones/μl
Shizuya [1]	Human		1:10			
Ioannou [22]	Human (PAC)	70ng (140)	1:10	15,000	2 (1st)	
Woo [5]	Sorghum	25ng (157)	1:10	13,500	2 (1st, 2nd)	68
Choi [3]	A. thaliana	20ng (100)	1:5	12,672	2 (1st)	100
Wang [23]	Rice	40ng (125)	1:10	11,000	2 (1st, 2nd)	55
Choi [4]	Rice (*japonica*)	40ng (150)	1:10	7,296	2 (2nd)	90
Zhang [4]	Rice (*indica*)	40ng (130)	1:5	14,208	3 (1st)	47
Kim [2]	Human	100ng (140)	1:10	96,000	70 (2nd)	
Frijters [14]	Lettuce	20-60ng (111)	1:10	53,000	6 (2nd)	88
Choi (unpublished)	Cotton	50ng (110)	1:15	51,353	7 (1st, 2nd)	40

Transformation and arraying

Transformation is carried out by electroporation. The transformation efficiency for BACs is between 40 and 1,500 transformants per μl of ligation product, or 20 to 1,000 transformants/ng DNA. There is an inverse relationship between the insert size and the transformation efficiency. A lower field strength (9–13 kV/cm) yields a higher average insert size but a lower number of clones [24].

The *E. coli* strain used for BAC library construction is DH10B (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G), which includes mutations that: (1) block restriction of foreign DNA by endogenous restriction endonucleases (*hsd*RMS); (2) block restriction of DNA containing methylated DNA (*mcr*A, *mcr*B, *mcr*C and *mrr*); (3) block recombination (*rec*A1); and (4) take up large DNA (*deo*R). Electroporation competent DH10B cell can be made in the laboratory or purchased commercially.

Recombinant clones are picked manually with toothpicks or robotically (e.g., Genetix Q-bot) into 384-well microtiter plates containing growth media. After incubation overnight at 37 °C duplicate copies of the library are produced and the library is ready for screening using hybridization or PCR.

Library characterization

Several tests can be done to determine the quality of a BAC library. In our laboratory we perform three basic tests to evaluate the genome coverage of a BAC library – average insert size, average number of clones hybridizing with single copy probes and chloroplast DNA content.

Determination of the average insert size of the library is assessed in two ways. First, during library construction every ligation is tested to determine the average insert size by assaying 20 to 50 BAC clones per ligation. DNA is isolated from recombinant clones using a standard mini preparation protocol, digested with *Not*I to free the insert from the BAC vector, and then sized by electrophoresis through a CHEF gel. After the library is completed (3 to 20X genome coverage) the insert size from a single random clone from every 384-well plate is determined as above. Figure 4 shows a typical analysis of rice BAC clones from a second size selection ligation.

To determine the genome coverage of the library we screen it by hybridization with single copy RFLP markers distributed randomly across the genome. Microtiter plates containing BAC clones were spotted onto 22 cm×22 cm Hybond N+ membranes by using a multitasking robot (Q-bot: Genetix, Inc., UK). Bacteria from 48 or 72 plates are spotted twice onto one membrane, resulting in 18,000 to 27,648 unique clones on each membrane in either a 4×4 or 5×5 grid pattern. Because each clone is present twice, false positives are eliminated and true positives are easily recognized and identified. An example autoradiogram image of the a BAC high-density filter of the TAMU *Arabidopsis* BAC library [3] hybridized with a cosmid RFLP probe is shown in Figure 5.
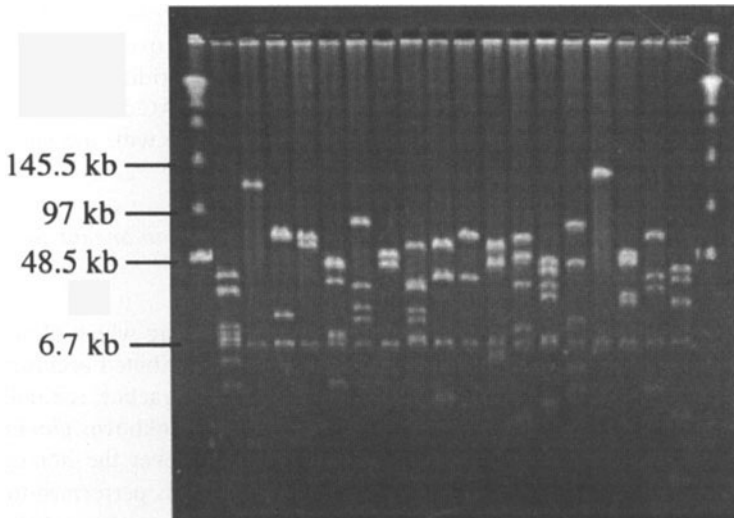
Fig. 4. Analysis of random rice (Lemont: *Oryza sativa* subsp. *japonica*) BAC clones by CHEF electrophoresis. Lanes 1 and 20 are lambda concatemer. Lanes 2–19 are alkaline lysis minipreps of recombinant BAC clones digested with *Not*I. Note: The 6.9 kb band in each lane is pBeloBAC11.
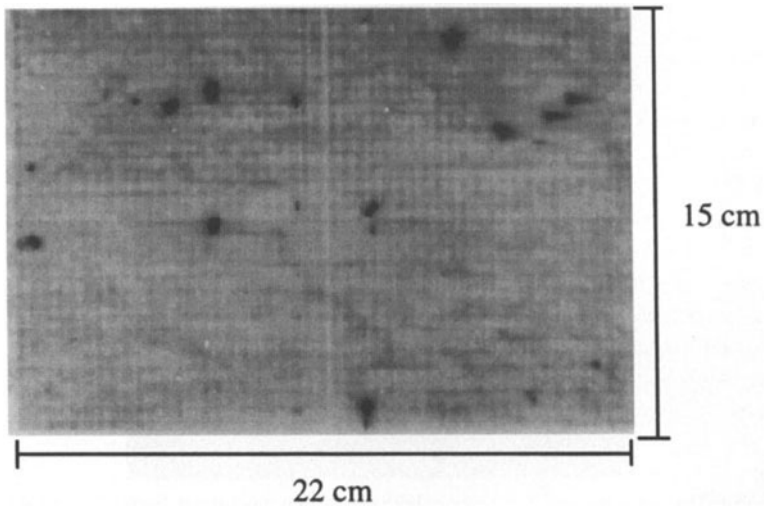
Fig. 5. BAC library screening by colony hybridization: An autoradiogram image of BAC high-density replica filter hybridized with a cosmid DNA probe (m262). TAMU *Arabidopsis* BAC clones (12,288) are screened on double-spotted (4×4) high density filters with a cosmid RFLP marker. Positive hybridization addresses (20 clones): 1P4, 2H13, 5I18, 9J14, 11N16, 14G8, 14G16, 16A16, 17C10, 20B2, 20O22, 21B4, 26H10, 26M2, 27B4, 27L21, 29H19, 30H22, 30P22, 31O3

Finally, the chloroplast DNA content in the BAC library is estimated by hybridizing three chloroplast genes (*ndhA*, *rbcL* and *psbA*) spaced evenly across the chloroplast genome to the library on high-density hybridization filters. Figure 6 shows such a hybridization to part of our cotton BAC library and shows that approximately 1.1% of the 32,729 clones hybridized with the chloroplast probes.

A mathematical analysis for the number of clones needed to anchor the target gene

An anchoring scheme refers to any method for determining which clones contain a given target gene. It is assumed that clones are distributed according to a homogeneous Poisson distribution along the genome. In practice, the collection of clones is often not randomly distributed because of unknown cloning bias and partial digestion rather than random shearing. However the homogenous distribution assumption is used in the mathematical analysis performed to determine the number of clones (Table 4) required to obtain a certain probability of having any DNA sequence represented in the genomic library [25].
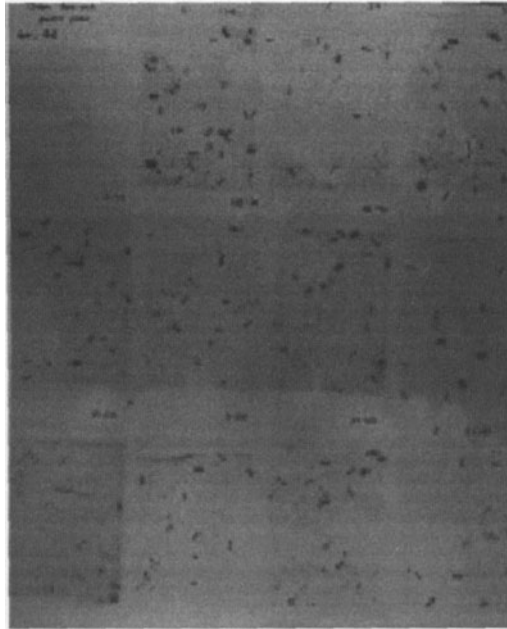
Fig. 6. BAC library screening by colony hybridization with chloroplast DNA. Cotton pBACwich clones (16,896 or 1,536 × 11) were screened on double-spotted high density filters with chloroplast DNA probes (Dr J. Mullet, Texas A&M University). Each filter (8 cm × 12 cm) was inoculated by duplicating each colony on the same filter with a 384 prong high density replicating tool (HDRT) from four 384-well microtiter plates using the Biomek 2000 robot (Beckman, USA) for a total of 1,536 BAC clones per filter.

Table 4. The number of clones required for a 99% probability of having any DNA sequence represented in a genomic library.

Common name	Scientific name	Genome size Mb/1C [26, 27]	Insert size 100 kb	Insert size 150 kb
Apple	*Malus x domestica*	769	3.5×10^4	2.4×10^4
Arabidopsis	*Arabidopsis thaliana*	100	4.6×10^3	3.1×10^3
Banana	*Musa* sp.	873	4.0×10^4	2.7×10^4
Barley	*Hordeum vulgare*	4,873	2.2×10^5	1.5×10^5
Canola	*Brassica napus*	1,182	5.4×10^4	3.2×10^4
Cassava	*Manihot esculenta*	760	3.5×10^4	2.3×10^4
Common bean	*Phaseolus vulgaris*	637	2.9×10^4	2.0×10^4
Cotton	*Gossypium hirsutum*	2,246	1.0×10^5	6.9×10^4
Lettuce	*Lactuca sativa*	2,639	1.2×10^5	8.1×10^4
Maize	*Zea mays*	2,504	1.2×10^5	7.7×10^4
Oat	*Avena sativa*	11,315	5.2×10^5	3.5×10^5
Onion	*Allium cepa*	15,290	7.0×10^5	4.7×10^5
Orange	*Citrus sinensis*	382	1.8×10^4	1.2×10^4
Pepper	*Capsicum annuum*	2,702	1.2×10^5	9.4×10^4
Rice	*Oriyza sativa* ssp. *indica & japonica*	431	2.0×10^4	1.3×10^4
Sorghum	*Sorghum bicolor*	760	3.5×10^4	2.3×10^4
Soybean	*Glycine max*	1,115	5.1×10^4	3.4×10^4
Sugarbeet	*Beta vulgaris* ssp. *esculenta*	758	3.5×10^4	2.3×10^4
Sugarcane	*Saccharum* sp.	3,000	1.4×10^5	9.2×10^4
Tobacco	*Nicotiana tabacum*	4,434	2.0×10^5	1.4×10^5
Tomato	*Lycopersicon esculentum*	953	4.4×10^4	2.9×10^4
Wheat	*Triticum aestivum*	15,966	7.4×10^5	4.9×10^5

The number of clones in the library is determined using the following equation:

$$N = \ln(1 - P)/\ln(1 - L/G) ,$$

where, $N=$ number of clones in library; $P=$ probability of obtaining a specific clone; $L=$ length of average clone insert in kilobasepairs; $G=$ haploid genome length in kilobasepairs.

In general 99% coverage represents four to five haploid genome equivalents. However, if such a library were used for the construction of complete physical maps or whole genome sequencing, one needs to generate very deep libraries with a P value of 99.9 to 99.99%.

In the following sections, we shall discuss the procedures used to construct, analyze, and manipulate BAC libraries from plants using examples for BAC libraries constructed in our laboratory.

Procedures

BAC vector preparation

Cell growth
1. Streak *E. coli* DH10B containing the BAC vector (pBeloBAC11) onto a LB agar plate containing 12.5 μg/ml chloramphenicol and grow at 37 °C overnight.
2. Inoculate 5 ml of LB media containing 30 μg/ml chloramphenicol with a single colony from step 1 and grow for 8 h at 37 °C.
3. Inoculate four 2.8 l culture flasks containing 1 l of LB chlorampenicol (30 μg/ml), prewarmed to 30 °C, with 1 ml/liter of culture from step 2. Grow at 30 °C with shaking (225 rpm) to a cell density of approximately 1×10^9 cells/ml ($A_{600} = 1.0$-1.2).
4. Harvest the cells by centrifugation at 4 °C for 15 min at 6,000 g (about 6,200 rpm in Beckman JA-14, Beckman, USA).

Solutions

LB plates: 1% Tryptone, 0.5% Yeast extract, 1% NaCl, 1.5% Agar.
   LB medium: 1% Tryptone, 0.5% Yeast extract, 1% NaCl.
   Add chloramphenicol (stock solution: 20 mg/ml in ethanol) at below 70 °C after autoclaving.

Plasmid purification

1. Extract BAC vector using Qiagen Maxi Plasmid Purification Protocol (Five QIAGEN-tip 500 columns for a 4 l preparation).
2. Dissolve the final plasmid pellet in 3.6 ml of TE, add 0.4 ml of 10 mg/ml of ethidium bromide, and transfer the DNA solution to a 15 ml polypropylene Falcon tube.
3. Add about 4.0 g of solid CsCl and adjust the density of the solution to 1.59 g/ml by additional CsCl or TE. The final volume should be about 5 ml.
4. Load solution into a Ultra-Clear centrifuge tube (Beckman order # 344057) and centrifuge the mixture to density equilibrium in a Beckman NVT90.1 rotor at 70,000 rpm for 24 h at 20 °C.
5. View the supercoiled plasmid and relaxed DNA bands with long-wavelength UV light and remove the lower band by puncturing the side of the tube with a 21-gauge syringe needle below the band. Carefully draw the plasmid DNA into a 1 ml syringe.
6. Extract the ethidium bromide from the DNA solution with one volume of ddH_2O-saturated isoamyl alcohol. Continue extracting with fresh ddH_2O-saturated isoamyl alcohol until all visible color is removed (5 to 6 times).
7. Dilute the extracted DNA sample with two volume units of TE and precipitate with 95% ethanol and centrifugation. Wash the pellet gently with 70% ethanol, recentrifuge, and air dry.
8. Resuspend the DNA pellet in 100 μl of TE and determine the DNA concentration by spectrophotometry and agarose gel electrophoresis using known DNA concentration standards.

Notes
8. The final yield is approximately 20 to 40 μg from 4 l of media.

Solutions

TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
   Water-saturated isoamyl alcohol.

Digestion and dephosphorylation

1. Digest pBeloBAC11 (10 μg) to completion with 100 units of *Hind*III or *Bam*HI (Gibco BRL, USA) and corresponding buffer with additional 4 mM spermidine at 37 °C for two to four hours.
2. Extract the digested DNA twice with phenol/chloroform (1:1), precipitate and wash with ethanol, and resuspend in 50 μl of 1X TA buffer (provided with HK phosphatase: Epicentre, USA) following the addition of 5 mM CaCl$_2$.
3. Dephosphorylate the DNA by adding two units of HK Phosphatase (Epicentre) per μg of DNA and incubating at 30 °C for 2 h.
4. Heat the reaction at 65 °C for 30 min to inactivate HK Phosphatase.
5. Ethanol-precipitate the dephosphorylated vector and dissolve in 100 μl of TE.

Notes
2. Restriction enzyme incubation for an extended time period or with high salt may result in exonuclease activity.
3. The digested BAC vector can be dephosphorylated with Shrimp Alkaline Phosphatase (SAP 1 unit/μg of DNA: USB, USA).

Solutions

10X *Hind*III buffer: 0.5 M Tris-HCl, pH 8.0, 0.1 M MgCl$_2$, 0.5 M NaCl.
10X *Bam*HI buffer: 0.5 M Tris-HCl, pH 8.0, 0.1 M MgCl$_2$, 1 M NaCl.
10X Spermidine-trihydrochloride: 40 mM in sterilized ddH$_2$O.
10X TA buffer: 330 mM Tris-acetate, pH 7.8, 660 mM K-acetate, 100 mM Mg-acetate, 5 mM DTT, and 1 mg/ml Bovine serum albumin.
Phenol/chloroform (1:1): Mix equal amounts of phenol and chloroform and equilibrate the mixture by extracting several times with 0.1 M Tris-HCl (pH 7.6).

Preparation of megabase-size DNA from plants

Nuclei preparation (This method was developed by [21] and modified by S. Choi).
1. Grind 30 to 50 g of the fresh or frozen tissue into a powder in liquid nitrogen with a mortar and pestle (do not over grind) and immediately transfer into an ice cold 500 ml beaker containing 200 ml ice-cold homogenizing buffer (HB) plus 0.15% β-mercaptoethanol (add before use) and 0.5% Triton X-l00 (20% stock in HB).
2. Repeat step 1, four to six times in order to obtain enough nuclei to embed.
3. Stir each mixture from step 1 with a magnetic stir bar for 20 min on ice.
4. Filter the mixture from step 3 into an ice-cold 250 ml centrifuge bottle through two layers of cheesecloth (American Fiber & Finishing, Inc., USA) and one layer of miracloth (Calbiochem order # 475855, USA) placed in a single large, wide mouth funnel. To obtain more nuclei, remove the cheesecloth and squeeze the remainder of the homogenate into the centrifuge bottle.
5. Pellet the filtered homogenate by centrifugation in a fixed-angle rotor at 1,800 g at 4 °C for 20 min (about 3,500 rpm in JA-14, Beckman).
6. Discard the supernatant fluid and gently resuspend the pellet in one to five ml of ice cold wash buffer with assistance of a small paint brush. After resuspension adjust the volume to 30 ml with wash buffer and transfer to a 50 ml round bottom Oakridge tube.
7. Pellet the nuclei by centrifugation at 1,800 g, 4 °C for 15 min in a swinging bucket centrifuge (about 2,800 rpm in GH-3.8 Horizontal rotor, Beckman).
8. Wash the nuclei pellet two additional times by resuspension in wash buffer followed by centrifugation at 1,800 g, 4 °C for 15 min. During the last washing step the resuspended nuclei are filtered through one layer of miracloth by gravity into new Oakridge tubes.
9. After the final wash, resuspend the pelleted nuclei in a small volume of HB (2 to 3 ml). Count the nuclei with a hemacytometer under a phase contrast microscope and adjust to approximately 4×10^7 nuclei/ml with the addition of HB (for plants with haploid genomic sizes of 500 to 1,000 Mb).

Notes
9. In practice, the optimum number of nuclei depends on the genomic size of the plant and for some plants species it can be difficult to count the nuclei. When preparing nuclei for the first time we optimize the nuclei concentration empirically. We suggest preparing the nuclei as described above and in the final step embed three different concentrations of nuclei (e.g., 1 ml concentrated nuclei + 3 ml of HB, 2 ml concentrated nuclei + 2 ml of HB, and 4 ml concentrated nuclei + 0 ml of HB).

Solutions

HB: 0.5 M Sucrose, 10 mM Trizma base, 80 mM KCl, 10 mM EDTA, 1 mM Spermidine, 1 mM Spermine, final pH 9.4–9.5 adjusted with NaOH.

   Wash buffer: HB + 0.15% β-mercaptoethanol (add before use) + 0.5% Triton X-100 (20% stock in HB).

Protoplast preparation (*Tomato*) (This method was developed by [20] and modified by Wing *et al.* [28])
1. Harvest 30 to 50 g of the young leaves and cut from midvein 5 to 10 times to make strips of 1 to 2 mm. The leaf is still intact but feathered.
2. Transfer approximately 4 g of leaves into protoplast buffer plus 1% cellulase and 0.05% pectolyase in a large Petri dish and shake gently for 4 h at room temperature.
3. Check the release of protoplasts using an inverted microscope. If 70% to 80% of lysed protoplasts are observed, remove the leaves and filter the liquid sequentially using 80 and 33 μm sieves into a flask.
4. Pellet the protoplasts at 40 g for 10 min (about 500 rpm in JA-14) and resuspend in 50 ml of protoplast buffer.
5. Repeat filtering as step 3 and centrifuging as step 4, and resuspend in a small amount (about 2 to 3 ml) of protoplast buffer.
6. Count an aliquot using a microscope and hemacytometer and adjust the final concentration to 4×10^7 protoplasts /ml of protoplast buffer.

Notes
Making protoplasts is specific for every plant species and needs to be optimized.

Solutions

Protoplast buffer: 0.5 M D-Mannitol, after autoclaving add 20 mM 2-N-Morpholine ethanosulfonic acid, adjust pH 5.6 with KOH.

Encapsulating in agarose microbeads (I) (This method was developed [28] for plant protoplasts and modified by Zhang *et al.* [21] for plant nuclei).

1. Prepare 1% to 1.2% low melting point (LMP) agarose (Seaplaque GTG, FMC, USA) solution in HB for nuclei (or protoplast buffer for protoplasts) and store in a 45 °C water bath.
2. Warm 15 ml of light mineral oil in a 50 ml Falcon tube to 45 °C in a water bath.
3. Place 150 ml of ice cold HB for nuclei (or protoplast buffer for protoplasts) in a 500 ml beaker into an ice bath on top of a magnetic stir plate and swirl the solution vigorously using a magnetic stir bar (#5 in Corning stirrer PC-620, USA).
4. Place the nuclei or protoplast solution in a 500 ml flask and prewarm to 45 °C in a water bath.
5. Pipet an equal volume of 1% to 1.2% LMP agarose in HB from step 1, kept in a 45 °C water bath, into the prewarmed nuclei or protoplast suspension and mix well but gently.
6. Add 20 ml of prewarmed light mineral oil at 45 °C from step 2 to the prewarmed nuclei or protoplast suspension, shake the mixture of the flask vigorously for 3 to 5 s, and immediately pour into the 500 ml beaker of step 3 containing the swirling 150 ml of ice cold HB (or protoplast buffer for protoplasts).
7. Continue swirling for 5 to 10 min on ice to break up any clumps and allow for the agarose microbeads to be more uniform in size.
8. Harvest the agarose microbeads by centrifugation at 600 g, 4 °C for 15 to 20 min in a swinging bucket centrifuge (about 1,800 rpm in Beckman GH-3.8).
9. Discard the supernatant fluid and resuspend all the pelleted microbeads in 5 to 10 volumes of ESP in a 50 ml Falcon polypropylene tube.

DNA purification in agarose

10. Incubate the beads in ESP for 24 h at 50 °C with gentle shaking to degrade the proteins.
11. Pellet the microbeads by keeping the beads at 50 °C without shaking for 1 h and discard the supernatant fluid.
12. Add new ESP for further 24 h-incubation.
13. Wash the beads six times for 1 hour per time with TE at 4 °C. The first three washes include PMSF (phenylmethyl sulfonyl fluoride) at 0.1 mM to inactivate the Proteinase-K.

Solutions

ESP: 0.5 M EDTA, pH 9.0-9.3, 1% Sodium lauroyl sarcosine, add 0.1 mg/ml Proteinase K before use.
   PMSF: 50 mM stock in isopropanol.

Encapsulating in agarose plugs (*II*)

1. Mix equal volumes of nuclei and 1% LMP (Seaplaque GTG) agarose in HB (or protoplast buffer for protoplasts) and aliquot into plug molds (BIO-RAD catalog # 170–3622) on ice.
2. Immerse the casted plugs in a large volume of ESP (at least ten times the volume of the agarose plugs, 1.0 to 0.5 mg/ml Proteinase K for plugs) in a 50 ml Falcon polypropylene tube and incubate at 50 °C overnight with gentle agitation.
3. Change the ESP one time and incubate for 5 h to overnight with agitation.
4. Equilibrate agarose-embedded DNA thoroughly with TE containing 1 mM PMSF to inactivate residual Proteinase K three times for 1 h each.
5. Wash three times with only TE.

Notes

The plugs can be chopped into fragments that are about the same size as beads without any appreciable DNA breakage, and then used for partial digestion [29].

Partial digestion

Wide window
1. Incubate 50 μl of microbeads or chopped pieces of half of a plug with a 45 μl mixture of (*Hind*III or *Bam*HI reaction buffer, 0.1 mg/ml Acetylated BSA, 4 mM Spermidine) on ice for 20 min.
2. Add 5 μl of *Hind*III or *Bam*HI freshly diluted in distilled water (e.g., 0 U, 1 U, 2 U, 4U, 8 U, 16 U, and 50 U/5 μl) and allow to diffuse into the beads for 10 min on ice.
3. Transfer the reaction mixture to a 37 °C water bath for partial digestion and incubate for 20 min.
4. Stop the reaction by adding 1/10 volume of 0.5 M EDTA, pH 8.0 and placing the tubes on ice.
5. Load the partially digested DNA on a 1% agarose gel in 0.5X TBE with a wide-bore tip and seal the wells with the same molten agarose as the gel.
6. Perform pulsed-field gel electrophoresis on a CHEF Mapper (BIO-RAD, USA) under the conditions of 6.0 V/cm, 90 s pulse, 0.5X TBE buffer, 12 °C for 18 h.
7. After checking the ethidium bromide stained gel from step 6, select the enzyme concentration giving a majority of DNA fragments ranging from about 300 to 600 kb. because of DNA trapping during the step 6 running conditions, we expect the actual size of the DNA fragments obtained from the 300 to 600 kb fraction to be between 50 to 300 kb in size (Figure 2).

Narrow window
8. From the information of the wide window digetsion, carry out another set of partial digestions to optimize the narrow window (Figure 3).
9. Repeat steps 1–7.

Notes
2. The amount of restriction enzyme and the digestion time depend on the number of restriction sites in the plant genome: usually 0 to 50 units for 5 min to 1 h.

Solutions

0.5X TBE: Trizma base 0.045 M, Boric acid 0.045 M, EDTA 1 mM, pH 8.3.

Preparing partially digested DNA for ligation-first size selection

1. Select the amount of enzyme giving the optimum digestion pattern from the narrow window and perform the digestion reaction on a large scale by carrying out a number of reactions (10 to 20 reactions) under the exact same conditions as previously determined (same volumes, same tubes, same dilution of enzyme, etc.).
2. Load the partially digested DNA on a 1% LMP agarose gel (Seaplaque, FMC) in 1X TAE with a wide-bore tip and seal with the same molten agarose.
3. Perform pulsed-field gel electrophoresis on a CHEF Mapper under the conditions of 6.0 V/cm, 90 s pulse, 1X TAE buffer, 12 °C for 18 h.
4. Cut DNA fragments ranging from about 300 to 600 kb from the gel and use for ligation or a second size selection.

Notes

4. When we use chopped DNA plugs (that have highly concentrated DNA) for a partial digestion we can use a shorter pulse time (20 to 40 s) to spread out the DNA on the gel. This condition avoids trapping smaller DNA segments so that a second size selection may not be necessary.

Solutions

1X TAE: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0.

Preparing partially digested DNA for ligation-second size selection
1. Dialyze the gel piece cut from the first size selection with TE + 50 to 100 mM NaCl.
2. Melt the gel piece cut from the first size selection at 65 °C for 5 min and pipet into the wells of a second size selection gel (1% LMP in 1X TAE) with a polyethylene transfer pipet (Fisher, USA).
3. Carry out a second size selection at 4.0 V/cm, 5 s pulse, 1X TAE buffer, 12 °C for 8 h.
4. Excise the DNA band in the compression zone, and dialyze with TE + 50 to 100 mM NaCl.

Ligation

1. Melt the dialyzed LMP DNA piece from 1st or 2nd size selection at 65 °C for 5 min, and transfer to a 45 °C water bath.
2. Add 1 unit of GELase (Epicentre, USA) per 100 μg of gel with GELase buffer and incubate at 45 °C for 1 h.
3. Check the concentration of the DNA solution by loading 10 μl on a 1% agarose minigel with ethidium bromide and running a quick electrophoresis at 60 V, for 1 h, in 0.5X TBE with uncut lambda DNA standard solutions (e.g., lambda 5 ng, 10 ng, 20 ng, 40ng, and 80 ng/well: Usually the concentration of the size selected DNA is between 0.5 and 2 ng/μl).
4. Ligate 50 to 200 ng of the size selected DNA to the dephosphorylated BAC vector in a molar ratio of 1:10 to 15 (size selected DNA to vector DNA; see Table 3 for details) in a total volume of 100 μl with 6 units of T4 DNA ligase (USB, USA) plus ligase buffer at 12 or 16 °C for 16 h.
5. Drop-dialyze the ligation solution on Millipore filters (filter type VS, 0.025 μm) with TE for 1 hour to remove the ligation buffer.

Notes
1. Strong *et al.* [30] described a electroelution method for purifying large, gel-fractionated DNA molecules that alleviates the need for melting the agarose and subsequent enzymatic agarose digestion.

Transformation

1. Transform 1 to 2.5 μl of the ligation material into 20 to 25 μl of *E. coli* DH10B competent cells (Gibco BRL, USA) by using the BRL Cell-Porator system (Gibco BRL) and the following settings: voltage 300 to 400 V; capacitance 330 μF; impedance low ohms; charge rate fast; voltage booster resistance 4 Kohms.
2. Transfer the electroporated cells to 15 ml culture tubes with 0.4 to 1 ml of SOC and shake at 220 rpm for 50 min at 37 °C.
3. Spread the SOC medium from step 2 onto one or two LB plates containing 12.5 μg/ml chloramphenicol, 50 μg/ml X-Gal (stock: 20 mg/ml in dimethylformamide) and 25 μg/ml IPTG (stock: 200 mg/ml in ddH$_2$O), and incubate at 37 °C for 20 to 36 h.
4. Pick white colonies into 384 well microtiter plates containing LB freezing medium.
5. Incubate the microtiter plates at 37 °C overnight and store at −80 °C.

Notes
1. The Bio-Rad Gene Pulser II (BIO-RAD) can be used under the conditions of 100 Ohms, 16 kV/cm, and 25 μFa. It may be advantageous to use a lower field strength in order to increase the average insert size. It has been demonstrated that a lower field strength (9 to 13 kV/cm) yields a higher average insert size but a lower number of clones [24].

Solutions

SOC: 2% Bacto tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM Glucose, pH 7.0 ± 0.1.

LB freezing medium: LB, 36 mM K$_2$HPO$_4$, 13.2 mM KH$_2$PO$_4$, 1.7 mM Na Citrate, 0.4 mM MgSO$_4$, 6.8 mM (NH$_4$)$_2$SO$_4$, 4.4% v/v Glycerol, 12.5 μg/ml Chloramphenicol.

BAC DNA insert isolation

1. Prepare 5 ml culture of LB medium containing 12.5 μg/ml chloramphenicol, inoculate a single colony, and incubate with shaking at 37 °C for 18 to 20 h.
2. Centrifuge the culture at 2,000 g at 4 °C for 15 min using a table top centrifuge (about 3,000 rpm in GH-3.8).
3. Pour off the supernatant fluid and resuspend the cell pellet in 0.2 ml of ice-cold GTE.
4. Transfer the suspension to a 1.5 ml microfuge tube, add 0.4 ml (freshly prepared) of 0.2 N NaOH+1% SDS solution, mix by inversion several times, and incubate on the bench for 5 min.
5. Add 0.3 ml of the potassium acetate stock and gently invert the mixture.
6. Centrifuge the mixture for 15 min at 12,000 g in a micro-centrifuge (13,000 rpm in Biofuge 13, Baxter, USA).
7. Remove 0.75 ml of the supernatant fluid without disturbing the pellet and transfer to a clean microfuge tube.
8. Add 0.6 volumes of cold isopropanol (0.45 ml) and centrifuge at 12,000 g for 15 min to pellet the DNA.
9. Remove the supernatant, rinse the pellet with 1 ml of cold 70% ethanol, and dry upside down until the pellet becomes transparent.
10. Add 40 μl of TE and digest 10 μl of this DNA solution with *Not*I to free the insert from the BAC vector.
11. Run 1% agarose gels in 0.5X TBE at CHEF conditions of 6 V/cm, initial switch time 5 s, final switch time 15 s and for 13 to 15 h (Figure 4).

Solutions

GTE: 50 mM Glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.1 mg/ml RNase.

Potassium acetate stock: 60 ml 5 M potassium acetate, 28.5 ml glacial acetic acid, 11.5 ml ddH$_2$O, pH 4.8–5.2

BAC library screening by hybridization

1. Inoculate Hybond N+ membranes (Amersham, UK) with a 384-prong High Density Replicating Tool (HDRT) from microtiter plates.
2. Place the membranes on LB agar plates containing 12.5 μg/ml chloramphenicol and incubate at 37 °C for 12 to 36 h until colonies of 1 to 2 mm diameter are obtained.
3. Remove the membranes and place, colony side up, on a pad of absorbent filter paper (Whatman Cat. No. 3030 700) soaked in the following solutions and for the specified time: (1) Solution 1 (0.5 N NaOH, 1.5 M NaCl) for 7 min; (2) Solution 2 (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0), 7 min; (3) Air dry for more than 1 h; (4) Solution 3 (0.4 N NaOH), 20 min; (5) Solution 4 (5X SSPE), 7 min; (6) Air dry overnight.
4. Prehybridize the filters at 65 °C for at least 4 h with hybridization buffer.
5. Exchange the prehybridization buffer with the fresh buffer and continue prehybridization for 2 to 4 h at 65 °C.
6. Add probes and hybridize for 18 to 36 h at 65 °C.
7. Wash the filters with 2X-0.1X SSC and 0.1% SDS 2 to 3 times for 20 min at 65 °C.
8. Blot with paper towels, wrap in plastic wrap, and expose for 24 to 72 h with a intensifying screen.

Notes
1. Using a Q-bot (Genetix, Inc., UK), 384-well microtiter plates containing BAC clones are spotted onto 22 cm × 22 cm Hybond N+ membranes. Bacteria from 72 plates are spotted twice onto one membrane, resulting in 27,648 unique clones on each membrane. Alternatively, the nylon filters (12 × 8 cm) can be inoculated with a 384 prong HDRT from microtiter plates using the Biomek 2000 robot (Beckman).
3. The membranes can be stored at room temperature for months.

Solutions

5X SSPE: 0.9 M NaCl, 50 mM Sodium phosphate, 5 mM EDTA, pH7.7.
Hybridization buffer: 0.5 M Sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 10 μg/ml sheared denatured salmon sperm DNA
2X SSC: 6M NaCl, 1M Citric acid-trisodium salt.

References

1. Shizuya H, Birren B, Kim U-J, Mancino V, Slepak T, Tachiri Y and Simon M (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using F-factor-based vector. Proc Natl Acad Sci USA 89: 8794–8797.
2. Kim U-J, Birren BW, Slepak T, Mancino V, Boysen C, Kang HL, Simon MI, Shizuya H (1996) Construction and characterization of a human bacterial artificial chromosome library. Genomics 34: 213–218.
3. Choi S, Creelman RA, Mullet JE, Wing RA (1995) Construction and characterization of bacterial artificial chromosome library of *Arabidopsis thaliana*. Weeds World 2: 17-20, and Plant Mol Biol Rep 13: 124–128.
4. Zhang HB, Choi S, Woo S-S, Li Z, Wing RA (1996) Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Molecular Breeding 2: 11–24 .
5. Woo S-S, Jiang J, Gill BS, Paterson AH, Wing RA (1994) Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*. Nucl Acids Res 22: 4922–4931.
6. Jiang J, Gill BS, Wang G-L, Ronald PC, Ward DC (1995) Metaphase and interphase fluorescence *in situ* hybridization mapping of the rice genome with bacterial artificial chromosomes. Proc Natl Acad Sci USA 92: 4487–4491.
7. Cai L, Taylor JF, Wing RA, Gallagher DS, Woo S-S, Davis SK (1995) Construction and characterization of a bovine bacterial artificial chromosome library. Genomics 29: 413–425.
8. Burke DT, Carle GF, Olson MV (1987) Cloning of large segments of exogenous DNA into yeast using artificial-chromosome vectors. Science 236: 806–812.
9. Burke DT (1990) YAC cloning: options and problems. GATA 7: 94–99.
10. Neil DL, Villasante A, Fisher RB, Vetrie D, Cox B and Tyler-Smith C (1990) Structural instability of human tandemly repeated DNA sequences cloned in yeast artificial chromosome vectors. Nucl Acids Res 18: 1421–1428.
11. Green ED, Riethman HC, Dutchik JE, Olson MV (1991) Detection and characterization of chimeric yeast artificial-chromosome clones. Genomics 11: 658–669.
12. Pierce JC, Sauer B, Sternberg N (1992) A positive selection vector for cloning high molecular weight DNA by the bacteriophage P1 system: Improved cloning efficacy. Proc Natl Acad Sci USA 89: 2056–2060.
13. Willetts N, Skurray R (1987) Structure and function of the F factor and mechanism of conjugation. In Neidhardt et al.(eds), *Escherichia coli* and *Salmonella typhimurium:* Cellular and Molecular Biology, Vol. 2, pp. 1110–1133. American Society for Microbiology, Washington, D.C.
14. Frijters ACJ, Zhang Z, van Damme M, Wang G-W, Ronald PC., Michelmore RW (1996) Construction of a bacterial artificial chromosome library containing large *Eco*RI and *Hind*III genomic fragments of lettuce. Theor Appl Genet 94: 390–399.
15. Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc Natl Acad Sci USA 93: 9975–9979.
16. O'Connor M, Peifer M, Bender W (1989) Construction of large DNA segments in *Escherichia coli*. Science 244: 1307–1312.
17. Bradshaw MS, Bollekens JA, Ruddle FH (1995) A new vector for recombination-based cloning of large DNA fragments from yeast artificial chromosomes. Nucl Acids Res 23: 4850–4856.
18. Larionov V, Kouprina N, Graves J, Chen XN, Korenberg JR, Resnick MA (1996) Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. Proc Natl Acad Sci USA 93: 491–496.
19. Larionov V, Kouprina N, Graves J, Resnick MA (1996) Highly selective isolation of human DNAs from rodent-human hybrid cells as circular yeast artificial chromosomes by transformation-associated recombination cloning. Proc Natl Acad Sci USA 93: 13925–13930.

20. Ganal MW, Tanksley SD (1989) Analysis of tomato DNA by pulse-field gel electrophoresis. Plant Mol Biol Rep 7: 17–41.
21. Zhang H-B, Zhao XP, Ding XD, Paterson AH, and Wing RA. (1995) Preparation of megabase-sized DNA from plant nuclei. Plant J 7: 175–184.
22. Ioannou PA, Amemiya CT, Garnes J, Kroisel PM, Shizuya H, Chen C, Batzer MA, de Jong PJ (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nature Genetics 6: 84–90.
23. Wang G-L, Holsten TE, Song W, Wang H-P, Ronald PC (1995) Construction of a rice bacterial artificial library and identification of clones linked to the Xa21 disease resistance locus. Plant J 7: 525–533.
24. Sheng Y, Mancino V, Birren B (1995) Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucl Acids Res 23: 1990-1996.
25. Clarke L, Carbon J (1976) A colony bank containing synthetic Col El hybrid plasmids representative of the entire *E. coli* genome. Cell 9: 91–100.
26. Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9: 208–219.
27. Goodman HM, Ecker JR, Dean C (1995) The genome of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 92: 10831–10835.
28. Wing RA, Rastogi VK, Zhang HB, Paterson AH, Tanksley SD (1993) An improved method of plant megabase DNA isolation in agarose microbeads suitable for physical mapping and YAC cloning. Plant J 4: 893–898
29. Wang Y-K, Schwarttz DC (1993) Chopped insert: a convenient alternative to agarose/DNA inserts or beads. Nucl Acids Res 21: 2528.
30. Strong SJ, Ohta Y, Litman GW and Amemiya CT (1997) Marked improvement of PAC and BAC cloning is achieved using electroelution of pulsed-field gel-separated partial digests of genomic DNA. Nucl Acids Res 25: 3959–3961.

Yeast as Versatile Tool in Transcription Factor Research

ANNEMARIE H. MEIJER, JAN SCHOUTEN, PIETER B.F. OUWERKERK
and J. HARRY C. HOGE
*Institute of Molecular Plant Sciences, Leiden University, Clusius Laboratory, P.O. Box 9505, 2300
RA Leiden, The Netherlands*

Introduction

Besides representing an important production organism in the biotechnology in-
dustry, the yeast *Saccharomyces cerevisiae* also constitutes a powerful organism
for studying the fundamental processes operative in cells of eukaryotes. This
stems from its ease of cultivation and handling, short generation time and ad-
vanced molecular genetics, and from the fact that the basic features of cellular
mechanisms and functions appear to be evolutionary conserved between yeast
and other eukaryotes. In recent years yeast has proven particularly useful for
analyzing the molecular components involved in initiation of gene transcription.
Here we describe what yeast has to offer to researchers interested in identifica-
tion and characterization of transcription factors. First some background infor-
mation will be given on yeast strains and vectors. Subsequently, the currently
available yeast techniques for transcription factor research will be briefly dis-
cussed. Finally, detailed step-by-step protocols for the successful application of
these techniques will be provided.

Yeast strains and vectors

S. cerevisiae divides by budding and can exist stably in a haploid or a diploid
state. The haploid cell is either of mating type *MATa* or *MATα* and two cells of
opposite mating type can fuse to form a diploid cell (*MATa/MATα*). These can
divide indefinitely by mitosis but, under conditions of carbon and nitrogen star-
vation, can undergo meiosis to produce four haploid spores that can be individu-
ally recovered and studied. For general information on yeast, its manipulation,
genetics and molecular biology see Guthrie and Fink [26] and Ausubel et al. [2]
and references therein.

Most laboratory strains are maintained as haploids that carry a number of
auxotrophic mutations, often in genes involved in the biosynthesis of amino ac-
ids (e.g. *HIS3*, *TRP1*, *LEU2* and *LYS2*) or nucleotides (e.g. *URA3* and *ADE2*).
Yeast vectors generally carry selectable marker genes that can complement spe-
cific auxotrophic mutations. Introduction of vectors with different selectable
markers in an appropriate host strain allows simultaneous propagation of mul-
tiple plasmids. When using yeast as a research organism, it is advisable to select
a strain with a multitude of stable (non-reverting) auxotrophic mutations (pref-
erably deletions) because the number of available markers may become rapidly

exhausted in practical work. Apart from the auxotrophic markers, several dominant markers can be applied in yeast. Among these are *APT1* [30], *CAT* [28] and *HPT* [24], that confer G418, chloramphenicol and hygromycin resistance, respectively. Some auxotrophic markers have additional useful applications. The *URA3* marker can be counterselected using 5-fluoro-orotic acid (5-FOA) because the encoded enzyme converts 5-FOA into a toxic compound. Similarly, the *LYS2* marker can be counterselected using α-amino-adipate (α-AA). The *HIS3* marker can be employed as a sensitive reporter gene because the activity of the enzyme it encodes can be titrated with the competitive inhibitor 3-amino-1,2,4-triazole (3-AT) in a concentration-dependent manner. This allows correction for leaky (basal) *HIS3* expression and fine-tuning to a desired stringency of selection conditions in genetic screens for *HIS3* expression. Another valuable reporter gene for yeast is the *Escherichia coli lacZ* gene, that can be assayed visually (blue/white colonies) with X-GAL or quantitatively using ONPG as substrate for the encoded enzyme (see [20] for a useful plasmid containing a *lacZ* reporter gene linked to the minimal yeast *CYC1* promoter). The *E. coli gusA* gene [51, 37] and the *Aequoria victoria GFP* gene [45, 55] can also be used as reporter genes in yeast.

All commonly used yeast vectors can also be propagated in *E. coli* and are generally composed of an *E. coli* plasmid backbone, a yeast selectable marker gene and yeast sequences that enable their maintenance in yeast. Integrative and non-integrative vectors can be distinguished. The integrative vectors (YIp) lack sequences for replication in yeast. Transformation of yeast occurs by integration into the nuclear genome via recombination between homologous yeast sequences in the vector and the chromosomal DNA. Prior to transformation, the integrative vector is usually linearized within the yeast sequences that are homologous to the intended site of integration in the nuclear genome. This directs the integration event to the site of cleavage and increases the frequency of transformation. The non-integrative vectors can be easily moved back and forth between *E. coli* and yeast. These vectors, often referred to as shuttle vectors, replicate as circular plasmids in the nucleus of yeast. Two general types are routinely used for non-integrative yeast transformation: centromere and episomal vectors. The centromere vectors (YCp) carry chromosomal yeast sequences for autonomous replication (*ARS*) and sequence segments from yeast chromosome centromeres (*CEN*) for mitotic and meiotic stability as well as for unbiased segregation. These plasmids are very stably maintained at a low copy number, usually 1–2 copies per cell, and are often used when low expression levels of transgenes are required. The episomal vectors (YEp) bear the origin of replication and a partition element from the yeast 2 μ circle plasmid that is endogenous in most laboratory yeast strains. In conjuction with gene products provided by 2 μ circles of the host strain, the partition element ensures equal distribution of replicated plasmid molecules between mother and daughter cells. YEp plasmids are usually maintained at a high copy number that varies between 10 and 40 copies per cell, and are therefore often used when the goal is to achieve high expression levels of transgenes. However, the copy number of YEp vectors can become

drastically diminished when containing a gene whose expression is deleterious to the host strain. Even under selective conditions this may result in plasmid retention in only a fraction of the yeast cells.

A variety of promoters are available for the expression of heterologous genes in yeast. The strong promoters of *PGK1* (for useful expression vectors see [43, 44]) and *ADH1* (for useful expression vectors see [29, 50]) are often used. While considered to be constitutive, their activity is affected by the carbon source in the medium (high in glucose medium, several-fold lower in non-glucose medium). The most commonly applied promoter for conditional gene expression is the tightly regulated *GAL1* promoter, that is repressed by glucose and can be up to 1000-fold induced if glucose is replaced by galactose (for useful expression vectors see [6, 19]). Other conditional promoters include the *CUP1* promoter (induction by copper ions in Cu^{2+}-resistant yeast strains) [36, 38] or engineered promoters that are controlled by tetracycline [22] or glucocorticoids [48, 13]. Because the consensus sequence surrounding the translational start codon is quite divergent between yeast and plants/animals [8], it may be wise to express foreign genes from yeast promoters as a translational fusion construction. Convenient sets of vectors have been developed for this purpose [19, R&D Systems]. For optimal gene expression, an efficient yeast terminator (e.g., *CYC1* terminator) is generally used in expression vectors. A simple check on whether heterologous proteins are expressed to a sufficient level in yeast is possible when producing the proteins in fusion with an epitope tag such as that derived from human influenza virus hemagglutinin. Details and references about foreign gene expression in yeast can be found in Romanos et al. [49].

To facilitate construction of cDNA libraries for expression in yeast, bacteriophage λ vectors are frequently used containing a linearized shuttle vector flanked by two *loxP* sites [16, 7, 41]. The cDNA fragments are inserted in sense orientation between an appropriate yeast promoter and terminator in the shuttle plasmid part of the λ vector and propagated as a λ-phage library in *E. coli*. Next, an *E. coli* strain producing the Cre-recombinase is lysogenically infected with this phage library. Due to interaction of the Cre-recombinase with the *loxP* sites, the shuttle plasmid molecules will automatically excise and circularize. Upon subsequent amplification in *E. coli* the shuttle plasmid library is ready for use in transformation of yeast.

Yeast techniques for transcription factor research

Heterologous transcription factor genes can be cloned in yeast by functional complementation of mutants or by employing genetic selection systems.

Cloning by functional complementation [5] involves transformation of a mutant yeast strain defective in a particular transcription factor with a cDNA library in a yeast expression vector, and subsequent application of a selection strategy for distinguishing colonies in which the transcription factor gene defect has been phenotypically restored. Obviously, this approach can only be em-

ployed when a viable yeast mutant for the transcription factor gene of interest is available and an appropriate selection scheme can be devised.

Cloning by genetic screening is based on the specific interaction in yeast between the transcription factor of interest and its known DNA-binding site, resulting in activation of a reporter gene whose expression is dependent on this interaction and can be selected for [9, 61, 31, 57]. It requires transformation of yeast with a TATA box-containing reporter (*HIS3* or *lacZ*) gene construction having upstream binding sites for the transcription factor. Subsequently, the obtained reporter strain is transformed with a cDNA library in a yeast expression vector and colonies expressing the reporter gene are selected. Preferably the reporter gene construction is integrated into the yeast genome instead of being maintained as a non-integrative plasmid because integration provides better stability and a constant gene copy number. The yeast expression vector used for construction of the cDNA library is designed either for producing non-hybrid proteins encoded by the cDNA or for producing hybrids of the cDNA-encoded protein and a strong transcription activation domain (one-hybrid system) from either the yeast Gal4 transcriptional activator (for useful vectors see Clontech Catalogue) or the *Herpes simplex* virus VP16 transcriptional co-activator protein (for useful vectors see [6]). The non-hybrid approach only allows cDNA cloning of transcription factors harbouring, in addition to their sequence-specific DNA-binding domain, a transcription activation domain that functions properly in yeast. The applicability of the one-hybrid system (Figure 1) is not restricted by this limitation and therefore generally favoured for protein-DNA interaction-based cDNA cloning of transcription factors in yeast.

The yeast one-hybrid system also facilitates rapid functional dissection of the DNA-binding domains of transcription factors that have been cDNA cloned in this system. This is done by retransforming the reporter strain with mutagenized versions of the transcription factor cDNA clone under study and subsequent analysis of reporter (*lacZ*) gene expression. By expressing a one-hybrid system-derived transcription factor cDNA clone as non-hybrid protein in the reporter strain it can be simply tested whether this protein possesses, in addition to its sequence-specific DNA-binding domain (BD), an intrinsic transcription activation domain (AD) that functions in yeast. If this were the case, mutational analysis can be used to delineate the location of crucial amino acids of its AD. Alternatively, its AD can be detected and then mapped by expressing the protein and derivatives thereof as fusion with a heterologous BD such as that of Gal4 (for useful vectors see Clontech Catalogue) in a yeast strain with a Gal4-responsive reporter gene. A recently described variant of the one-hybrid system is the reverse one-hybrid system [58] that employs a counterselectable *URA3* gene constuction as the reporter gene. It allows convenient detection of mutations or molecules that dissociate the interactions between a DNA-binding protein and its binding site identified in the one-hybrid system.

Yeast is also useful for identifying proteins that interact with a transcription factor of interest, such as other DNA-binding proteins or co-activators [12, 14, 52]. For this the two-hybrid system [17, 11, 4, 18, 27, 15, 60, 3] is used (Figure

One-hybrid screening
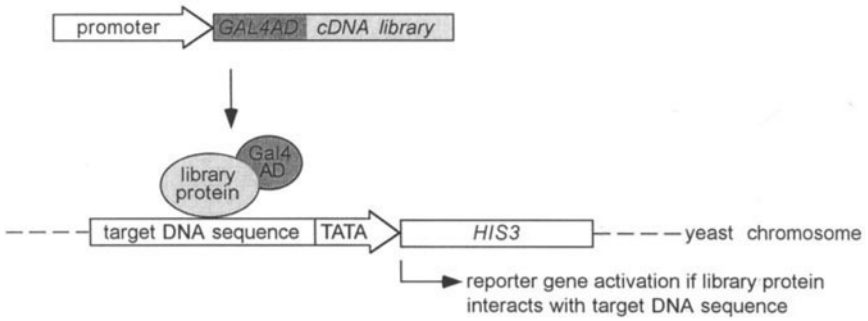
Two-hybrid screening
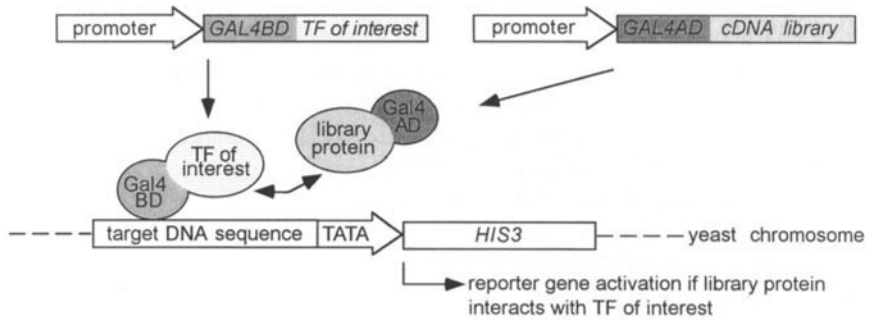
Target site selection
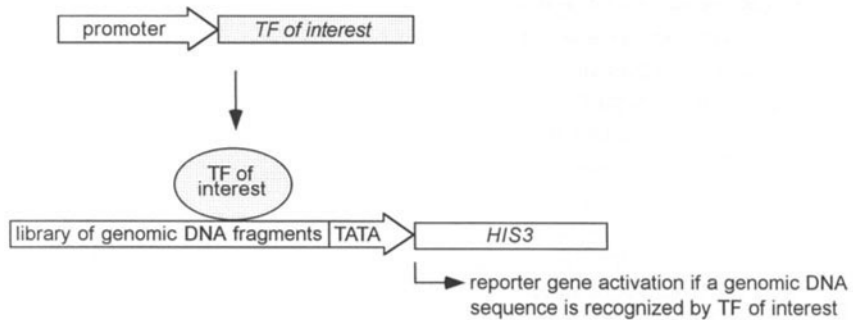
Fig. 1. Schematic overview of yeast one-hybrid, two-hybrid and target site selection systems as used in transcription factor research. TF: transcription factor; Gal4AD, Gal4BD: activation and binding domains, respectively, of the yeast transcription factor Gal4. Details are described in the text.

1). This system exploits the ability of a pair of interacting proteins X and Y to form a complex that can trigger expression of a reporter gene when X is produced as a fusion with a DNA-binding domain (X-BD hybrid) and Y as a fusion with a transcription activation domain (Y-AD hybrid). The BD directs the X-DB fusion protein to cognate upstream binding sites in the reporter gene. As a result of non-covalent interactions between the X and Y moieties of X-BD and Y-AD, the AD of the Y-AD fusion protein becomes tethered to the reporter gene, thereby activating its expression. Several two-hybrid systems have been developed for studying interactions between known proteins and for cDNA cloning of proteins that interact with a given protein. The DNA-binding and transcription activation domains most commonly used in these systems are the binding domains of yeast Gal4 and *E. coli* LexA, and the activation domains of Gal4 and *H. simplex* virus VP16. Occasionally, use is made of a bacterial activation domain, called B42, that is weaker than the strong activation domains of Gal4 and VP16. The reporter (*HIS3*, *LEU2*, *lacZ*) genes usually contain several copies of the binding sites of the corresponding BD's. Because of its nuclear localization signal the Gal4 BD is naturally routed to the yeast nucleus for binding to its target sites. The LexA BD, that lacks such a localization signal, must enter the yeast nucleus by passive diffusion, but engineered versions for active nuclear import are available [3]. The Gal4, VP16, and B42 AD's are usually employed in conjunction with an added nuclear localization signal. Two-hybrid experiments with the Gal4 BD require the use of mutant yeast strains that are defective in both *GAL4* and *GAL80* function. For the LexA BD such special yeast strains are not necessary. Contrary to the Gal4 BD, the LexA BD therefore permits conditional expression of two-hybrid proteins via the Gal4-dependent *GAL1* promoter. X-BD and, Y-AD hybrids are generally constructed with the BD or AD at the amino terminus because this simplifies in-frame fusion. If desired, however, both the BD and AD can also be fused at the carboxy terminus. In two-hybrid screenings of cDNA libraries the protein of interest (bait) and the cDNA-encoded proteins (prey) are invariably expressed as BD and AD fusions, respectively. The bait protein-BD fusion is generally constitutively expressed to ensure continuous occupancy of its target site in the reporter gene, while the prey protein-AD fusions are either constitutively or conditionally expressed. Useful strains, vectors, libraries and other reagents for the yeast two-hybrid system are commercially available (Clontech, Invitrogen, Origene, Stratagene).

Besides the two-hybrid system, a modified one-hybrid system can be used for identification of proteins interacting with a particular transcription factor. Here the transcription factor of interest or a derivative thereof is expressed as a non-hybrid protein that binds to cognate upstream reporter gene sequences, and is subsequently used as bait for cDNA expression library-encoded prey-AD hybrid proteins that interact with the transcription factor and thereby trigger or boost reporter gene activity [25, 56].

Various modifications of the two-hybrid system have been described. These include systems for studying RNA-protein interactions [53], ligand-protein interactions [34], and phosphorylation-dependent protein-protein interactions [46],

as well as a system for identifying molecules or mutations that disrupt protein-protein interactions [58, 59]. For details the reader is referred to the cited publications. Recently, a novel yeast two-hybrid system for detecting protein-protein interactions was developed that is not based upon the transcriptional activation of a reporter gene. This system [1], designated SOS-recruitment system (SRS), exploits the finding that the human Ras guanylyl nucleotide exchange factor (GEF), hSos, can substitute for the yeast Ras GEF, Cdc25, to allow cell survival and proliferation. In a temperature-sensitive *cdc-25-2* mutant yeast strain, the hSos-mediated rescue depends on recruitment of hSos to the plasma membrane. In the SRS this recruitment is mediated by the interaction between two hybrid proteins. The known protein (X) is produced as a fusion with a truncated hSos variant that cannot rescue the *cdc-25-2* ts mutant, and the sought-after proteins (Y) as fusions with a meristoylation signal sequence that confers membrane-targetting. Interaction between X and Y directs hSos to the plasma membrane and yields yeasts colonies that can grow at the non-permissive temperature. Because the SRS is not based on transcriptional activation, this system provides unique opportunities for studying interactions involving transcriptional repressors.

The versatility of yeast as tool for transcription factor research is furthermore demonstrated by its use in genetic selection of binding sites and putative target genes of transcription factors [62, 10, 35, 39, 21, 40]. Target site selection in yeast (Figure 1) requires the construction of a library of DNA fragments cloned upstream of a TATA box-containing reporter gene in a yeast vector. The DNA fragments can either be oligonucleotides of random sequence or can be derived from genomic DNA, dependent on the application of the library for binding site selection or selection for putative target genes. In a screening, the DNA-reporter library is introduced into a yeast strain harboring an expression construction (activator) of the transcription factor of interest. Candidate target DNA sequences can subsequently be identified by selecting for activator-dependent reporter gene expression. If desired, the expressed activator could consist of the DNA-binding portion of the transcription factor of interest fused to a strong transcription activation domain of a heterologous transcription factor. This would enable selection of target sequences of DNA-binding proteins that do not possess a sufficiently strong activation function or act as transcriptional repressors. It is necessary to have the activator expressed under control of a regulatable promoter and/or resident on a counterselectable vector so as to distinguish activator-dependent positive clones from clones activated by an endogenous yeast factor. We have recently described two bacteriophage λ vectors with automatic subcloning capability to facilitate DNA library construction [40].

Procedures

Culturing of yeast

Yeast can be grown at 30 °C in rich culture medium (YPD or YAPD) or in selective medium (SD) containing the appropriate supplements. For solid medium add 2% Bacto-agar (Difco, 0140-01) before autoclaving. Adenine, uracil, and amino acids can be added before autoclaving the medium or added to the medium after autoclaving from sterile 100× stock solutions. Yeast strains can be stored at −80 °C in 20% glycerol.

Media and additives:

YPD:　2% Bacto-peptone (Difco, 0118-17-0)
　　　1% Bacto-yeast extract (Difco, 0127-17-9)
　　　2% D-glucose
　　　autoclave

YAPD:　YPD containing 20 mg/l adenine hemisulfate

SD:　0.67% yeast nitrogen base without amino acids (Difco, 0919-15-3)
　　　2% D-glucose
　　　autoclave

100× stock solutions of supplements:		
	adenine hemisulfate:	2 g/l
	L-histidine:	2 g/l
	L-leucine:	3 g/l
	L-lysine-HCl:	3 g/l
	L-methionine:	2 g/l
	L-tryptophan:	2 g/l
	uracil:	2 g/l

One-hybrid screening protocol

The given procedure makes use of a cDNA library for expression of proteins fused to the C-terminal end of the Gal4 activation domain. With minor modifications the same procedure is suited for screening cDNA libraries expressing non-hybrid or other hybrid proteins.

Steps in the procedure
  1. Construct the required reporter gene construction and cDNA library. The reporter gene construction should consist of the *HIS3* gene preceded by a yeast TATA box and one or more copies[1] of the target DNA sequence of interest. For making this construction, we recommend the pHIS3 reporter gene cassettes described by Meijer et al. [40]. Reporter gene constructions in pHIS3 can be transferred to the pINT1 integration vector [40] that is designed for integration of the reporter gene construction at the non-essential yeast *PDC6* locus. A convenient vector for construction of the cDNA library is λACTII [41], a bacteriophage λ vector with Cre-*loxP*-mediated automatic subcloning properties, that allows unidirectional cloning of cDNAs synthesized by means of the ZAP cDNA synthesis kit (Stratagene). The λACTII-derived yeast/*Escherichia coli* plasmid (pACTII) directs constitutive expression of hybrids between the Gal4 transcription activation domain (Gal4 AD) and the cDNA-encoded protein in yeast. pACTII contains a 2 μ ori and a *LEU2* selection marker for yeast and an *Ap* resistance gene for *E. coli*. The following parts of this procedure are based on the use of the pHIS3/pINT1 vector system and a pACTII cDNA library.
  2. Integrate the reporter gene construct into the genome of a suitable yeast strain such as Y187 (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, met⁻, gal4Δ, gal80Δ, URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*; Clontech). Prior to transformation, the pINT1 integration vector is linearized by *SacI* and/or *NcoI* digestion. For transformation follow the LiAc procedure (see below), select transformants on YAPD plates containing 150 μg/ml of the antibiotic G418, and restreak these at least once on fresh G418 plates[2]. We advise to construct also a number of control yeast strains to determine the specificity of reporter gene activation. These strains could contain a reporter gene construction without upstream binding sites or reporter gene constructions with mutated, non-functional target sites.
  3. To determine whether the reporter strain shows background growth on his-lacking medium perform a 3-AT[3] titration: streak colonies on SD plates lacking his (for Y187: SD + ade, trp, leu, met) and containing 0, 1, 2, 5, 10 or 25 mM 3-AT. For reference

also streak on an SD plate containing his[4]. Incubate the plates for 3–7 days at 30 °C and score growth. If background growth on his-lacking medium is observed[5], add 3-AT during library screenings to a concentration of 2–5 mM above the lowest concentration of 3-AT with which background growth could be inhibited in the titration experiment[6].

4. Inoculate a few colonies of the reporter strain in 50 ml of YAPD medium and grow overnight with shaking at 30 °C.

5. Dilute the overnight culture to an OD_{600} of approximately 0.25 in YAPD and grow this culture for another 3 h at 30 °C with shaking. A culture volume of 100 ml is sufficient to perform 10 small scale library transformations that should result in a total of at least 10^6 transformants.

6. Follow the LiAc procedure (steps 3–11) to prepare competent cells and to perform the desired number of identical transformation reactions with 1 μg of cDNA library plasmid each[7].

7. Resuspend the transformed yeast cells in 200 μl of H_2O per transformation.

8. Determine the transformation efficiency for two of the identical transformations by plating 100 μl of a 10^{-2} and a 10^{-3} dilution on SD medium lacking leu (for Y187: SD + ade, trp, met, his). The expected number of transformants per transformation reaction is at least 10^5 when using Y187.

9. Plate each transformation reaction on 3 standard plates or on a large (150 mm) plate with SD medium lacking leu and his and containing the appropriate concentration of 3-AT as determined in step 3 (for Y187: SD + ade, trp, met + x mM 3-AT).

10. Incubate plates at 30 °C for at least 3 days and up to 10 days.

11. Streak putative positives on fresh plates containing the same medium as used during library screening.

12. An optional step when using Y187 is to perform a colony filter lift assay for β-galactosidase activity. Y187 contains a lacZ reporter gene with upstream Gal4 target sites. Activation of this reporter gene may indicate that the cDNA library clone encodes a non-specific DNA-binding protein.

13. Isolate the library plasmid from each putative positive according to the plasmid isolation procedure. If a large number of positives has been obtained, it is useful to try to narrow down the number of clones that need to be analyzed further. Identical or overlapping clones can be identified on the basis of restriction and cross-hybridization patterns.

14. Retransform the yeast reporter strain and the control yeast strains (see step 2) with the library plasmid and select transformants on SD plates lacking leu (see step 8). Also transform each strain with the empty library vector.

15. Check growth of the transformants in a 3-AT titration experiment (see step 3) to compare the strength of reporter gene activation among the different positives. Use the strain containing the empty vector as a control. True positives should specifically activate the reporter strain with wild-type target sites.

16. As first steps in the subsequent analysis of cDNAs that encode putative DNA-binding proteins we recommend to perform partial sequence analysis and database searches to detect possible homologies with described DNA-binding proteins[8]. Furthermore it should be verified that the cDNA-encoded protein is indeed capable of binding the target site, for example by producing *in vitro* translated proteins using the TNT® coupled reticulocyte lysate system (Promega) and testing these in electrophoretic mobility shift assays (protocols described elsewhere). Alternatively, purified GST or HIS-tagged fusion proteins can be used.

Notes

1. A convenient method for multimerization of DNA elements is described by Ouwerkerk and Memelink [47].

2. Transformants should turn pink on YAPD medium due to the *ade2-101* mutation; ignore small white colonies as these carry a spontaneous mitochondrial mutation. Do not select transformants on SD medium as this would require a much higher G418 concentration. An optional step is to verify the integration of the reporter gene construct by Southern analysis with the *PDC6* gene and the reporter gene as probes. Yeast chromosomal DNA can be isolated with the Nucleon MiY kit (Amersham).

3. 3-AT (3-amino-1,2,4-triazole, Fluka 09540) is a competitive inhibitor of the His3 enzyme. 3-AT is added to the medium (at ca. 60 °C) just before pouring plates from a filter-sterilized 1 M stock solution (stored at 4 °C).

4. G418 selection is no longer required from this stage onwards, as the integrants are stable.

5. Background growth on SD medium lacking his and containing 3-AT may result from activation of the reporter gene by an endogenous yeast factor. If growth can be inhibited by low concentrations of 3-AT, in our experience it usually does not interfere with the identification of DNA-binding library proteins in a one-hybrid screening. However, one must be aware of the possibility of isolating artefacts that do not bind DNA, but enhance the activity or abundance of the endogenous yeast factor.

6. The optimal 3-AT concentration is somewhat dependent on plating density. Therefore it can be useful to fine tune further the 3-AT concentration under the conditions as used in a library screening.

7. Although it is possible to scale up the reaction volume rather than to perform identical small scale transformations, this may require adjustment of the incubation times in the LiAc procedure to obtain the same transformation efficiency.

8. Apart from cDNA clones encoding hybrids of the library protein and the Gal4 AD, we also obtained from our screenings a significant number of positive cDNA clones of which the protein-coding sequence was not in frame with that of the Gal4 AD. Instead these clones contained a full length protein-coding sequence, were translated from their own ATG start codon, and harboured an intrinsic transcriptional activation function.

Two-hybrid screening protocol

A number of variants of the two-hybrid system have been developed as described in the introduction. Here, a protocol is given for a Gal4-based two-hybrid library screening. Obviously, a similar procedure can be applied to investigate interactions between two known proteins.

Steps in the procedure
1. Construct the required bait vector and cDNA library. A suitable bait vector is pGBT9 (2 μ ori, *TRP1* selection marker; Clontech), in which the ADH1 promoter directs (relatively low[1]) constitutive expression of the bait translationally fused to the Gal4 DNA-binding domain (BD/bait). A suitable vector for the cDNA library is pACTII which is described in step 1 of the one-hybrid screening procedure. The following parts of this procedure are based on the use of a bait vector such as pGBT9 with *TRP1* marker and a cDNA library in a vector such as pACTII (see step 1 in the one-hybrid procedure) containing a *LEU2* marker gene.
2. Introduce the bait vector into a suitable yeast strain such as CG1945 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4-542, gal80-538, cyh^{r}2, lys2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::GAL4$_{17\text{-}mers(\times 3)}$-CYC1$_{TATA}$-lacZ*; Clontech). For transformation follow the LiAc procedure, select transformants on SD medium lacking trp (for CG1945: SD + his, lys, ade, leu) and restreak these at least once on fresh plates.
3. To verify that the BD/bait protein is not capable of autonomous reporter gene activation, perform a 3-AT[2] titration experiment. Streak colonies of the BD/bait strain and a control strain (CG1945 containing an empty pGBT9 vector[3]) on SD plates lacking his and trp (for CG1945: SD + lys, ade, leu) and containing 0, 5, 10, 25, 50 or 75 mM 3-AT. For reference also streak on an SD plate containing his. Incubate the plates for several days up to a week at 30 °C and compare growth of the BD/bait and control strains. If autonomous activation of the *HIS3* reporter gene occurs and cannot be inhibited by a relatively low concentration of 3-AT (up to 25 mM), it is advisable to try to construct an alternative version of the bait protein in which the activation function has been deleted. Obviously, this may also result in elimination of a potential interaction domain.
4. Inoculate a few colonies of the BD/bait strain in 50 ml of selective medium (see step 2) and grow overnight with shaking at 30 °C.
5. Dilute the overnight culture to an OD$_{600}$ of approximately 0.25 in YAPD and grow this culture for another 3 h at 30 °C with shaking[4].

A culture volume of 100 ml is sufficient to perform 10 small scale library transformations that should result in a total of 10^5-10^6 transformants.

6. Follow the LiAc procedure (steps 3–11) to prepare competent cells and to perform the desired number of identical transformation reactions with 1 μg of cDNA library plasmid each[5].

7. Resuspend the transformed yeast cells in 200 μl of H_2O per transformation.

8. Determine the transformation efficiency for two of the identical transformation reactions by plating 100 μl of a 10^{-2} and a 10^{-3} dilution on SD medium lacking leu and trp (for CG1945: SD + his, ade, lys). The expected number of transformants per transformation reaction is between 10^4 – 10^5 when using CG1945.

9. Plate each transformation reaction on 3 standard plates or on a large (150 mm) plate of SD medium lacking leu, trp, his and containing sufficient 3-AT (as determined in step 3) to suppress background growth of the BD/bait strain (for CG1945: SD + ade, lys, x mM 3-AT).

10. Incubate plates at 30 °C[6] for at least 3 days and up to 10 days.

11. Streak putative positives on fresh plates containing the same medium used during library screening. A 3-AT titration may also be performed at this stage to compare the strength of reporter gene activation among the different positives.

12. Check positives from step 11 for activation of the second reporter gene (*lacZ*) according to the β-galactosidase filter assay procedure (use the BD/bait strain as a negative control). If the *lacZ* reporter, that contains Gal4 binding sites in a different promoter context, is not activated, the yeast colony is likely to be a false positive. However, because expression of the *lacZ* reporter in CG1945 is rather weak, there is a risk of discarding a false negative.

13. Isolate the library plasmid from each putative positive according to the plasmid isolation procedure. Be aware that both the BD/bait and AD/library plasmids will be isolated by this procedure, because both carry the *Ap* resistance gene. Distinguish between these two plasmids by restriction analysis. If a large number of positives has been obtained, it is useful to try to narrow down the number of clones that need to be analyzed further. Identical or overlapping clones can be identified on the basis of restriction and cross-hybridization patterns.

14. Perform co-transformations of CG1945 with the following combinations of plasmids:
    (a) BD/bait plasmid   +   AD/library plasmid
    (b) BD plasmid without insert (pGBT9) +   AD/library plasmid
    (c) BD plasmid expressing an
        unrelated protein   +   AD/library plasmid

    Plate transformants on the same medium used during library screening and incubate for 3–10 days at 30 °C. For true positives only combination (a) should allow growth.

    Alternatively, these tests can be performed by assaying *lacZ* reporter gene activity. In that case it is recommended to co-transfer the plasmids to a yeast strain with a more sensitive *lacZ* reporter gene than CG1945, such as strain Y187 (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, met⁻, gal4Δ, gal80Δ, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*; Clontech). For co-transformation of Y187, plate the cells on medium lacking leu and trp (SD + ade, met, his), incubate at 30 °C for 3 days, then restreak transformants for β-galactosidase filter assays. The use of the *lacZ* reporter gene also allows quantification[7] of the two-hybrid interactions (see protocol).

15. An optional test to verify further the interaction is to clone the library insert in the BD plasmid and the bait in the AD plasmid and test these plasmids in a two-hybrid assay.

16. As first steps in the subsequent analysis of cDNAs that encode putative interacting proteins we recommend to perform partial sequence analysis and database searches, and to verify the interaction by an independent method, for example *in vitro* [3].

Notes
1. Major differences exist in the expression levels driven by the *ADH1* promoter in the various vectors for Gal4-based two-hybrid assays. This is due to the fact that some vectors (e.g. the BD-vector pAS2-1 from Clontech) contain the 1500 bp full length *ADH1* promoter that directs very high expression, whereas others contain the 410 bp truncated *ADH1* promoter that directs lower expression. However, there are also differences in the expression levels of different vectors with the truncated *ADH1* promoter, because in some plasmids the expression is enhanced by upstream vector sequences. Vectors with a truncated *ADH1* promoter directing relatively low expression are the BD-vectors pGBT (Clontech) and pBD-GAL4Cam (Stratagene) and the AD-vectors pGAD424, pGAD10 (Clontech) and pAD-GAL4 (Stratagene), whereas the AD-vectors pACT2 (Clontech) and pACTII [41] give relatively high expression. Use of the high expression vectors may result in isolation of more artefacts. Therefore, most groups presently seem to prefer to use a low expression vector, at least for the bait construct. Some laboratories have even switched to the use of single copy ARS/CEN vectors instead of 2 μ vectors [32]. On the other hand certain known interactions could not be detected with low expression vectors [33].
2. See note 3 in one-hybrid screening procedure.
3. The yeast strains described for two-hybrid screenings are leaky for *HIS3* expression to different extents. CG1945 is slightly leaky and requires 5 mM 3-AT to suppress

background growth. Other strains (e.g., Y190, Clontech) may require as much as 25 mM 3-AT to suppress background growth. See also note 6 in one-hybrid procedure.

4. We have experienced that after overnight growth in liquid medium (even under selective conditions) a large percentage of the yeast cells (up to > 95%) may have lost the BD/bait plasmid. This is probably due to toxicity of certain BD/bait plasmids. We therefore recommend to check for possible loss of the BD/bait construction by plating dilutions of an overnight culture on selective and non-selective plates and comparing the number of colonies. If loss of the BD/bait construction occurs, it will be necessary to co-transform the BD/bait construction together with the library in step 6. However, co-transformation generally results in lower transformation efficiencies.

5. See note 7 in the one-hybrid screening procedure.

6. It can be useful also to screen at 20 °C instead of 30 °C, because others have found that certain interactions could only be detected at this lower temperature [32].

7. An important consideration when comparing the strengths of different interactions is that the levels to which different BD or AD hybrid proteins are produced may vary, due to differences in mRNA or protein stability. This can be checked by Western blot analysis using monoclonal antibodies against the Gal4 BD and AD domains (Clontech).

Target site screening protocol

The target site selection procedure can be applied both for random binding site selection and for identification of natural target genes. In either case a library is required of DNA fragments (respectively oligo-nucleotides of random sequence or genomic DNA fragments of 500 – 1000 bp) cloned upstream of a reporter gene on a yeast/*E. coli* shuttle plasmid. The library is introduced into a yeast strain containing an ex-pression construction (activator) of the transcription factor of interest. If desired, the activator can be a hybrid protein consisting of the DNA-binding portion of this transcription factor and a strong heterologous transcriptional activation domain (e.g., Gal4 or VP16). To be able to distinguish true positives (that are activator-dependent) from clones activated by an endogenous yeast factor, it is necessary that the acti-vator is expressed under control of an inducible promoter and/or on a counterselectable vector. The described procedure is an example of a possible strategy for target site screening. It is based on the use of the *HIS3* reporter gene and an activator under the control of the galac-tose-inducible *GAL1* promoter and on a vector (*URA3* marker) that is counterselectable with 5-fluoro orotic acid (5-FOA). A useful addition to the procedure below would be to include the *ADE5* gene as a sec-tor marker on the activator plasmid as described by Liu et al. [35]. This plasmid facilitates discrimination between true and false positives, as true positives appear as red colonies, whereas false positive colonies that are not dependent on the activator construction show the appear-ance of white sectors.

Steps in the procedure
1. Construct the required reporter library and an expression con-struction of the transcription factor of interest (activator). The ac-tivator must be expressed under control of the *GAL1* promoter on a vector with the *URA3* marker gene. Suitable vectors are de-scribed in [19]. Convenient vectors for construction of the reporter library are λHIS3B and λHIS3BS [40], bacteriophage λ vectors with Cre-*loxP*-mediated automatic subcloning properties that contain a *HIS3* reporter gene preceded by a yeast TATA-box and a unique *Bam*HI site for cloning of genomic *Sau*3A1 fragments. The λHIS3B/λHIS3BS-derived yeast/*E. coli* plasmids (YCpHIS3B/ YCpHIS3BS) contain a *TRP1* selection marker for yeast and an *Ap* resistance gene for *E. coli*.
2. Introduce the activator construction into a suitable yeast strain such as YPH500 (*MAT*α, *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1* [54]). For transformation follow the LiAc pro-cedure, select transformants on SD medium lacking ura (for

YPH500: SD + lys, ade, trp, his, leu), and restreak these at least once on fresh plates.

3. Inoculate a few colonies of the activator strain into 50 ml of selective medium (see step 2) and grow overnight with shaking at 30 °C.

4. Dilute the overnight culture to an OD_{600} of approximately 0.25 in YAPD and grow this culture for another 3 hours at 30 °C with shaking[1]. A culture volume of 100 ml is sufficient to perform 10 small scale library transformations that should result in a total of 10^5–10^6 transformants.

5. Follow the LiAc procedure (steps 3–11) to prepare competent cells and to perform the desired number of identical transformation reactions with 1 μg of λHIS3B/λHIS3BS-derived reporter library plasmid each[2].

6. Resuspend the transformed yeast cells in 200 μl of H_2O per transformation.

7. Determine the transformation efficiency for two of the identical transformation reactions by plating 100 μl of a 10^{-2} and a 10^{-3} dilution on SD medium lacking ura and trp (for YPH500: SD + lys, ade, his, leu). The expected number of transformants per transformation reaction is 10^4-10^5 when using YPH500.

8. Plate each transformation reaction on, for example, 10 large (150 mm) plates of SD medium lacking ura and trp. A low plating density (approximately 1000 colonies per plate) is required for replica plating in the following steps.

9. Incubate plates at 30 °C for 3 days.

10. To identify activator-dependent his[+] colonies, replica plate the colonies on (1) SD medium lacking ura, trp and his and containing 3-AT (SD + lys, ade, leu, × mM 3-AT) and (2) SDgal medium lacking ura, trp and his and containing 3-AT (SD with 2% galactose instead of glucose + lys, ade, leu, × mM 3-AT)[3].

11. Incubate plates at 30 °C for 3 days up to 10 days. Colonies that appear on glucose medium are false positives, as these are activator-independent. Colonies that appear only on galactose medium are putative positives. Note that growth of yeast on galactose is always slower than on glucose[4].

12. To confirm the activator-dependent growth of the putative positives from step 11, replica plate or streak these colonies on (1) SDgal lacking ura, trp and his and containing 3-AT (SD with 2% galactose instead of glucose + lys, ade, leu, × mM 3-AT) and (2) SDgal lacking trp and his and containing 3-AT and 5-FOA (SD with 2% galactose instead of glucose + ura, lys, ade, leu, × mM 3-AT, 0.1% 5-FOA[5]).

13. Incubate plates at 30 °C up to 2 weeks (growth on 5-FOA medium may be very slow). Colonies that appear on 5-FOA medium are

false positives, because the activator plasmid is counterselected. Activator-dependent putative positives grow only on 5-FOA-lacking galactose medium.

14. Isolate the library plasmid from each putative positive according to the plasmid isolation procedure.

15. Retransform the yeast activator strain with the library plasmid and select transformants on SD plates lacking ura and trp (see step 7).

16. Streak transformants on SDgal plates lacking ura, trp and his and containing 3-AT (see step 10). A 3-AT titration may also be performed at this stage to compare the strength of reporter gene activation among the different positives.

17. If the capability of the reporter plasmid to allow growth of the activator strain on galactose-containing medium lacking histidine is confirmed in step 16, proceed with analysis of the putative target site isolated. In brief this analysis could entail the following. Sequence the DNA fragments containing the putative target sites. Perform database searches and discard fragments showing homology to known coding regions or containing repetitive DNA sequences. Make alignments of the various fragments isolated and search for a putative consensus binding site. Perform electrophoretic mobility shift assays or footprinting experiments with the isolated DNA fragments to confirm that the transcription factor of interest is capable of binding the DNA fragment *in vitro*. Clone the DNA fragment upstream of a plant reporter gene and test if this construct is inducible by the transcription factor (if necessary fused to a strong transcription activation domain) in transient assays or in stable transformants. If these tests are positive and when screening for natural target sites, the corresponding gene should be isolated by screening a genomic DNA library with the isolated DNA fragment. Further clues that the isolated gene is a true target of the transcription factor can be obtained by checking for co-localization of the target gene product and the transcription factor and by investigating whether the putative target gene is up or down regulated in transgenic tissues harbouring sense or antisense expression constructions of the transcription factor.

Notes
1. Check for possible loss of the activator plasmid: see note 4 in two-hybrid screening procedure.
2. See note 7 in one-hybrid screening procedure.
3. To avoid isolation of reporter gene plasmids with low affinity binding sites and to reduce the number of clones activated by an endogenous yeast factor, 3-AT is added during selection. We recommed to start with 50 mM 3-AT. If desired this concentra-

tion may be lowered or increased. See also note 3 in one-hybrid screening proce-
dure.

4. Growth on galactose medium can be improved by the addition of 2% raffinose. Raffi-
nose itself does not interfere with galactose induction or glucose repression of the
GAL1 promoter.

5. Dissolve 5-FOA (Duchefa, Haarlem, The Netherlands, F0176) directly in the culture
medium (at ca. 60 °C) just before pouring plates.

LiAc-mediated transformation of yeast

The introduction of DNA into yeast can be accomplished by various methods, such as electroporation, spheroplast transformation, and LiAc-mediated transformation. The LiAc-procedure is the preferred method for library screenings as it is simple and reproducible and results in high transformation efficiencies. Depending on the yeast strain used, we generally obtain 10^4-10^6 transformants/μg DNA with the procedure described below. Please note that in library screenings it is more important to maximize the total number of transformants per transformation than to obtain the highest possible efficiency. Thus, when using for example 10 ng of DNA per transformation the efficiency may be as high as 10^7-10^8/μg, but the total number of transformants obtained will be significantly lower than in a transformation with 1 μg of DNA. The procedure described below is modified from Gietz et al. [23]. Useful tips may also be found at the internet homepage of the Gietz laboratory (http://www.umanitoba.ca/faculties/medicine/human_genetics/gietz).

Steps in the procedure
1. Inoculate a few yeast colonies in 50 ml of YPD (or YAPD for yeast strains carrying an *ade1* or *ade2* mutation) medium and grow overnight with shaking at 30 °C. When the yeast strain already contains an episomal vector, grow the culture in selective medium.
2. Dilute the overnight culture to an OD_{600} of approximately 0.25 in YPD[1] (or YAPD) and grow this culture for another 3 h at 30 °C with shaking[2].
3. Transfer the culture to 50 ml tubes and centrifuge at 2500 rpm for 2 min in a swing out table top centrifuge at room temperature.
4. Discard the supernatant fluid, resuspend the cells in 50 ml H_2O by vortexing and centrifuge as in step 3.
5. Discard the supernatant fluid and resuspend the cells in 1 ml of freshly prepared 1xTE/1xLiAc.
6. Transfer the resuspended cells to an eppendorf tube, centrifuge for 30 s at full speed and resuspend the pellet in 250 μl 1×TE/1×LiAc. The yeast cells are now competent for transformation and immediate use is recommended for optimal efficiency[3].
7. For transformation, mix 1 μg of plasmid DNA and 25 μg of Yeastmaker carrier DNA[4] (Clontech) with 50 μl of competent cells. Prior to use the carrier DNA should be boiled for 10 min and then placed on ice. For co-transformation of two or more plasmids use 2–3 μg of each plasmid. Integration vectors should be linearized prior to transformation.
8. Add 300 μl of freshly prepared 40%PEG/1×TE/1×LiAc and vortex to mix.

9. Incubate at 30 °C for 30 min with shaking.
10. Transfer tubes to a 42 °C waterbath and incubate for 15 min[5].
11. Centrifuge cells for 30 s in an eppendorf centrifuge and resuspend the pellet in 50–200 μl of H_2O.
12. When selecting for an auxotrophic marker, plate cells on SD medium lacking the appropriate amino acid. When selecting for an antibiotic resistance marker gene, grow the cells for 4–6 h in YPD (or YAPD) medium at 30 °C with shaking, then centrifuge the cells and plate on YPD (or YAPD) medium containing the appropriate antibiotic.

Notes
1. It is recommended to use YPD also for cultures grown overnight in selective medium, as this may result in higher competence.
2. For optimal transformation efficiency it is important that the cells are harvested during the exponential growth phase (OD_{600} 0.4-0.8). Therefore, it may be necessary to adjust the incubation time for certain yeast strains.
3. When high transformation efficiency is not required, it is possible to use competent cells stored at -80 °C in 20% glycerol/1xTE/1xLiAc or to prepare competent cells directly from the overnight culture or by resuspending a few yeast colonies from an YPD or SD plate in 1xTE/1xLiAc.
4. Yeastmaker carrier DNA (Clontech) is sheared herring testes DNA. We recommend to use the commercial carrier DNA preparation, as the quality (average size range) of the carrier DNA is an important factor in determining transformation efficiency. Avoid repetitive freeze-thawing of the carrier DNA.
5. The optimal conditions for transformation are strain-dependent. For some yeast strains the efficiency may be improved by lengthening or shortening the 42 °C incubation step or by adjusting the temperature of this step (vary between 38 and 42 °C).

Solutions

10×TE stock:
0.1 M Tris/HCl
10 mM EDTA
pH 7.5
autoclave

10×LiAc stock:
1 M LiAc
pH 7.5, adjust with diluted acetic acid
autoclave

50% PEG stock:
50% polyethylene glycol (PEG) 4000 (Merck, 807490) autoclave; solution is hygroscopic: store in small portions in well-closed bottles

1×TE/1×LiAc:
prepare fresh from 10×TE and 10×LiAc stocks and sterile H_2O

40%PEG/1×TE/1×LiAc:
prepare fresh from 50% PEG, 10×TE and 10×LiAc stocks

Plasmid isolation from yeast

Steps in the procedure
1. Grow a 10 ml overnight culture in selective medium.
2. Centrifuge 3–4 × 1.5 ml of the culture in an eppendorf tube for 30 s at full speed.
3. Resuspend the pellet in 200 μl of 0.9 M sorbitol/ 50 mM EDTA (pH 8.0) containing 4 mg/ml freshly added lyticase (from *Arthrobacter luteus*, 750 U/mg, Sigma L-4025).
4. Incubate for 60 min at 30 °C to produce spheroplasts.
5. Centrifuge for 5 min at 3000 rpm[1] in an eppendorf centrifuge.
6. Use the pellet to perform a standard alkaline lysis plasmid DNA miniprep as normally used for plasmid isolation from *E. coli*. Dissolve the plasmid DNA in 20 μl of TE.
7. Use 2–10 μl for transformation of chemically competent *E. coli* cells or electroporate 1–2 μl to electro-competent *E. coli* cells[2].
8. Isolate DNA from the *E. coli* transformants by a standard plasmid DNA miniprep procedure[3].

Notes
1. Centrifugation at higher speed will results in premature lysis of spheroplasts.
2. For isolation of multicopy (2 μ) plasmids transformation of chemically competent cells (transformation efficiency $\geq 10^6/\mu$g DNA) is usually satisfactory. For single copy (ARS/CEN) plasmids it may be necessary to use electro-competent cells with a higher transformation efficiency.
3. When isolating plasmids from positive yeast colonies resulting from a library screening, it is advisable to check the plasmid DNA preparations from several *E. coli* transformants by restriction endonuclease analysis, because of the possibility that the yeast cells may have been harboring more than one library plasmid. Also be aware of the fact that recombination between different vectors may occur in yeast.

Filter assay for β-galactosidase activity

Steps in the procedure
1. Plate or restreak yeast transformants[1] on selective medium and incubate for 1-3 days at 30 °C.
2. Lift the yeast cells with a filter of reinforced nitrocellulose[2] (Optitran BA-S 85, 0.45 μm, Schleicher and Schuell). Proceed directly with the filter or – for maximum sensitivity – incubate the filter overnight on a fresh plate of selective medium.
3. Place the filter in a polystyrene tray and pour liquid nitrogen over the filter.
4. Let the filter thaw, then pour liquid nitrogen over the filter again.
5. Let the filter thaw, then place it in a petridish on a Whatman filter paper soaked in Z-buffer containing freshly added β-mercaptoethanol (50 mM) and X-GAL (0.3 mg/ml).
6. Incubate at 30 °C for 15 min up to several hours and check periodically for blue staining[3].

Notes
1. Positive control plasmids are available, for example, from Clontech.
2. Normal nitrocellulose can be used, but easily cracks when frozen.
3. Overnight incubation sometimes results in false positives.

Solutions

Z-buffer: 60 mM Na_2HPO_4
              40 mM NaH_2PO_4
              10 mM KCl
              1 mM $MgSO_4$
              adjust to pH 7.0
              autoclave

X-Gal stock: 30 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in N,N-dimethylformamide
              store at -20 °C

Quantitative β-galactosidase assay

Steps in the procedure
1. Grow yeast cultures in selective medium to an OD of 0.5 – 0.7 and determine the exact OD_{600}. Alternatively, resuspend colonies from fresh plates of selective medium in water to an OD of approximately 0.6 and determine the exact OD_{600}. When assaying larger numbers of cultures, it is convenient to use a microtiter plate reader for the OD600 measurements. For accuracy, assay each culture in duplicate or triplicate.
2. Spin down 0.5 ml of the culture or resuspended yeast cells in an eppendorf tube by centrifugation for 30 sec at full speed.
3. Carefully remove the supernatant solution, then wash the pellet in 1 ml of Z-buffer and centrifuge again for 30 s at full speed.
4. Carefully remove the supernatant solution and resuspend the pellet in 100 μl of Z-buffer.
5. Freeze the tube in liquid nitrogen for 1 min, then thaw in a 37 °C water bath.
6. Immediately after thawing, repeat step 5 once.
7. Add 700 μl of Z-buffer containing 50 mM β-mercaptoethanol.
8. Start the incubation by addition of 160 μl of ONPG[1] solution, record the time, vortex and place the tube in a 30 °C waterbath or heat block. It is recommended to start the different reactions at, for example, 30 s intervals. Also set up a blank reaction with 100 μl of Z-buffer instead of yeast suspension.
9. Mix the reactions by inversion approximately every 15 min to prevent sedimentation of the yeast cells and check for development of a yellow color.
10. Stop reactions that have turned yellow by the addition of 400 μl of 1 M Na_2CO_3, mix and record the time.
11. Centrifuge the tubes in an eppendorf centrifuge for 5 min at full speed.
12. Carefully transfer the supernatant solutions to cuvettes or to the wells of a microtiter plate and determine the absorbance at 420 nm. Use the blank reaction as reference for calibration. The assay is linear at $A_{420} < 1$.
13. Calculate the β-galactosidase activity in Miller units [42], according to the following formula (1 unit is defined as the amount which hydrolyzes 1 μmol of ONPG per min):
β-galactosidase units = $1000 \times A_{420}/(t \times V \times OD_{600})$
t = incubation time (min)
V = culture volume assayed (0.5 ml)

Notes

1. More sensitive detection of β-galactosidase activity is possible by using CPRG or Galacton-Star™ (Clontech) as substrate. We have no experience with this, but assay procedures are described in the Clontech Yeast Protocols Handbook.

Solutions

Z-buffer: see description in the β-galactosidase filter assay procedure

ONPG solution: 4 mg/ml o-nitrophenyl-β-D-galactopyranoside in Z-buffer prepare fresh before use

References

1. Aronheim A, Zandi E, Henneman H, Elledge SJ and Karin M (1997) Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Mol Cell Biol 17: 3094– 3102.
2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1997) Current Protocols in Molecular Biology. Chapter 13: *Saccharomyces cerevisae*. John Wiley & Sons.
3. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1997) Current Protocols in Molecular Biology. Chapter 20: Analysis of Protein Interactions. John Wiley & Sons.
4. Bartel PL, Chien CT, Sternglanz R and Fields S (1993) Using the two-hybrid system to detect protein-protein interactions. In: Cellular Interactions in Development: A Practical Approach (Hartley DA ed.) pp. 153–179. Oxford University Press.
5. Becker DM, Fikes JD and Guarente L (1991) A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. Proc Natl Acad Sci USA 88: 1968–1972.
6. Bonner JJ (1991) Vectors for the expression and analysis of DNA-binding proteins in yeast. Gene 104: 113–118.
7. Brunelli JP and Pall ML (1993) A series of yeast/*Escherichia coli* λ expression vectors designed for directional cloning of cDNAs and Cre/*lox*-mediated plasmid excision. Yeast 9: 1309–1318.
8. Cavener DR and Ray SC (1991) Eukaryotic start and stop translation sites. Nucl Acids Res 19: 3185–3192.
9. Chan JY, Han X-L and Kan YW (1993) Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. Proc Natl Acad Sci USA 90: 11371–11375.
10. Chang YC and Timberlake WE (1992) Identification of *Aspergillus brlA* response elements (BREs) by genetic selection in yeast. Genetics 133: 29–38.
11. Chien CT, Bartel P, Sternglanz R and Fields S (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA 88: 9578–9582.
12. Claret FX, Hibi M, Dhut S, Toda T and Karin M (1996) A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature 383: 453-457.
13. Clausen M, Lamb CJ, Megnet R and Doerner PW (1993) Hormone-inducible expression and metal affinity chromatography of recombinant proteins in *Saccharomyces cerevisiae*. Anal Biochem 212: 537–539.
14. Davies B, Egea-Cortines M, de Andrade Silva E, Saedler H and Sommer H (1996) Multiple interactions amongst floral homeotic MADS box proteins. EMBO J 15: 4330–4343.
15. Durfee T, Becherer K, Chen PL, Yeh SH, Yang Y, Kilburn AE, Lee WH and Elledge SJ (1993) The retinablastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes & Dev 7: 555–569.
16. Elledge SJ, Mulligan JT, Ramer SW, Spottswood M and Davis RW (1991) λYES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc Natl Acad Sci USA 88: 1731–1735.
17. Fields S and Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340: 245–247.
18. Fields S and Sternglanz R (1994) The two-hybrid system: An assay for protein-protein interactions. Trends Genet 10: 286–292.
19. Foreman PK and Davis RW (1994) Cloning vectors for the synthesis of epitope-tagged, truncated and chimeric proteins in *Saccharomyces cerevisiae*. Gene 144: 63–68.
20. Forsburg SL and Guarente L (1988) Mutational analysis of upstream activation sequence 2 of the *CYC1* gene of *Saccharomyces cerevisae*: a HAP2-HAP3-responsive site. Mol Cell Biol 8: 647–654.

21. Friedman-Einat M, Einat P, Snyder M and Ruddle F (1996) Target gene identification: Target specific transcriptional activation by three murine homeodomain/VP16 hybrid proteins in *Saccharomyces cerevisiae*. J Exp Zool 274: 145–156.

22. Gari E, Piedrafita L, Aldea M and Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. Yeast 13: 837–848.

23. Gietz D, St Jean A, Woods RA and Schiestl RH (1992) Improved method for high efficiency transformation of yeast. Nucl Acids Res 20: 1425.

24. Gritz L and Davies J (1983) Plasmid-encoded hygromycin B resistance: The sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisae*. Gene 25: 179–188.

25. Gstaiger M, Knoepfel L, Georgiev O, Schaffner W and Hovens CM (1995) A B-cell coactivator of octamer-binding transcription factors. Nature 373: 360–362.

26. Guthrie C and Fink GR (1991) Guide to Yeast Genetics and Molecular Biology. Methods Enzymology. Volume 194. Academic Press, San Diego.

27. Gyuris J, Golemis EA, Chertkov H and Brent R (1993) Cdi1, a human G1- and S-phase protein phosphatase that associates with Cdk2. Cell 75: 791–803.

28. Hadfield C, Cashmore AM and Meacock PA (1986) An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. Gene 45: 149–158.

29. Hadfield C, Cashmore AM and Meacock PA (1987) Sequence and expression characteristics of a shuttle chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. Gene 52: 59–70.

30. Hadfield C, Jordan BE, Mount RC, Pretorius GHJ and Burak E (1990) G418 resistance as a dominant marker and reporter for gene expression in *Saccharomyces cerevisiae*. Curr Genet 18: 303–313.

31. Kim SY, Chung HJ and Thomas TL (1997) Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J 11: 1237–1251.

32. Kohalmi SE, Reader LJV, Samash A, Nowak J, Haughn GW, Crosby WL (1998) Identification and characterization of protein interactions using the yeast 2-hybrid system. In: Plant Molecular Biology Manual 2nd Edition, Suppl. III (Gelvin SB, Schilperoort RA, eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands, M1: 1–30.

33. Legrain P, Dokhelar M-C and Transy C (1994) Detection of protein-protein interactions using different vectors in the two-hybrid system. Nucl Acids Res 22: 3241–3242.

34. Licitra EJ and Liu JO (1996) A three-hybrid system for detecting small ligand-protein interactions. Proc Natl Acad Sci USA 93: 12817–12821.

35. Liu J, Wilson TE, Milbrandt J and Johnston M (1993) Identifying DNA-binding sites and analyzing DNA-binding domains using a yeast selection system. Methods 5: 125–137.

36. Macreadie IG, Horaitis O, Verkuylen AJ and Savin KW (1991) Improved shuttle vectors for cloning and high-level Cu^{2+}-mediated expression of foreign genes in yeast. Gene 104: 107–111.

37. Marathe SV and McEwen JE (1995) Vectors with the *gus* reporter gene for identifying and quantitating promoter regions in *Saccharomyces cerevisiae*. Gene 154: 1844–1849.

38. Mascorro-Gallardo JO, Covarrubias AA and Gaxiola R (1996) Construction of a *CUP1* promoter-based vector to modulate gene expression in *Saccharomyces cerevisiae*. Gene 172: 169–170.

39. Mastick GS, McKay R, Oligino T, Donovan K and López AJ (1995) Identification of target genes regulated by homeodomain proteins *in Drosophila melanogaster* through genetic selection of Ultrabithorax protein-binding sites in yeast. Genetics 139: 349–363.

40. Meijer AH, Ouwerkerk PBF and Hoge JHC (1998) Vectors for transcription factor cloning and target site identification by genetic selection in yeast. Yeast 14: 1407–1416.

41. Memelink J (1997) Two yeast/*Escherichia coli* Lambda/plasmid vectors designed for yeast one- and two-hybrid screens that allow directional cDNA cloning. Trends Genet 13: 376.

42. Miller JH (1972) Experiments in Molecular Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

43. Minet M and Lacroute F (1990) Cloning and sequencing of a human cDNA coding for a multifunctional polypeptide of the purine pathway by complementation of the *ade2-101* mutant in *Saccharomyces cerevisiae*. Curr Genet 18: 287–291.

44. Minet M, Dufour M-E and Lacroute F (1992) Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J 2: 417–422.

45. Niedenthal RK, Riles L, Johnston M and Hegemann JH (1996) Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. Yeast 12: 773–786.

46. Osborne MA, Dalton S and Kochan JP (1995) The yeast tribrid system – Genetic detection of trans-phosphorylated ITAM-SH2-interactions. Biotechnology 13: 1474–1478.

47. Ouwerkerk PBF and Memelink J (1997) A simple method for directional multimerization of DNA sequences. Trends Genet 13: 207.

48. Picard D, Schena M and Yamamoto KR (1990) An inducible expression vector for both fission and budding yeast. Gene 86: 257–261.

49. Romanos MA, Scorer CA and Clare JJ (1992) Foreign gene expression in yeast: A review. Yeast 8: 423–488.

50. Riesmeier JW, Willmitzer L and Frommer B (1992) Isolation and characterization of a sucrose carrier DNA from spinach by functional expression in yeast. EMBO J 11: 4705–4713.

51. Schmitz UK, Lonsdale DM and Jefferson RA (1990) Application of the β-glucuronidase gene fusion system to *Saccharomyces cerevisae*. Curr Genet 17: 261–264.

52. Schultz TF and Quatrano RS (1997) Characterization and expression of a rice *RAD23* gene. Plant Mol Biol 34: 557–562.

53. SenGupta DJ, Zhang B, Kraemer B, Pochart P, Fields S and Wickens S (1996) A three-hybrid system to detect RNA-protein interactions *in vivo*. Proc Natl Acad Sci USA 93: 8496–8501.

54. Sikorski RS and Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19–27.

55. Siemering KR, Golbik R, Sever R and Haseloff J (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. Curr Biol 6: 1653–1663.

56. Sieweke MH, Tekotte H, Frampton J and Graf T (1996) MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. Cell 85: 49–60.

57. Ulmasov T, Hagen G and Guilfoyle TJ (1997) ARF1, a transcription factor that binds to auxin reponse elements. Science 276: 1865–1868.

58. Vidal M, Brachmann RK, Fattaey A, Harlow E and Boeke JD (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and protein-DNA interactions. Proc Natl Acad Sci USA93: 10315–10320.

59. Vidal M, Braun P, Chen E, Boeke JD and Harlow E (1996) Genetic characterization of a mammalian protein-protein interaction by using a yeast reverse two-hybrid system. Proc Natl Acad Sci USA 93: 10321–10326.

60. Vojtek AB, Hollenberg SM and Cooper JA (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 74: 205–214.

61. Wang MM and Reed RR (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364: 121–126.

62. Wilson TE, Fahrner TJ, Johnston M and Milbrandt J (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. Science 252: 1296–1300.

Section N:
Plant recombination systems
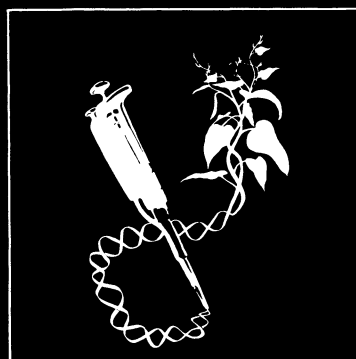
**Plant Molecular
Biology Manual**

Cre-*lox* Directed Integration of Transgenes into the Tobacco Genome

Henrik Albert[1] and David W. Ow[2] *

[1]Tropical Fruit, Vegetable, and Sugarcane Research Laboratory, USDA/ARS, 99–193 Aiea Heights Dr., Aiea, HI 96701, USA;
[2]Plant Gene Expression Center, USDA/ARS, 800 Buchanan St., Albany, CA 94710, USA
* *Corresponding author (ow@pgec.ars.usda.gov)*

Introduction

Several site-specific recombination systems have been tested for genome manipulations in plants [17]. One particular application is to direct the insertion of DNA into recombination sites previously placed into the genome [2]. Because it is a commonly held belief that expression variation results from "position effects" (the random chromosome locations of transgenes), integrating DNA into a defined chromosome location is thought to reduce transgene expression variability among independent transformants. There is recent indication that this "position effect" does not account for all of the variability of transgene expression [16]. However, site-specific DNA integration remains an attractive transformation tool for additional reasons: It can produce transgenic plants that predominantly harbor single copy and precise molecules, i.e., molecules without the rearrangements and truncations frequently seen with other transformation systems [1]. To have single copy insertions may by itself eliminate one of the factors most frequently linked to multicopy associated transgene silencing [13, 18].

The site-specific DNA integration experiments described by Albert et al. [1] rely on use of the Cre-*lox* recombination system from bacteriophage P1 [9]. It consists of two components: a 38 kDa Cre recombinase and a 34 basepair (bp) *lox* recombination site. The *lox* site is composed of an asymmetrical 8 bp spacer sequence flanked by 2 copies of a 13 bp sequence in opposite orientations (Figure 1). In this paper the 13 bp inverted repeat will be referred to as the right and left elements. These elements are the recognition and binding sites for the Cre protein, with one Cre monomer binding to each element. Cre binding to the elements is a cooperative process, so that binding of a Cre monomer at one element promotes the binding of another Cre monomer at the other [3, 6, 12].

Cre-*lox* directed transgene insertion involves a recombination reaction between a chromosomal *lox* site and a circular transgene construct containing a second *lox* site. A first requirement is to generate transgenic plants containing a chromosomally integrated *lox* site. A plant line with an integration site, or 'target', may be used for many experiments, so verification of the integrity of the chromosomal target is essential. We have used the binary vector pBIN19 [4] and *Agrobacterium*-mediated transformation to generate our transgenic target tobacco lines. Because pBIN19 carries the *nptII* gene as a selection marker, a dif-

A.

	Left element	8 bp spacer	Right element
loxP	ATAACTTCGTATA	GCATACAT	TATACGAAGTTAT
		X	
loxP	ATAACTTCGTATA	GCATACAT	TATACGAAGTTAT
lox66	ATAACTTCGTATA	GCATACAT	TATACGAAcggta
		X	
lox71	taccgTTCGTATA	GCATACAT	TATACGAAGTTAT
lox76	ATAACTTCGTATA	GCATACAT	TATACGcccggta
		X	
lox75	taccgggCGTATA	GCATACAT	TATACGAAGTTAT

B.

Fig. 1. (A) Nucleotide sequence of the wild-type *lox* site (*loxP*) and two pairs of modified *lox* sites. Lower case letters indicate deviation from *loxP* sequence. (B) Symbol for *lox* used in this paper show outer rectangles representing the right and left elements (13 bp inverted repeat) and the arrowhead representing the orientation of the central spacer (8 bp).

ferent selectable marker gene was used to select Cre-*lox* mediated integration of additional DNA. For this purpose, we used the *hpt* gene (hygromycin phosphotransferase) that confers resistance to hygromycin [7]. Upon verification of a satisfactory target plant line, circular transgene constructions containing a *lox* site can then be integrated into the chromosomal *lox* site. The product of the recombination is the integrated linearized construction flanked by two *lox* sites (Figure 2). Because Cre-*lox* recombination is freely reversible, the two *lox* sites flanking the transgene can recombine again to excise the intervening DNA. Recombination between two *lox* sites closely linked on the same molecule is more likely than recombination between *lox* sites on independent molecules. Therefore excision is favored over the insertion reaction. This means that integration events may occur only transiently in the presence of Cre recombinase.

To obtain a stably integrated molecule, two approaches have been used to abolish Cre activity after site-specific integration. In the first approach, Cre activity is provided through the transient expression of a *cre* construction. Target plant cells are co-transformed with two constructs: the insertion plasmid construction containing a *lox* site, and the *cre* expression construction lacking a *lox* site. The *cre* construction provides Cre recombinase for the *lox*-containing insertion plasmid to recombine with the chromosomal site. Unless the *cre* con-
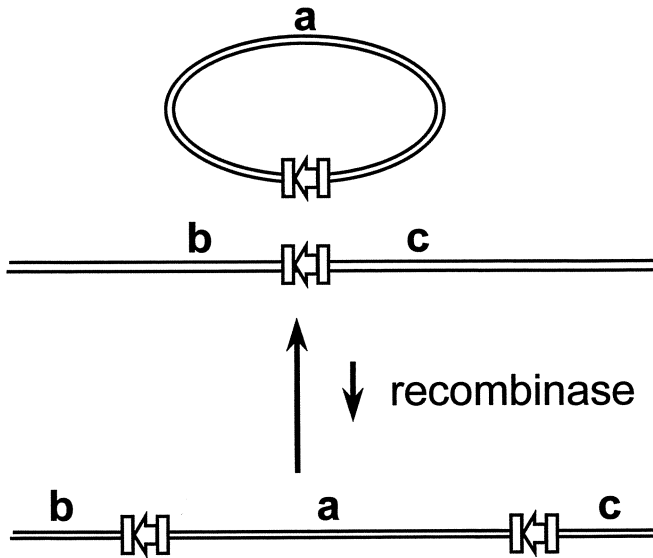
Fig. 2. Recombination between a chromosomal *lox* site and a *lox* site-containing plasmid. Arrows between the two states indicate that excision is favored over integration.

struction integrates stably into the genome, it will be lost upon cell division and thereby deplete Cre recombinase activity. An example of this has been shown through the transient expression of pMM23, a *35S-cre* plasmid, where *35S* is the cauliflower mosaic virus 35S RNA promoter [14]. In the second approach, the chromosomal target construction contains a *35S-lox-cre* construction where the target *lox* site is situated between the promoter and *cre* coding region. Insertion into the target *lox* site displaces the *cre* gene from its promoter and results in termination of *cre* expression (Figure 3).

A supplementary strategy for stabilizing Cre-*lox* insertions has been the use of modified *lox* sites engineered to make the recombination reaction a directional process. Development of *lox* sites that contain base changes in one element but retain the wild-type sequence for the other element and which are still recognized by Cre have been described [1]. The nucleotide sequences for the wild-type *lox* site (*loxP*) and two pairs of modified *lox* sites that were successfully used in plant transformation are shown in Figure 1. Recombination between one of these modified sites and a complementary site, i.e., a *lox* site with base changes in the opposite element, produces one *lox* site which is fully wild-type and the other with base changes in both elements (Figure 4). This 'double mutant site' is poorly recognized by Cre, and hence excision is inhibited. Using complementary modified *lox* sites in the chromosomal target construction and in the insertion plasmid serves to stabilize these integration events.

As the substrate for Cre recombinase is double-stranded DNA, transgene constructions for integration into a chromosomal *lox* site can be introduced by direct DNA transformation. In tobacco and other systems where regeneration from
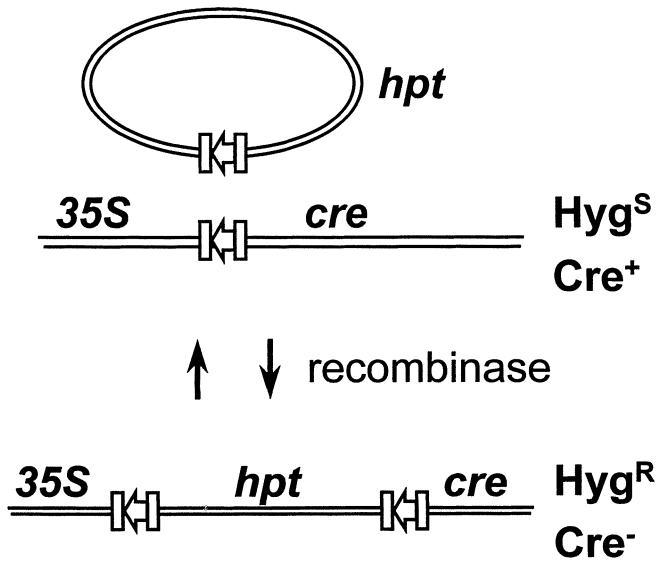
Fig. 3. Control of Cre activity by promoter displacement. The *cre* gene is expressed from the chromosomal target construction. An insertional recombination event separates the *cre* coding region from its promoter (*35S*) and terminates *cre* expression.
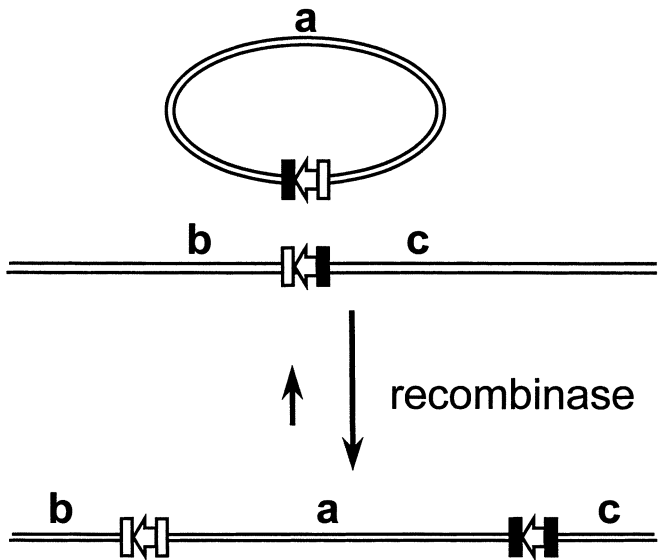
Fig. 4. Stabilization of Cre-*lox* insertion events by use of modified *lox* sites. Recombination between one *lox* site containing a modified left element (filled rectangle) and a 'complementary' *lox* site containing a modified right element produces a 'double-mutant' *lox* site that is poorly recognized by Cre. Thus, the integrated molecule is less prone to excise.

protoplasts has been worked out, polyethylene glycol-(PEG) mediated transformation of protoplasts is an effective approach. The protocol below is adapted from the published work of numerous researchers [5, 14, 15, 19, 20].

The configuration of the selectable marker can be designed so that random insertions of the transgene construction do not ordinarily give rise to resistant calli. The chromosomal target construction contains a *lox* site immediately downstream of a promoter, and an insertion plasmid contains a *lox* site immediately upstream of a promoterless selectable marker gene (Figure 5). Intermo-
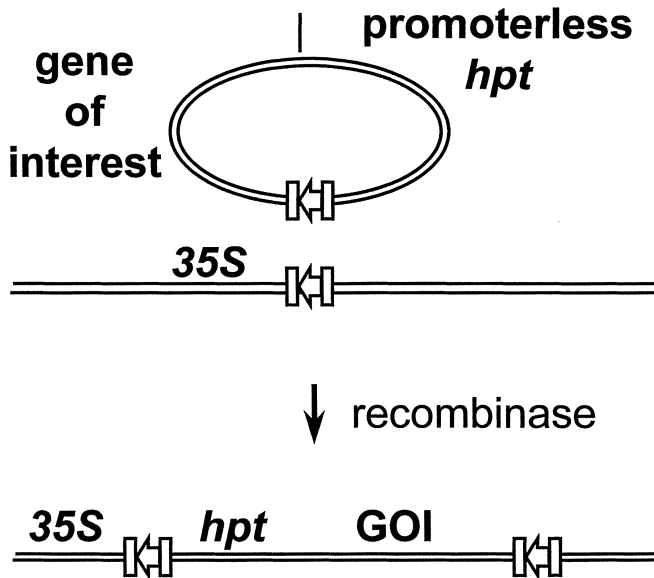
Fig. 5. Use of a promoterless selectable marker gene to reduce recovery of random insertion events. The insertion plasmid contains the 'gene of interest' (GOI) and a promoterless selectable marker gene (*hpt*). Cre mediated insertion places the selectable marker gene immediately downstream of the chromosomal target promoter (*35S*) to confer a resistance phenotype.

lecular Cre-*lox* recombination places the selectable marker gene immediately downstream of the target site promoter, resulting in transcription of the selectable marker and manifestation of the resistance phenotype. Insertion plasmids that integrate elsewhere in the genome would not produce the resistance phenotype unless the insertion plasmid breaks upstream of the *lox* site and then fuses *hpt* to an endogenous plant promoter. This combination of events would be less probable than site-specific integration. Indeed, fusion of the insertion plasmid to endogenous promoters was not found among 58 hygromycin resistant plants analyzed [1].

Procedures

Maintenance of target plant lines

The following example is for Nicotiana tabacum cv. Wisconsin 38.
Transgenic tobacco plants harboring chromosomal *lox* target sites
are grown axenically by standard tissue culture procedures. Grow the
plants in Magenta boxes on MSNT medium with 0.8% Bacto-Agar at
26 °C with 16 h of light per day. Transfer stem apices every four to six
weeks to maintain healthy growing plants.

Isolation of protoplasts and PEG transfection

1. Harvest young healthy leaves about 3–4 cm long.
2. Place 5 to 8 leaves per petri dish containing 15 ml protoplasting
   enzyme solution. 8 leaves typically yield ca. 10^7 protoplasts.
3. Stack the leaves 2 deep and cut into strips about 2 mm wide, per-
   pendicular to the mid-rib, using a sterile scalpel.
4. Seal the plates with parafilm and agitate on a platform shaker at
   the lowest speed (ca. 80 rpm) for 10 min.
5. Incubate 14 to 18 h at room temperature in the dark.
6. Agitate on platform shaker at lowest speed (ca. 80 rpm) for
   10 min.
7. Gently pipette leaf debris up and down in a wide-bore 25 ml pi-
   pette to release protoplasts. Transfer digestion mix to sterile 100
   mesh nylon screen to filter debris.
8. Transfer filtered protoplast solution to 50 ml conical polypropy-
   lene tube and centrifuge 20 min, 200 g, at room temperature in a
   swinging bucket rotor to float the protoplasts.
9. Transfer the floating protoplasts to a fresh 50 ml conical polypro-
   pylene tube. This step requires patience and practice. A relatively
   narrow 5 ml pipette with an automatic pipetter works best. Touch
   the pipette tip to the surface of the liquid and gently move the pi-
   pette up while drawing in protoplasts until the pipette leaves the
   surface. Repeat until all of the protoplasts have been harvested,
   but keeping the volume less than 10 ml.
10. Dilute the protoplasts with 4 volumes W5 and mix gently. Pellet
    the protoplasts for 5 min., 200 g, at room temperature.
11. Remove the supernatant fluid and gently resuspend the proto-
    plasts in 40 ml W5. Pellet as in step 10.
12. Repeat the W5 wash as in step 11.
13. Resuspend the pellet in 10 ml W5 and count the protoplasts in a
    hemocytometer.
14. Aliquot 2×10^6 intact protoplasts into 50 ml conical polypropy-

lene tubes for each transformation. Add MaMg to 10 ml and pellet as above.

15. Remove the supernatant solution and resuspend with 0.5 ml MaMg. Total volume would be ca. 0.6 ml.

16. (optional) Heat shock cells 5 min in a 45 °C water bath. Remove tubes and place at room temperature. Heat shock has been reported to improve transient expression, but we see reduced protoplast regeneration.

17. After 10 to 15 min, or directly if not using heat shock, gently mix in transformation DNA. Typically, we use 10 μg supercoiled plasmid DNA plus 50 μg carrier DNA. If co-transforming with a *cre* expression plasmid, include an additional 10 μg of this DNA, for a total of 70 μg. Make certain that all of the DNA samples are aseptic. The total DNA used is typically in a volume of less than 30 μl.

18. Gently add 0.6 ml 40% PEG in MaMg and swirl gently.

19. Incubate at room temperature for 25 min, gently swirling occasionally.

20. Slowly and gently, dilute protoplasts with 10 ml K_3A media. We add 2 ml dropwise, swirling as we add. Then wait at least 10 min before the next addition of another 2 ml. This slow dilution is VERY tedious, but in some cases, it seems to have a major effect on protoplast regeneration.

21. Transfer protoplasts to petri dishes (we use NUNC). Add an additional 10 ml K_3A and gently mix. Protoplasts should now be at approx. 5×10^4/ml, if 50% survived the PEG treatment. At this point you can check the protoplasts on an inverted microscope. Many shrunken (evacuolated) or broken cells indicate problems. Seal the plates with parafilm; incubate in the dark (without shaking) at 26 °C.

Selection and regeneration of transformants

1 After 1 week, the protoplasts should have undergone wall regeneration and several cell divisions. Transfer protoplasts to a 50 ml conical polypropylene tube (save the plate and keep covered, as many protoplasts will be stuck to the plate) and add an equal volume of the wash solution. Mix gently and pellet for 7 min, 200 g, at room temperature.

2 Remove the supernatant fluid except ca. 0.5 ml. Resuspend pellet gently and transfer to the original plate. If the protoplasts look very dense, divide them into 2 plates. Add 20 ml 1% Seaplaque low melting agarose in 1X K_3A at 37–40 °C. Swirl gently to distribute protoplasts evenly. Allow agarose to gel.

3. Cut protoplast embedded agarose into quarter portions with a sterile spatula. Transfer 1/8 of the amount to a fresh plate containing

30 ml K$_3$A for a non-selected control (this will gauge protoplast viability). Add 30 ml K$_3$A with selection agent (we use hygromycin B at 20 μg/ml). Use sterile spatula to loosen and float agarose sections. Seal plates with parafilm and rotate at 80 rpm on a platform shaker, in the dark at 26 °C.

4. Renew liquid media every week. Many micro-calli will be loosened from the agarose and will be free in the medium; use caution to minimize the loss of these micro-calli.

5. Resistant calli (ca. 1-2 mm) should become visible by 3 to 6 weeks. These can be transferred to solid shooting media (MSNTS) with selection (such as hygromycin B at 20 μg/ml) and moved to a growth chamber with 16 h light, 26 °C.

6. Shoots that arise can be transferred to solid MSNT in magenta boxes. We do not add a selection agent to the rooting medium.

7. To confirm stable maintenance of a selectable marker gene, a 'shooting assay' may be used. Remove a leaf from a putative transgenic plant, place it on solid MSNTS with selection, and cut the leaf into small (ca. 0.5 to 1.0 mm square) pieces. Use a leaf from an untransformed plant as a negative control. Transformed leaf pieces will develop callus along the cut edges within ca. 2 weeks, and shoots will develop within a few weeks thereafter.

8. PCR with primers flanking the upstream and downstream *lox* sites of the integration junction can be used to confirm targeted insertion. DNA gel blots can be used to analyze copy number, construction integrity, and the presence of random integration events.

Media and solutions

K$_3$AS

final concentration	*amount for 1 liter*
1X MS salts	4.3 g
3.13 mM CaCl$_2$ · 2H$_2$O	0.46 g
1.67 mM xylose	0.25 g
400 mM sucrose	137.0 g
1X B$_5$ vitamins	1.0 ml 1000X stock
10.8 μM NAA	1.0 ml 2 mg/ml stock
2.22 μM BA	25.0 μl 20 mg/ml stock

K_3A

final concentration	amount for 1 liter
1X MS salts	4.3 g
3.13 mM $CaCl_2 \cdot 2H_2O$	0.46 g
1.67 mM xylose	0.25 g
300 mM sucrose	102.96 g
400 mM mannitol	72.8 g
1X B_5 vitamins	1.0 ml 1000X stock
5.38 μM NAA	0.5 ml 2 mg/ml stock
888 ηM BA	10.0 μl 20 mg/ml stock
452 ηM 2,4D	0.5 ml 200 μg/ml stock

For K_3AS and K_3A: Adjust to pH 5.8 with 1 N KOH, adjust to 1000 ml with H_2O, filter sterilize with 0.45 micron filter. MS salts (GIBCO BRL #11117-066), NAA = α-naphthaleneacetic acid (GIBCO BRL #21570-015), BA = Benzyladenine (GIBCO BRL #16105-017); 2,4-D = 2,4-dichlorophenoxyacetic acid (Sigma #D7299). NAA and BA stocks dissolved in DMSO, 2,4-D stock dissolved in ethanol. These solutions are stable at room temperature for at least one month.

W5

final concentration	amount for 1 liter
154 mM NaCl	9.00 g
125 mM $CaCl_2 \cdot 2H_2O$	18.37 g
5 mM KCl	0.37 g
5 mM glucose	0.90 g

Adjust pH to 5.8 with 1 N KOH, adjust to 1000 ml with H_2O, filter sterilize with 0.45 micron filter. Stable at room temperature for at least one month.

Protoplasting enzyme solution
1% Cellulase R10 (Serva #16419)
0.2% Macerozyme (Serva #28302)

Dissolve in K$_3$AS, pH to 5.8 with 1 N KOH, centrifuge 2000 g, 5 min to pellet insoluble material and filter sterilize through 0.45 micron filter. Prepare fresh for each use.

2X MaMg

final concentration	*amount for 100 ml*
0.8 M mannitol	14.6 g
30 mM MgCl$_2$	0.61 g
0.2% w/v MES	0.20 g

Adjust to pH to 5.6 with 1 N KOH, adjust to 100 ml with H$_2$O, filter sterilize with 0.45 micron filter. Stable at room temperature for at least one month. MES = 2-(N-morpholino) ethane sulfonic acid (Sigma #M8250).

Polyethylene glycol solution
40% w/v PEG, MW 3,350 (Sigma #P-3640) in MaMg
Dissolve PEG in 2X MaMg, adjust total volume to make MaMg 1X. Check to see pH is 5.6–7.0, adjust with KOH or HCl if necessary. This solution may be frozen in 10 ml aliquots and stored at −20° C. Thaw and filter sterilize with 0.45 micron filter on day of use.

1000X B5 vitamins

final concentration	*amount for 100 ml*
8.12 mM nicotinic acid	0.1 g
29.6 mM thiamine HCl	1.0 g
48.6 mM pyridoxine HCl	1.0 g
555 mM myoinositol	10.0 g

Adjust to 100 ml with H$_2$O, filter sterilize with 0.45 micron filter. Store frozen at −20° C. Nicotinic acid (Sigma #N4126), thiamine HCl (Sigma #T3902), pyridoxine HCl (Sigma #P9755), myoinositol (Sigma #I3011).

Carrier DNA
Prepare a 10 mg/ml solution of calf thymus DNA (Sigma #D1501). Sonicate until average size is 0.5 to 2 kb. Phenol extract, chloroform extract and ethanol precipitate. Alternatively, Sigma #D8661 may be used as supplied.

Wash solution

final concentration   *amount for 500 ml*

0.2 M $CaCl_2 \cdot 2H_2O$ 14.7 g
0.5% w/v MES   2.5 g

Adjust pH to 5.8 with 1 N KOH, adjust to 500 ml with H_2O. Stable at room temperature for at least one month. MES = 2-(N-morpholino) ethane sulfonic acid (Sigma #M8250).

MSNT Agar

final concentration   *amount for 1 liter*

1X MS salts   4.3 g
87.6 mM sucrose   30.0 g
1X B5 vitamins   1.0 ml 1000X stock

Dissolve in 800 ml H_2O, adjust pH to 5.7-5.9 with 1 N NaOH or KOH. Add 8 g Bacto-Agar, adjust to 1000 ml and autoclave.

MSNTS Agar

final concentration   *amount for 1 liter*

1X MS salts   4.3 g
87.6 mM sucrose   30.0 g
1X B5 vitamins   1.0 ml 1000X stock
537 ηM NAA   50 μl 2 mg/ml stock
4.44 ηM BA   50 μl 20 mg/ml stock

Dissolve in 800 ml H_2O, adjust pH to 5.7-5.9 with 1 N NaOH or KOH. Add 8 g Bacto-Agar, adjust to 1000 ml and autoclave.
     MS salts (GIBCO BRL #1117-066), NAA = α-naphthaleneacetic acetic acid (GIBCO BRL #21570-015), BA = Benzyladenine (GIBCO BRL #16105-017); NAA and BA stocks dissolved in DMSO.

References

1. Albert H, Dale EC, Lee E. and Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J. 7: 649–659.
2. Albert H and Ow DW (1998) Recombinase-mediated gene integration in plants. In: Jain S.M., Brar D.S. and Ahloowalia B.S. (eds.), Somaclonal Variation and Induced Mutation in Crop Improvement. Kluwer Academic Publishers, Dordrecht, pp. 503–518.
3. Argos P, Ly A, Abremski K, Egan JB, Ljungquist EH, Hoess RH, Kahn ML, Kalionis B, Narayana SVL, Pierson LS, Sternberg N and Leong JM (1986) The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J 5: 433–440.
4. Bevan M (1984). Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12: 8711–8721.
5. Bilang R, Klöti A, Schrott M and Potrykus I (1994) PEG-mediated direct gene transfer and electroporation. In: Gelvin SB and Schilperoort RA (eds.), Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, pp. A1:1–16.
6. Bruckner RC and Cox MM (1986) Specific contacts between the FLP protein of the yeast 2-micron plasmid and its recombination site. J Biol Chem 261: 11798–11807.
7. Elzen Pvd, Townsend J, Lee K and Bedbrook J (1985) A chimeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol Biol 5: 299–302.
8. Fukushige S and Sauer B (1992) Genomic targeting with a positive-selection *lox* integration vector allows highly reproducible gene expression in mammalian cells. Proc Natl Acad Sci USA 89: 7905–7909.
9. Hoess RH and Abremski K (1990) The Cre-*lox* recombination system. In: Eckstein F and Lilley DMJ (eds.), Nucleic Acids and Molecular Biology. Springer-Verlag, Berlin, pp. 99–109.
10. Jaenisch R, Jähner D, Nobis P, Simon I, Löhler J, Harbers K and Grotkopp D (1981). Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. Cell 24: 519–529.
11. Lacy E, Roberts S, Evans EP, Burtenshaw MD and Constantini FD (1983). A foreign β-globin gene in transgenic mice: Integration at abnormal chromosomal positions and expression in inappropriate tissues. Cell 34: 343–358.
12. Mack A, Sauer B, Abremski K and Hoess R (1992). Stoichiometry of the Cre recombinase bound to the *lox* recombining site. Nucl Acids Res 20: 4451–4455.
13. Matzke MA and Matzke AJM (1995). How and why do plants inactivate homologous (trans)genes? Plant Physiol 107: 679–685.
14. Morgan MK and Ow DW (1995) Polyethylene glycol-mediated transformation of tobacco leaf mesophyll protoplasts: An experiment in the study of Cre-*lox* recombination. In: Maliga P, Klessig DF, Cashmore AR, Gruissem W and Varner JE (eds.), Methods in Plant Molecular Biology, Cold Spring Harbor Laboratory Press, 10 Skyline Drive/Plainview/NY 11803-2500, pp. 1–17.
15. Negrutiu I, Dewulf J, Pietrzak M, Botterman J, Rietveld E, Wurzer-Figurelli EM, Ye D and Jacobs M (1990) Hybrid genes in the analysis of transformation conditions: II. Transient expression vs stable transformation – analysis of parameters influencing gene expression levels and transformation efficiency. Physiol. Plantarum 79: 197–205.
16. Ow DW, Day C, Holappa L, Lee E, Kobayashi J, Albert H and Koshinsky H (1997). Expression of site-specific transgenes. Supplement to Plant Mol. Biol. Rep. 15: abstract #210.
17. Ow DW and Medberry SL (1995). Genome manipulation through site-specific recombination. Crit. Rev. Plant Sci. 14: 239–261.
18. Park Y-D, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM and Matzke MA (1996). Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J. 9: 183–194.

19. Paszkowski J and Saul M (1986). Direct gene transfer to plants. Meth Enzym 118: 668–684.
20. Potrykus I and Shillito RD (1986). Protoplasts: isolation, culture, plant regeneration. Methods Enzymol. 118: 549–578.

Section O:
Plant cytoskeleton
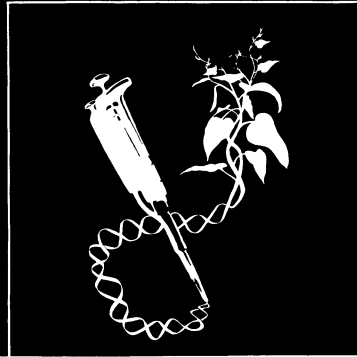
Plant Molecular Biology Manual

Gene Transfer Mediated by Site-Specific Recombination Systems

L. ALEXANDER LYZNIK[1,2], DAVID PETERSON[1], ZUO-YU ZHAO[1], XUENI GUAN[1], BEN BOWEN[1], BRUCE DRUMMOND[1], GRACE ST. CLAIR[1], LAURA TAGLIANI[1] and CHRIS BASZCZYNSKI[1]

[1]*Department of Crop Protection , Pioneer Hi-Bred International, Inc. P.O. Box 1004, Johnston, IA 50131, USA;*
[2]*Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47906, USA*

Introduction

Stable genetic transformation depends on DNA recombination, a process defined as a physical exchange of genetic information between interacting DNA molecules. DNA recombination reactions are conveniently divided into three categories: homologous recombination, site-specific recombination, and all others pejoratively described as illegitimate recombination. Current plant transformation techniques primarily rely on the least understood mechanisms, that is, illegitimate recombination. As a result, genetic transformations are unpredictable with respect to the efficiency of transformation and reliability of transgene expression. The use of homologous recombination in DNA integration processes (described as gene targeting) is still a very inefficient process despite substantial ongoing research efforts to develop such techniques both for animal and plant applications [1, 2]. Site-specific recombination may bridge the gap between random, unpredictable illegitimate integration and future fully controllable genomic DNA manipulations based on homologous recombination or other mechanisms. There is already available evidence that site-specific recombination can increase the efficiency of genetic transformation, improve its reliability, and reduce the extent of modifications imposed on genomic DNA during the transformation process.

Site-specific recombination systems

There are numerous site-specific recombination systems identified in prokaryotic and lower eukaryotic organisms [3]. The text-book example of such a system is the bacteriophage λ integration/excision system. Although probably the best studied, the system is not practical because of a complex regulatory mechanism involved in the recombination process. Two other recombination systems classified within the λ integrase family of site-specific recombinases, the Cre/*lox* system of bacteriophage P1 [4] and the FLP/*FRT* system of the yeast 2 μm plasmid [5], are relatively simple, requiring just two components, the recombinase and its cognate recognition sites, to perform recombination reactions. These

two systems are the most popular in plant, animal, and insect genetic transformation experiments [6].

The mode of action

FLP and Cre recombinases are highly-specific double-strand DNA binding proteins having sizes of 48 kDa and 38.5 kDa, respectively. They bind to 13 bp binding elements of recognition target sites, either *FRT* – the *F*LP *r*ecognition *t*arget, or *lox* – the *l*ocus *o*f *c*rossing-over recognized by Cre. Both sites contain an 8 bp core region flanked by the binding elements in an inverted-repeat orientation. The *FRT* site contains an additional directly-repeated binding element of unknown function (Figure 1). The direction of the recombination sites is determined by the asymmetry of the spacer region. The recombinases (Cre as well as FLP) are capable of binding to their target integrated into chromosomes of other species and catalyzing the recombination reaction. This has been shown in all heterologous organisms tested to date including bacteria, insects, mammalian, and plant cells.
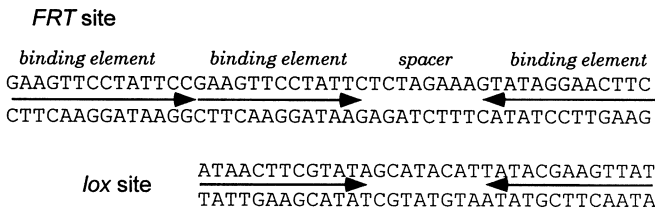
FRT site

Fig. 1. The structures of site-specific recombination target sequences recognized either by FLP recombinase – the *FRT* site or Cre recombinse – the *lox* site.

The first step in the recombination reaction involves binding of recombinase monomers and a major bending of the target sites that presumably facilitates DNA cleavage and strand exchanges (bending-deficient mutants of FLP protein exhibit reduced activity at the *FRT* sites) [7, 8]. Two FLP monomers are required to bind to two symmetry elements of the *FRT* site to produce a 144° bend in the DNA. DNA cleavage results from the nucleophilic attack of the catalytic-center tyrosine OH group on the phosphodiester bond at the border of the spacer region and symmetry elements. The recombinase becomes covalently attached to the DNA and the 5'OH end of the spacer region is exchanged with the analogous strand of the partner target site to form a "Holliday"-like intermediate. The Holliday structure is resolved by a second round of transesterification reactions.

The outcome of the recombination reaction depends on the orientation and position of the target sites. If two recombination sites are positioned in the same orientation, activity of the site-specific recombinase results in deletion of the DNA sequences between such sites (Figure 2). Site-specific recombination can invert DNA segments flanked by recombination sites that are in the opposite orientation relative to each other. The recombination reactions catalyzed by FLP or Cre proteins are reversible and the direction of such a reaction is not con-

trolled by other factors. Thus, the products of recombinational excision can re-integrate to restore the original substrates of the reaction, albeit at a reduced rate. Such reactions constitute an essential component of strategies designed to use site-specific recombination for genetic transformation protocols.
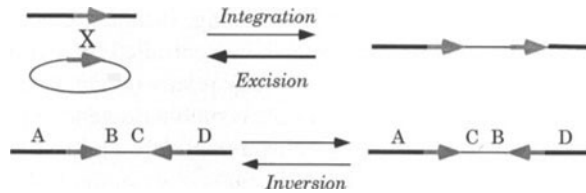
Fig. 2. Site-specific recombinases catalyze DNA integration, excision, or inversion depending on the orientation and position of the target sites.

Choice of site-specific recombination system

Despite numerous similarities between the FLP and the Cre site-specific recombination systems, their performances vary in different experimental setups. The original work on site-specific recombination in plants was done using the Cre/*lox* system in tobacco and *Arabidopsis* [9–11]. Since then, the FLP/*FRT* system was tested in tobacco, maize, and rice [12, 13]. Sonti *et al.* [14] reported constitutive expression of FLP recombinase in *Arabidopsis* but two other research groups encountered difficulties in the production of stably transformed *Arabidopsis* plants with constitutive expression of the *FLP* gene [13, 15]. Additional work is required to clarify whether strong expression of FLP recombinase indeed affects *Arabidopsis* growth. There is also an indication that the expression of Cre recombinase causes deviant phenotypes and retarded growth of tomato plants [16].

Cre recombinase is the popular choice in mammalian systems while both FLP and Cre recombinase have found successful applications in plants and flies [6]. As pointed out by Buchholz *et al.* (1996) [17], different thermostabilities of FLP and Cre recombinases may contribute to the different performance of these proteins in different systems. In *in vitro* recombination assays, the optimum temperature range for FLP-mediated recombination is 23 °C–30 °C (about 80% excision) while Cre-mediated recombination is most efficient at 37 °C–42 °C (about 40% excision). In addition, the FLP recombinase is virtually inactivated by incubation at 37 °C and above, indicating significant thermolability of this protein.

There are other factors that remain to be established such as recombinase affinity to DNA binding sites or possible differences in the mechanism of recombination between Cre and FLP proteins. Indeed, crystallographic studies of the Cre-*lox* complex suggest that the mechanism of DNA strand cleavage catalyzed by Cre protein differs from that of FLP [18].

Applications

Site-specific recombination provides a powerful tool for precise modifications of eukaryotic genomes. A basic paradigm of such procedures is to integrate a fragment of foreign DNA that contains a site-specific recombination site and, subsequently, to use the site-specific recombinase to make additional rearrangements in a fully controllable manner. The timing, location, efficiency, and outcome of the site-specific recombination can be controlled by a variety of factors including mutation of recombination sites, their relative orientation to each other, or regulation of the level and specificity of recombinase gene expression. Some of the approaches to making these systems useable are discussed below.

Excision

Excision of chromosomal fragments catalyzed by site-specific recombinases is the most straightforward application of site-specific recombinations. This application has been used very successfully in complementing gene targeting experiments in mammalian cells [19]. Used either to remove unnecessary DNA sequences from the targeted loci or to activate/inactivate targeted endogenous genes (recombination-activated gene expression-RAGE), the site-specific excisions have proven to be an invaluable genetic tool. These procedures have reached a level of sophistication where, for instance, an induced lethal genetic mutation of DNA polymerase β, could be limited only to mouse T cells by the action of Cre recombinase in which expression was controlled by the *lck* promoter [20]. Tissue-specific expression of site-specific recombinases has been used in numerous studies with both Cre and FLP recombinases [reviews: 6, 21]. For example, the tissue-specific RAGE strategy in plant cells involves the Cre-mediated activation of β-glucuronidase in developing tobacco embryos [22]. In this case, the Cre recombinase was fused to the promoters of the bean β-phaseolin or the soybean β-conglycinin. By crossing a plant containing a *cre* gene and a plant containing an inactivated *gusA* gene, the developmental pattern of GUS expression in tobacco seeds was observed in F1 progeny.

Chromosomal site-specific excisions are naturally efficient enough that they do not need to be selected for providing that a genetic transformation procedure produces a high percentage of transformed cells. For example, transient transformation of mouse cells with the pIC-Cre vector generated deletions of the *neo* gene from a gene targeted locus (the interleukin 2 receptor γ chain gene) in 4% of the viable cells after transformation [23]. Lyznik *et al.* [24] reported 2–3% frequency of excisions in maize cells (without selection) as a result of protoplast re-transformation with the *FLP* recombinase gene. In yet another variation of the system, a fusion between Cre and a green fluorescent protein (GFP) allowed recovery of site-specific deletions in transiently transformed stem cells by fluorescence-activated cell sorting [25]. Up to 95% of the fluorescent cells showed DNA excison events.

In fact, re-transformation with a site-specific recombinase gene may not even be necessary if the recombinase gene is chromosomally integrated but kept silent during the original transformation and selection procedures. Such an experimental system has elegantly been documented in transgenic mice carrying *lox* target sites and the *cre* recombinase gene driven by a male germ line specific promoter (the mouse protamine *Prm1* promoter). Upon mating transgenic males to wild-type females, all transmitted target alleles were recombined in progeny of some of the transgenic lines [26].

Finally, chromosomal excisions can be induced by cross breeding animals or cross pollination of plants where one parent contains site-specific target sites and the other parent contains actively expressed cognate site-specific recombinase. Such a strategy frequently produces up to 100% excisions in the progeny receiving both genes (Zuo-Yu Zhao, pers. Comm.) [10, 27, 28].

Integration

The site-specific recombinations are reversible reactions. Although excision is favored over integration, the product of integrative recombination can be stabilized providing that the recombinase activity is limited in time, or the product itself is no longer recognized as a substrate for the excision reaction (Figure 3). Sauer and Henderson [29] observed integration of the *tk* gene into the *lox*P locus in transgenic mouse cells at frequencies of 5–15% as compared to the frequency of random integration of the same gene. O'Gorman *et al.* [30] reported 50% integration efficiency (compared to random integration) for the FLP-catalyzed integration in monkey kidney cells. Using the same experimental procedures and the same cell line, Schlake and Bode [31] confirmed O'Gorman's results and demonstrated that a double-reciprocal crossover is also possible at the target site using two mutant *FRT* sites in plasmid DNA molecules (Figure 3C). This procedure resulted in the replacement of a chromosomal DNA fragment with a plasmid DNA fragment flanked by two *FRT* sites. A similar strategy has been applied to replace segments of the mouse genome surrounded by heterospecific *lox*P sites [32]. Mouse NIH 3T3 cells were co-transfected with the double *lox* plasmid and the Cre expression plasmid. The successful replacement of a designated chromosomal gene segment was observed in about 1% of the total number of viable cells plated, and it was more frequent than random, illegitimate recombination events. In yeast, an altered spacer region of the *lox*P site has been used to target endogenous *lox*-like sequences that naturally occur in the genome, thus achieving a specific targeting into chromosomal loci [33].

An alternative to the transient expression of the recombinase gene is to provide the recombinase protein instead. Baubonis and Sauer [34] tested such a possibility by introducing purified Cre recombinase directly into cultured human cells. Under optimal conditions, about 100 Cre-mediated integration events were recovered compared to an average of 791 random integrations of the selectable marker gene. The efficiencies of integrative recombination was affected more by the position of *lox*P sites than the number of integration sites.
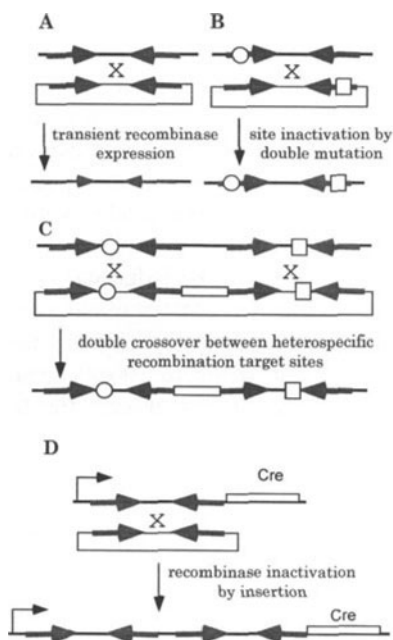
Fig. 3. Experimental strategies used for insertion of foreign DNA into the site-specific recombination targets integrated into genomic DNA. (A) The integration event is stabilized by depletion of recombinase protein in transiently transformed cells. (B) The integration event is stabilized because binding of recombinase protein to the double-mutated target site is severely impaired. (C) The integration event is stabilized because two heterospecific recombination sites do not recombine with each other. (D) The integration event is stabilized by inactivation of the recombinase gene as a result of the integration process. See text for more details.

The FLP/*FRT* system was used to integrate chromosome-mobilized DNA to specific target sites in *Drosophila* chromosomes [35]. In this refined genetic study, the stability of integration was achieved by heat-shock inducible expression of FLP or tissue-specific expression of a FLP gene controlled by the promoter of the β2-tubulin gene. This latter promoter is specifically expressed in the male germline. Tissue-specific expression of FLP protein was more efficient than the heat-shock treatment, producing up to 5% integration events in the crosses used to monitor DNA mobilization. Interestingly, heat-shock inducible expression of the FLP recombinase has been demonstrated in maize and *Arabidopsis* [15, 36].

In yet another embodiment of site-specific integration strategies, the activity of FLP recombinase was controlled chemically by fusion of the FLP protein to a steroid hormone receptor domain. It has previously been documented that fusion of the steroid hormone receptor domain to other proteins can render inducibility of their activities by steroid hormones [37]. Both Cre and FLP coding sequences have been fused to the ligand-binding domains of steroid receptors to regulate recombinase activity [38, 39]. Successful targeting of the integrated

FRT site using the FLP recombinase fused to the human oestrogen receptor was demonstrated by Logie and Stewart (1995). The recombination activity was rapidly induced by administration of estradiol, dexamethasone, or dihydrotestosterone to E25B2 transgenic cells containing the *FRT* recombination substrate. The induced activity of the FLP recombinase caused integration of vector sequences into the *FRT* locus at frequencies up to 25% compared to random integrations.

Site-specific integration into *lox* sites placed in the plant genome has been reported as well [40]. The authors tested mutant *lox* sites (to reduce excision activities after successful integrations) or transient expression of the *cre* gene and concluded that DNA integration at the target locus were less frequent when mutant *lox* sites were used (Figures 3A, 3B). The frequency of Cre-mediated integrations using transient expression of the *cre* gene was in the range of 10 to 100% as compared to random integration, making this procedure a workable option for introducing foreign DNA into pre-determined sites of the tobacco genome. By comparison, the same strategy using a pair of mutant *lox* sites provided evidence of targeted integration in the range of 2-16% compared to random integration in ES cells [41].

Chromosomal rearrangements

Chromosome rearrangements induced by site-specific recombination systems can be considered a powerful mutagenic procedure allowing precise mutation of chromosomal structures. Recombinase-mediated chromosomal rearrangements have been reported in many experimental systems including yeast, *Drosophila*, mammalian cells, and plants (see reviews: [21, 42]). While the FLP/*FRT* system has been successfully used in *Drosophila* [43], the Cre/*lox* system has been used for plant chromosome manipulations [44]. The interchromosomal recombination of *lox* sites induced by cross-pollination with tobacco plants containing active Cre recombinse led to the balanced translocation of chromosomal segments. The translocation products of the site-specific recombination were verified by a reciprocal exchange of DNA segments adjacent to the two *lox* sites.

To generate chromosomal inversions and deletions, Medberry *et al.* [45] used a modified *Ds* transposon containing *lox* sites. The Ac transposase-mediated transposition of the *Ds-lox* elements relocated the *lox* sites to new loci on the tobacco chromosome. Subsequent introduction of the Cre recombinase by cross-pollination with Cre-expressing plants induced deletion and inversion events in tobacco. However, many chromosomal rearrangements were not transmitted to progeny. An analogous system has been applied to generate chromosomal rearrangements in *Arabidopsis* [46]. Two inversion events were characterized between *lox* sites placed 5.6 and 16.5 cM from their original T-DNA *lox* site. One of them (the smaller one) was transmitted to subsequent generations as a single trait.

A combination of the maize *Ac/Ds* transposition system and the Cre/*lox* site-specific recombination system has been used to place *lox* sites on closely linked loci of tomato chromosome 6 [16]. Subsequent crossing of the transgenic plants

containing the *lox* sites to the Cre expressing plant led to isolation of one prog-
eny plant containing a chromosomal inversion of the 130 kb fragment located
between the *lox* sites. *In vitro* Cre-lox recombination demonstrated an apparent
maximum cleavage efficiency of 50 % per chromosomal *lox* site. Large dele-
tions of chromosomal segments from 200 kb up to 3–4 centimorgans have been
documented in ES cells using Cre-mediated recombination [47, 48].

Future prospects

Site-specific recombination systems offer the possibility to gain control over the
genomic integration process and to orchestrate rearrangements of genomic DNA
fragments. As applications of site-specific recombination become more sophis-
ticated and more site-specific recombination systems become available, such
systems may contribute to advanced genetic analysis of genome function in eu-
karyotic organisms. Thus, we have in our hands a molecular biology tool to study
the physiological relevance of gene expression (switching selected genes on and
off in a tissue-specific fashion), to analyze a developmental pattern of gene ex-
pression, to investigate a functional/structural organization of eukaryotic ge-
nomes (moving segments of chromosomes from one location to another), or to
generate precisely engineered hybrid genomes containing well-defined chromo-
somal fragments placed in pre-determined locations.

The potential applications of site-specific recombination are numerous.
Among some practical uses of the systems, we shall describe experimental ap-
proaches to using the yeast FLP/*FRT* recombination system as a component in
maize genetic transformation procedures in order to provide more control over
the DNA integration process. The flow chart illustrates a general strategy for use
of this system (Figure 4) and a complete description of the development of this
technology will be described elsewhere (Peterson *et al.*, manuscript in prepara-
tion).
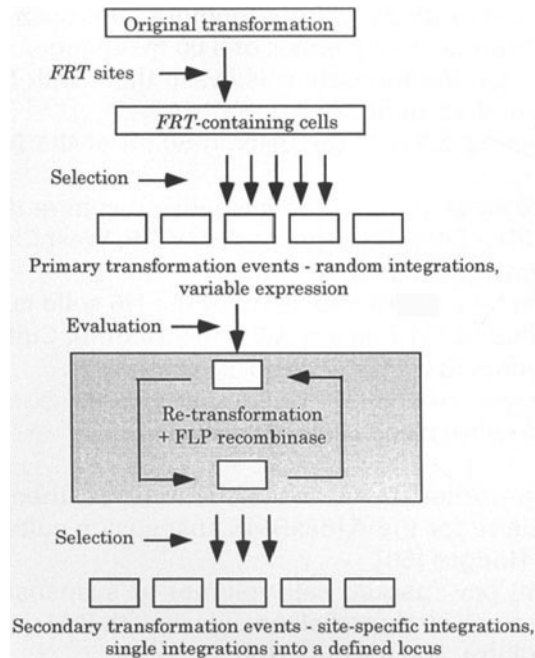
Fig. 4. A flow chart of transformation experiments proposed in this chapter.

Protocols

Experiments described in this section use site-specific recombination reactions catalyzed by FLP recombinase in maize cells. The procedures are experimental and there are possibilities for substantial improvements as discussed in the previous and following sections.

Suspension cultures

The suspension cultures have been established from immature embryos of the A188xBMS or A188xB73 genotypes. Establishment of suspension cultures of maize from immature embryos has been described elsewhere [49].
1. Culture suspended cells in 250 ml Erlenmeyer flasks containing 45 ml of MS culture medium (see solutions) on a gyratory shaker at 120 rpm, 26 °C, in the dark.
2. Subculture 5–10 ml pcv (packed cell volume) into 45 ml of fresh MS culture medium weekly.
3. Use suspension cells at the late logarithmic growth phase for protoplast isolation or prepare cells for transformation experiments by particle bombardment as follows:
   3.1. Transfer the suspension cells onto a 700 μm nylon mesh,

wash cells with N6 culture medium, and push them through the screen using a plunger of a 60 ml syringe.

3.2. Collect passed-through cells, wash them with N6 culture medium, and vacuum dry.

3.3. Resuspend 2.5 g of the cells in 50 ml of the N6 culture medium.

3.4. Filter 5 ml aliquots of the suspension culture through 5.5 cm glass fiber filter discs (grade 691, VWR, West Chester, PA) and vacuum dry cells on the filters.

3.5. Transfer the filters into 10 ml of the N6 solid culture medium solidified in Petri dishes (60 mm × 20 mm). Culture overnight in the dark at 26 °C.

Transient protoplast transformation assay

The protocol presented below is the adopted PEG-mediated transformation procedure for the A188xBMS suspension cultures described in Lyznik and Hodges [50].

1. Collect 5 ml pcv (packed cell volume) of suspension cells on a 90 μm nylon cell strainer (Falcon), wash with PIM (protoplast incubation medium), and vacuum dry.

2. Transfer cells into 20 ml of the digestion solution (PIM containing 2 % cellulase and 0.25 % pectinase, pH 5.9) (Worthington Biochemical, Freehold, NJ) and incubate for 3 hrs at room temperature (slow shaking at 40 rpm is optional).

3. Filter the digestion mixture through a 50 μm nylon screen and pellet the protoplasts at 50 g for 15 min.

4. Resuspend the protoplast pellet in 8 ml of 9 % Ficoll solution, transfer 4 ml of the protoplast suspension into a new tube and overlay with the same volume of TM (transformation medium). Centrifuge both tubes for 10 min. at 75 g.

5. Collect the band of protoplasts at the interface between Ficoll and TM, count viable protoplasts (dilute a sample of the protoplast suspension in TM containing 2.5 μm/ml fluoroscein diacetate), and adjust the concentration of protoplasts to 1×10^7 protoplasts per ml with TM.

6. Mix plasmid DNA (1–5 μg) with 0.2 ml of protoplast suspension and 0.2 ml of 50 % PEG in F-medium. Incubate at room temperature for 20 min.

7. Transfer the incubation mixture onto agarose blocks formed by 2 ml of the LMP-PCM solution placed in the 12–well microculture plate. Incubate overnight at room temperature in the dark without shaking.

8. Collect protoplasts from the agarose blocks into 1.5 ml microcentrifuge tubes, wash the agarose blocks with an additional 0.5 ml of

PCM. Centrifuge protoplast suspension at 50 g for 10 min and suspend pellet in a marker gene extraction buffer (marker gene activity assays are described elsewhere in this manual).

The use of a FLP-mediated excisional recombination reaction is recommended to test vector constructions containing the *FRT* sites and to evaluate the performance of FLP-expression vectors. Mono-molecular excisions catalyzed by the recombinase are more efficient than
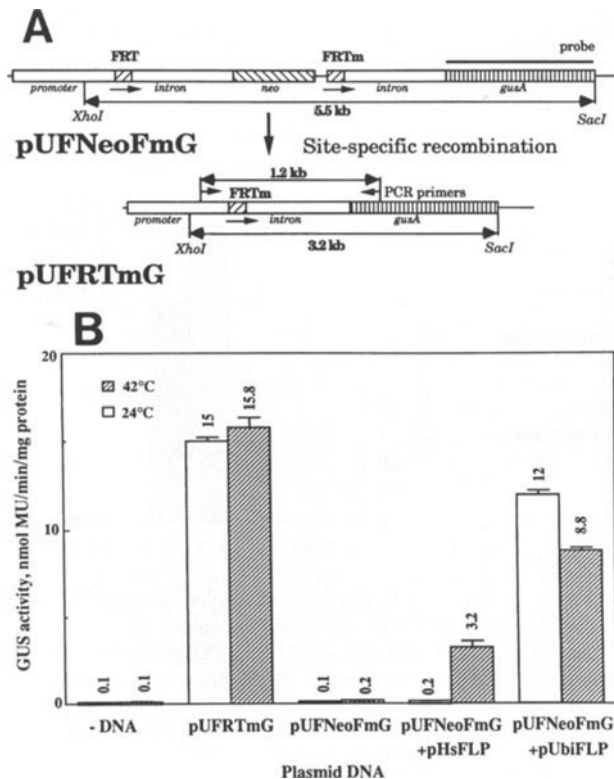
Fig. 5. Transient expression test for evaluation of the FLP/*FRT* system in maize protoplasts. (A) Vector design for testing site-specific recombination catalyzed by the FLP protein. The *FRT* sites were ligated into the *Bgl*II site of the first exon of the maize ubiquitin promoter. FLP-mediated excision eliminates the intron and the *neo* gene coding sequences located between two *FRT* sites in the direct repeat orientation. (B) Maize protoplasts were co-transformed with two vectors, one containing the *FRT* sites (pUFNeoFmG) and the other one containing the FLP gene under control of the maize ubiquitin promoter (pUbiFLP) or the soybean *Gmhsp 17.5* heat-shock gene promoter (pHsFLP). The GUS activity observed in protoplasts co-transformed with the pair of vectors pUF-NeoFmG/pUbiFLP and incubated at 24 °C indicates that the FLP/*FRT* system is functional in maize cells. An induction of GUS activity in protoplasts co-transformed with the pair of vectors pUF-NeoFmG/pHsFLP and incubated at 42 °C, but not at 24 °C, indicates that the FLP protein synthesis may be effectively regulated by the heat-shock treatment in maize protoplasts. Originally published in Lyznik *et al.* [36].

bi-molecular integration reactions, thus yielding a stronger recombination signal. The test plasmids contain a marker gene inactivated by a DNA fragment flanked by the *FRT* sites positioned between a promoter and the coding sequence of the marker gene (Figure 5A). Co-transformation of protoplasts with the test plasmid DNA and the FLP expression vector should activate expression of the marker gene provided that the *FRT* sites are functional. An example of the functional test of the FLP/*FRT* site-specific recombination system is shown in Figure 5B.

Similarly, FLP-mediated integration may be monitored in transient assays if *FRT* sites are positioned on two separate DNA molecules. The system allows testing different integration strategies as discussed in the introduction section (Figure 6).
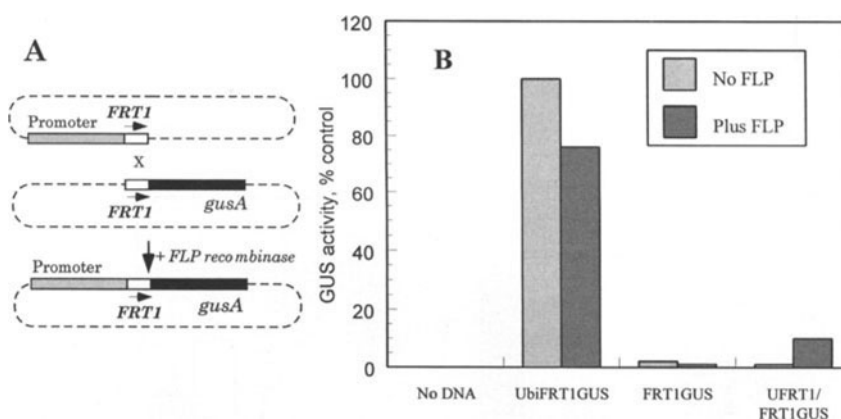
Fig. 6. Transient expression test for evaluation of the integration strategies in maize protoplasts. (A) Maize protoplasts are co-transformed with two vectors containing the *FRT* sites and the FLP expression vector. Integrative recombination catalyzed by the FLP protein should result in the activation of a marker gene expression (the *gusA* gene). (B) In this particular experiment, about 10% of the control GUS activity was observed in protoplasts co-transformed with the FLP expression vector (Plus FLP) and the integration substrates (UFRT/FRTGUS).

Generation of FRT-containing transgenic lines

It is prudent also to verify that experimental recombination target can be recognized and recombined by the recombinase protein while integrated into chromatin. Any developed stable transformation protocol may be used to introduce the *FRT* sites into maize genomic DNA. The choice depends on the final goal of the experimentation, whether transgenic plants or just transgenic cells are required. Multiple integrations of the *FRT* sites may also complicate subsequent re-transformation experiments. Thus the generation of single integration events is highly desirable (Figure 7).
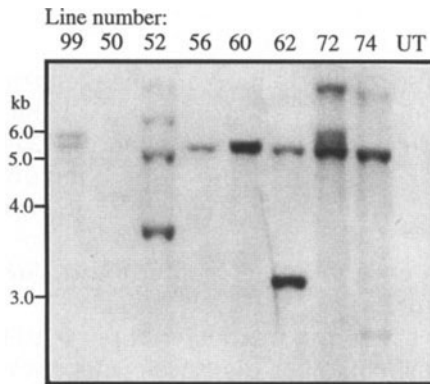
Fig. 7. Selection of *FRT*-containing transgenic material for the FLP-mediated excision experiments. Genomic DNA samples were digested with *Xba*I and *Sac*I to produce the 5.5 kb DNA fragment hybridizing to the *gusA* probe. Line number 56 was selected for further studies (taken from Hodges and Lyznik, 1996).

The following additional steps are required to recover stably transformed protoplasts after the PEG-mediated transformation described above:

1. Dilute the PEG-containing transformation mixture (step 6, the transient transformation protocol) with 6 ml of F-medium over 30 min. Pellet protoplasts by centrifugation at 50 g for 10 min.
2. Resuspend the protoplast pellet in 1 ml of PCM, count the number of viable protoplasts, and adjust the concentration to 1×10^6 protoplasts per 1 ml of PCM.
3. Plate 0.2 ml of the protoplast suspension onto 0.8 μm Millipore filters (Bedford, MA) placed over the feeder cell layer (6 ml of PCM-LMP containing 0.5 ml pcv of feeder cells – small clusters of the same suspension culture used for protoplast isolation). Culture protoplasts for 1 week in the dark at 24 °C.
4. Transfer Millipore filters with protoplasts onto fresh feeder plates for an additional 1 week incubation. Start selection 2 weeks after transformation by transferring the callus onto MS 2D medium supplemented with a selectable agent, for instance, 100 mg/ml kanamycin sulfate. Continue growing calli until transgenic cells can be easily identified and subcultured.

Maize transformation by particle bombardment

Alternatively, stable transformation of maize cells may be achieved by particle bombardment of suspension cells of the A188xB73 maize genotype [51] as described by Tomes *et al.* 195 [52].

1. Maintain rapidly growing friable embryogenic callus on N6P solid media at 28 °C in the dark. Subculture to fresh medium every 11–14 days.
2. Harvest callus cells in liquid medium and sieve cells through a 710 μm screen.
3. Suspend cells at 250 mg fresh weight per 5 ml liquid medium.
4. Use 5 ml of suspension to distribute cells onto a 5.5 cm glass fiber filter disc (grade 691, VWR, West Chester, PA) *via* vacuum filtration using a 4.7 cm glass microanalysis holder.
5. Transfer discs with cells onto solid N6-608 medium and incubate overnight.
6. Following overnight incubation, remove the cell-containing filters from the medium and briefly vacuum dry to remove the free liquid. Transfer the cell-containing filters into Petri dishes containing an additional dry paper filter (VWR, West Chester, PA).
7. Sonicate 1.0 μm gold particles (Analytical Scientific Instruments) briefly, pipette 50 μl gold particles (0.03 μg/μl) into a microcentrifuge tube immediately after sonication. Spin the particles down and discard the supernatant fluid. Tungsten particles can also be used effectively.
8. Re-suspend particles in 100 μl H$_2$O.
9. Add 10 μl of DNA at 0.1 μg/μl, 100 μl of 2.5M CaCl$_2$ and 20 μl of 0.1 M spermidine.
10. Sonicate briefly, and centrifuge the mixture for 10 s. at 10,000 rpm.
11. Sterilize the microcarrier (VWR, West Chester, PA) with 95% EtOH and air dry. Soak the 650 psi rupture discs (VWR, West Chester, PA) in 100% isopropanol.
12. Dilute the DNA-coated gold particles with 120 μl of EtOH and pipette 10 μl onto each microcarrier.
13. Bombard cells (delivery system – BioRad, Hercules, CA) under partial vacuum (28 in Hg) with 650 psi bombardment pressure at 9 cm target distance.
14. Transfer the bombarded cells into Petri dishes containing the N6-608 solid medium for 2–3 days incubation at 28 °C in the dark.
15. Incubate cells for an additional 6–7 days on medium containing a selection agent such as the N6R medium if the *bar* gene were used as a selectable marker gene.
16. Collect cells from the filter discs into tubes containing 5 ml of the melted N6W medium cooled to 50 °C and supplemented with a

selectable agent (for example, 1–3 mg/l bialaphos).

17. Mix well and overlay 2.5 ml cell-agar mixture into the Petri plates containing the same medium.

18. Culture cells at 28 ºC in the dark. Transformed colonies may be observed 6–8 weeks after bombardment.

Recombination-activated gene expression (RAGE) in maize.

Once the *FRT* sites are established in the maize genome, subsequent introduction and expression of the FLP protein can lead to the activation of gene expression and/or the elimination of unwanted foreign DNA sequences such as selectable marker genes.
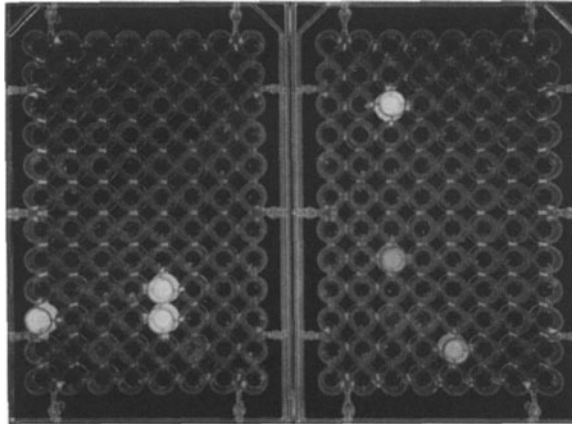
Fig. 8. Transient expression of the FLP gene leads to activation of expression of the *gusA* gene in maize protoplasts. Multiwell screening procedure for determining GUS activity (recombination events) in randomly chosen microcalli grown without selection for four weeks after transformation. Three to four percent of viable, calli-forming cells showed GUS activity indicative of the recombination event.

There are a number of methods including re-transformation, chemical/tissue-specific activation of recombinase gene expression, or cross breeding, that can used to introduce site-specific recombinase function into cells containing the pre-integrated recombination sites. As a result, the activation, inactivation, or exchange of foreign gene(s) expression is easily accomplished. In many instances, if re-transformation procedures are used, a subsequent selection for transformation events, but not necessarily for recombination events, needs to be performed. On the other hand, if the recombination event is linked to the activation of a selectable marker gene, the re-transformation event and the recombination event may be selected at the same time. The example used in this section illustrates the site-specific recombination of genome-integrated *FRT* sites. It utilizes transient expression of the FLP protein in re-transformed maize protoplasts for the exchange of foreign gene expression – the *neo* gene is removed and the *gusA* gene is activated. Because the efficiencies of transformation and site-specific recombination were relatively high, selection for transformation or recombination activity is not necessary. Recombination
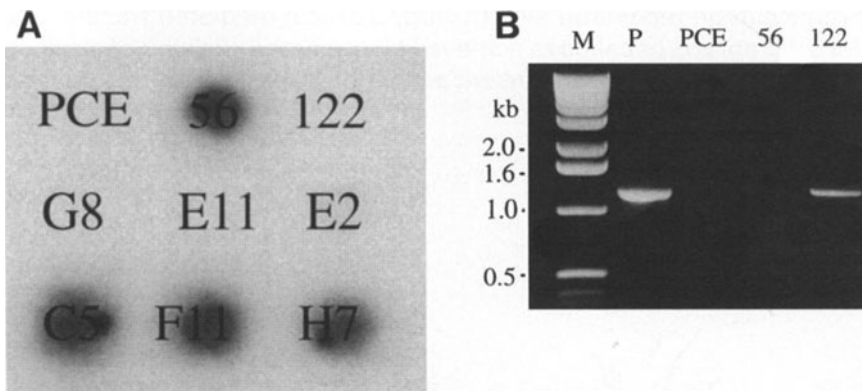
Fig. 9. NPTII activity (A) and PCR analysis of genomic DNA (B) in GUS-positive calli derived from line 56 following re-transformation with the pUbiFLP vector. (A) NPTII activity was assayed using the dot-blot method as described elsewhere [53]. Untransformed callus (PCE) and a number of re-transformed, GUS-positive calli (122, G8, E11, E2) showed no NPTII activity in protein extracts. Some lines (C5, F11, H7) retained NPTII activity probably because of an incomplete excision reaction. (B) PCR analysis. The recombination product should generate a 1.2 kb amplification fragment using a set of primers indicated in Figure 5A. Lane 3 and 4 represent PCE (untransformed) and line 56, respectively. Lane 5 shows amplification of the expected product of the site-specific recombination reaction that took place in line 122. Lane 1 shows the product of the positive control plasmid DNA amplification (pUFRTmG) (from Hodges and Lyznik, 1996 (A) and Lyznik *et al.*, 1996 (B)).
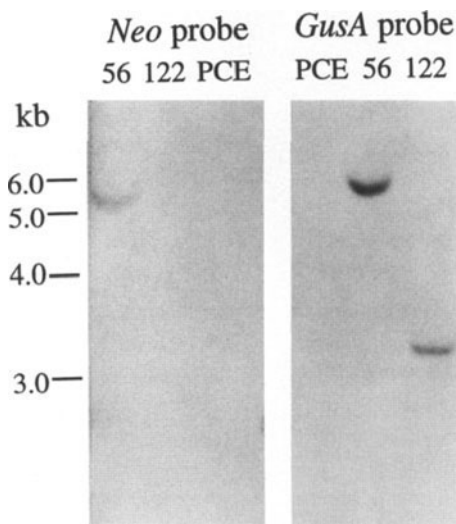
Fig. 10. Southern blot analysis of genomic DNA from line 56 re-transformed with the FLP-expression vector (line 122). A band of 3.2 kb hybridizing to the *gusA* probe is the product of a site-specific recombination reaction (published in Lyznik *et al.*, 1996).

events can be recovered also through particle mediated transformation of embryos or callus to achieve FLP mediated excision of a selectable marker gene and concurrent activation of other reporters such as the GFP gene.

Stable transformation and regeneration of maize plants

The following section presents examples of the evaluation and application of site-specific recombination reactions in whole plant and breeding experiments. To conduct these studies, stable transformation experiments can be conducted as outlined below, followed by regeneration of plants as described here and elsewhere [54].

1. Immature embryos of the "Hi-II" maize germplasm [51] are excised and placed scutelar side up onto N6P solid media.
2. Following 3–5 days incubation at 28 °C the embryos are transferred to the same media supplemented with an additional 90 g/l sucrose 3-4 hours before bombardment.
3. Follow the particle bombardment procedure described above.
4. 2–3 days after bombardment embryos are transferred to N6R solid media and sub-cultured every 11–14 days. Transformed colonies may be observed 6–8 weeks after bombardment.
5. When ready to regenerate plants, transfer tissue to selective medium containing indole-3-acetic acid (1 mg/ml), zeatin (0.5 mg/ml) and abscisic acid (1 mM) and 60 g/l sucrose
6. Following 2 weeks culture in the dark at 26 °C mature somatic embryos are transferred to hormone-free 1/2 strength MS based media and transferred into light to allow for embryo germination and plantlet development.
7. Developing plantlets are cultured in test tubes on hormone-free medium, and advanced to the greenhouse once sturdy shoot and root systems are formed.

The functionality of the FLP/*FRT* system in plants and recovery of viable fertile plants carrying either functional FLP recombinase, or introduced gene sequences flanked by *FRT* sites, or both can be demonstrated. To validate the successful inheritance of introduced DNA sequences containing functional *FRT* sites, events created as described above using a construction such as that described in Figure 5A can be regenerated to normal fertile plants in the greenhouse [54], and self- or cross-pollinated by standard methods to produce seed. That the *FRT* sites are heritable and also still capable of participating in FLP-mediated recombination reactions can be confirmed by culturing embryos or cells from the progeny and transforming the tissues with a plasmid carrying a functional FLP gene (such as pUbiFLP). Transient expression of FLP is generally adequate to demonstrate expression of GUS or other reporter genes resulting from successful recombination of the sequences between *FRT* sites. Table 1 summarizes data on GUS expression following microprojectile bombardment of a FLP expressing plasmid into tissues from progeny of different events, and demonstrates that functional *FRT* sequences are heritable in

Table 1. FLP expressing plasmid was introduced by microprojectile bombardment into embryonic tissues from progeny of T0 plants containing genome-integrated *FRT* sites, and samples were stained for GUS activity. The number of GUS+ progeny indicates the number of bombarded embryos showing GUS activity originating from site-specific recombination.

Event No.	Number of progeny examined	Number of GUS+ progeny
1	40	3
2	143	32
3	38	10
4	54	28
5	30	9
6	47	31
7	92	36

maize. The variation in expression among the progeny events is no different from what is generally observed following direct transformation with a functionally active GUS gene.

A high proportion of transformation events ($>65\%$) normally express functional FLP recombinase, as determined by transient assays where a vector such as that shown in Figure 5A is introduced into the FLP-containing events leading to expression of a reporter gene following recombination. The functionality and heritability of FLP recombinase in plants and their progeny is independently verified. Plants are regenerated in the greenhouse and observed for normal development as well as pollen morphology. Pollinations are made and embryos or cells are cultured as described above. Transient transformation of the tissues with a plasmid such as that shown in Figure 5A yields expression of GUS resulting from inheritance of functional FLP recombinase activity in the progeny. With normal Mendelian inheritance, 50% of the progeny would be expected to contain the FLP gene. Examination of progeny from three primary events which showed 100% normal pollen morphology yielded 146 FLP+ events out of 300 progeny tested. In cases where pollen morphology was variable, fewer FLP-expressing progeny were generally recovered.

Other experiments, involving various crosses between FLP and *FRT*-containing plants, can further substantiate the utility of this system or others, such as Cre/lox, for conducting effective recombination reactions in maize and other plant species.

Solutions

Protoplast transformation

1. *Protoplast incubation medium PIM*: 4.3 g/l MS salts, 10 ml/l thiamine·HCl stock solution (0.05 g/l), 20 ml/l 2,4-D stock solution (100 mg/l), 36.4 g/l mannitol, 40 ml/l $CaCl_2 \cdot 2H_2O$ stock solution (294 g/l), pH 5.9 adjusted with 0.1 N KOH.

2. *Protoplast culture medium PCM*: 4.3 g/l MS salts, 10 ml/l thiamine·HCl stock solution (0.05 g/l), 20 ml/l 2,4-D stock solution (100 mg/l), 36.4 g/l mannitol, 0.25 g/l glucose, 20 ml/l coconut water, pH 5.9 adjusted with 0.1 N KOH.

3. *Protoplast culture medium PCM-LMP*: PCM medium supplemented with 8 g/l low-melting-point agarose (Gibco BRL/Life Technologies, Gaithersburg, MD).

4. *Transformation medium TM*: 19.5 g/l MES 2(N-morpholino)ethanesulfonic acid, 36.4 g/l mannitol, 40 ml/l $CaCl_2 \cdot 2H_2O$ stock solution (294 g/l), pH 5.5 adjusted with 0.1 N KOH.

5. *Transformation solution PEG*: 50 g PEG (polyethylene glycol) in 50 ml 2x F-medium.

6. *F-medium*: 8.2 g/l NaCl, 372 mg/l KCl, 105 mg/l Na_2HPO_4, 900 mg/l glucose, 65 ml/ l $CaCl_2 \cdot 2H_2O$ stock solution (294 g/l), pH 7.2.

7. *9% Ficoll solution:* PCM containing 9% (w/v) Ficoll (Ficoll 400, Sigma Chemical, St. Louis, MO).

8. *Fluoroscein diacetate stock solution*: 50 mg/100 ml of acetone.

9. *MS 2D*: 4.3 g/l MS salts, 0.1 g/l myo-inositol, 5 ml/l MS vitamins stock solution (0.1 g/l nicotinic acid, 0.1 g/l thiamine·HCl, 0.1 g/l pyridoxine·HCl, 0.4 g/l glycine), 4 ml/l 2,4-D stock solution (0.5 mg/ml), 20 g/l sucrose, pH 5.9 adjusted with 0.1 N KOH.

Particle bombardment

1. *MS culture medium*: 4.3 g/l MS salts, 0.1 g/l myo-inositol, 5 ml/l MS vitamins stock solution (0.1 g/l nicotinic acid, 0.02 g/l thiamine·HCl, 0.1 g/l pyridoxine·HCl, 0.4 g/l glycine), 4 ml/l 2,4-D stock solution (0.5 mg/ml), 30 g/l sucrose, pH 5.6 adjusted with KOH.
2. *N6 culture medium*: 4 g/l Chu (N6) basal salts (Sigma C-1416, Sigma Chemical, St. Louis, MO), 1 ml/l Eriksson's vitamin mix (Sigma-1511, Sigma Chemical, St. Louis, MO), 1.25 ml/l thiamine-HCl stock solution(0.4 mg/l), 20 g/l sucrose, 2 ml/l 2,4-D stock solution (0.5 mg/ml), pH 5.8 adjusted with KOH.
3. *N6 solid medium*: same as the N6 culture medium but supplemented with 2 g/l Phytagel (Sigma Chemical, St. Louis, MO)
4. *N6P solid medium*: 4 g/l Chu (N6) basal salts (Sigma C-1416, Sigma Chemical, St. Louis, MO), 1 ml/l Eriksson's vitamin mix (Sigma-1511, Sigma Chemical, St. Louis, MO), 1.25 ml/l thiamine·HCl stock solution (0.4 mg/l), 30 g/l sucrose, 2 mg/l 2,4-D, 690 mg proline, 850 mg $AgNO_3$, 3 g/l Phytagel (Sigma Chemical, St. Louis, MO), pH 5.8 adjusted with KOH
5. *N6-608 solid medium*: 4 g/l Chu (N6) basal salts (Sigma C-1416, Sigma Chemical, St. Louis, MO), 1 ml/l Eriksson's vitamin mix (Sigma-1511, Sigma Chemical, St. Louis, MO), 1.25 ml/l thiamine·HCl stock solution(0.4 mg/l), 30 g/l sucrose, 690 mg proline, 850 mg $AgNO_3$, 3 g/l Phytagel (Sigma Chemical, St. Louis, MO), pH 5.8 adjusted with KOH.
6. *N6R solid medium*: 4 g/l Chu (N6) basal salts (Sigma C-1416, Sigma Chemical, St. Louis, MO), 1 ml/l Eriksson's vitamin mix (Sigma-1511, Sigma Chemical, St. Louis, MO), 1.25 ml/l thiamine·HCl stock solution(0.4 mg/l), 30 g/l sucrose, 2 mg/l 2,4-D, 850 mg AgNO3, 3 g/l Phytagel (Sigma Chemical, St. Louis, MO), pH 5.8 adjusted with KOH.
7. *N6W solid medium*: modified N6R solid medium containing 6 g/l SeaPlague agarose (FMC BioProducts, Rockland, ME) instead of 3 g/l Phytagel (Sigma Chemical, St. Louis, MO).

References

1. Puchta H (1998) Towards targeted transformation in plants. Trends Plant Sci 3: 77–78.
2. Shastry BS (1998) Gene disruption in mice: Models of development and disease. Mol Cell Biochem 181: 163–179.
3. Esposito D and Scocca JJ (1997) The integrase family of tyrosine recombinases: Evolution of a conserved active site domain. Nucl Acids Res 25: 3605–3614.
4. Sauer B anc Henderson N (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci USA 85: 5166–5170.
5. Futcher AB (1988) The 2 μm circle plasmid of *Saccharomyces cerevisiae*. Yeast 4: 27–40.
6. Odell JT and Russell SH (1994) *Use of site-specific recombination systems in plants*. In: *Homologous Recombination in Plants* Paszkowski J, (ed), Kluwer Academic Publishers, Dordrecht. pp. 219–265.
7. Luetke KH and Sadowski PD (1995) The role of DNA bending in Flp-mediated site-specific recombination. J Mol Biol 251: 493–506.
8. Chen JW, Evans B, Rosenfeldt H, Jayaram M (1992) Bending-incompetent variants of Flp recombinase mediate strand transfer in half-site recombinations: Role of DNA bending in recombination. Gene 119: 37–48.
9. Dale EC and Ow DW (1990) Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene 91: 79–85.
10. Odell J, Caimi P, Sauer B and Russell S (1990) Site-directed recombination in the genome of transgenic tobacco. Mol Gen Genet 223: 369–378.
11. Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234: 49–59.
12. Lyznik LA, Mitchell JC, Hirayama L, Hodges TK (1993) Activity of yeast FLP recombinase in maize and rice protoplasts. Nucl Acids Res 21: 969–975.
13. Lloyd AM, Davis RW (1994) Functional expression of the yeast FLP/FRT site-specific recombination system in *Nicotiana tobacum*. Mol Gen Genet 242: 653–657.
14. Sonti RV, Tissier AF, Wong D, Viret JF and Signer ER (1995) Activity of the yeast FLP recombinase in *Arabidopsis*. Plant Mol Biol 28: 1127–1132.
15. Kilby NJ, Davies GJ, Snaith MR and Murray JA (1995) FLP recombinase in transgenic plants: Constitutive activity in stably transformed tobacco and generation of marked cell clones in *Arabidopsis*. Plant J 8: 637–652.
16. Stuurman J, de Vroomen MJ, Nijkamp HJ and van Haaren MJ (1996) Single-site manipulation of tomato chromosomes *in vitro* and *in vivo* using Cre-*lox* site-specific recombination. Plant Mol Biol 32: 901–913.
17. Buchholz F, Ringrose L, Angrand PO, Rossi F, Stewart AF (1996) Different thermostabilities of FLP and Cre recombinases: Implications for applied site-specific recombination. Nucl Acids Res 24: 4256–4262.
18. Guo F, Gopaul DN and van Duyne GD (1997) Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. Nature 389: 40-46.
19. Rajewsky K, Gu H, Kuhn R, Betz UA, Muller W, Roes J, and Schwenk F (1996) Conditional gene targeting. J Clin Invest 98: 600–603.
20. Gu H, Marth JD, Orban PC, Mossmann H and Rajewski K (1994) Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science 265: 103–106.
21. Sauer B (1994) Site-specific recombination: Developments and applications. Curr Opin Biotechnol 5: 521–527.
22. Odell JT, Hoopes JL and Vermerris W (1994) Seed-specific gene activation mediated by the Cre/*lox* site-specific recombination system. Plant Physiol 106: 447–458.
23. DiSanto JP, Muller W, Guy-Grand D, Fischer A and Rajewsky K (1995) Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor γ chain. Proc Natl Acad Sci USA 92: 377–381.

24. Lyznik LA, Rao KV, Hodges TK (1996) FLP-mediated recombination of *FRT sites* in the maize genome. Nucl Acids Res 24: 3784–3789.

25. Gagneten S, Le Y, Miller J and Sauer B (1997) Brief expression of a GFP*cre* fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. Nucl Acids Res 25: 3326–3331.

26. O'Gorman S, Dagenais NA, Qian M and Marchuk Y (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc Natl Acad Sci USA 94: 14602–14607.

27. Lasko M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL and Westphal H (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci USA 89: 6232–6236.

28. Dale EC and Ow DW (1991) Gene transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci USA 88: 10558–10562.

29. Sauer B and Henderson N (1989) Cre-stimulated recombination at *loxP*-containing DNA sequences placed into the mammalian genome. Nucl Acids Res 17: 147–161.

30. O'Gorman S, Fox DT and Wahl GM (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science 251: 1351–1355.

31. Schlake T and Bode J (1994) Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry 33: 12746–12751.

32. Bethke B and Sauer B (1997) Segmental genomic replacement by Cre-mediated recombination: Genotoxic stress activation of the p53 promoter in single-copy transformants. Nucl Acids Res 25: 2828–2834.

33. Sauer B (1996) Multiplex Cre/*lox* recombination permits selective site-specific DNA targeting to both a natural and an engineered site in the yeast genome. Nucl Acids Res 24: 4608–4613.

34. Baubonis W, Sauer B (1993) Genomic targeting with purified Cre recombinase. Nucl Acids Res 21: 2025–2029.

35. Golic MM, Rong YS, Petersen RB, Lindquist SL and Golic KG (1997) FLP-mediated DNA mobilization to specific target sites in *Drosophila* chromosomes. Nucl Acids Res 25: 3665–3671.

36. Lyznik LA, Hirayama L, Rao KV, Abad A and Hodges TK (1995) Heat-inducible expression of FLP gene in maize cells. Plant J 8: 177–186.

37. Picard D (1994) Regulation of protein function through expression of chimaeric proteins. Curr Opin Biotechnol 5: 511–515.

38. Logie C, Stewart AF (1995) Ligand-regulated site-specific recombination. Proc Nat Acad Sci USA 92: 5940–5944

39. Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD and Reth M (1996) Inducible site-directed recombination in mouse embryonic stem cells. Nucl Acids Res 24: 543–548.

40. Albert H, Dale EC, Lee E and Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J 7: 649–659.

41. Araki K, Araki M and Yamamura K (1997) Targeted integration of DNA using mutant *lox* sites in embryonic stem cells. Nucl Acid Res 25: 868-872.

42. Ow DW (1996) Recombinase-mediated chromosome engineering in plants. Curr Opin Biotechnol 7: 181–186.

43. Golic KG (1991) Site-specific recombination between homologous chromosomes in *Drosophila*. Science 25: 958–961.

44. Qin M, Bayley C, Stockton T and Ow DW (1994) Cre recombinase-mediated site-specific recombination between plant chromosomes. Proc Natl Acad Sci USA 91: 1706–1710.

45. Medberry SL, Dale E, Qin M and Ow DW (1995) Intra-chromosomal rearrangements generated by Cre-*lox* site-specific recombination. Nucl Acids Res 23: 485–490.

46. Osborne BI, Wirtz U and Baker B (1995) A system for insertional mutagenesis and chromosomal rearrangement using the Ds transposon and Cre-*lox*. Plant J 7: 687–701.

47. Li LP, Schlag PM and Blankenstein T (1997) Transient expression of SV40 large T antigen by Cre/*LoxP*-mediated site- specific deletion in primary human tumor cells. Hum Gene Ther 8: 1695–1700.

48. Ramirez-Solis R, Liu P, Bradley A (1995) Chromosome engineering in mice. Nature 378: 720–724.

49. Sellmer JC, Ritchie SW, Kim IS and Hodges TK (1994) *Initiation, maintenance, and plant regeneration of type II callus and suspension cultures.* In: *The Maize Handbook.* Freeling M, Walbot V (eds), Springer-Verlag, New York, pp. 671–676.

50. Lyznik LA and Hodges TK (1994) *Polyethylene glycol-mediated DNA uptake into maize protoplasts. In: The Maize Handbook.* Freeling M, Walbot V, (eds), Springer-Verlag, New York, pp. 603–609.

51. Armstrong CL, Green CE, Phillips RL (1991) Development and availability of germplasm with high Type II culture formation response. Maize Genet. Coop Newslett 65: 92–93.

52. Tomes DT, Ross MC and Songstad DD (1995) *Direct DNA transfer into intact plant cells via microprojectile bombardment. In: Plant Cell Tissue and Organ Culture: Fundamental Methods.* Gamborg OL, Philips GC (eds), Springer-Verlag, Berlin, pp. 197–213.

53. Peng J, Wen F and Hodges TK (1993) A rapid method for qualitative assay of both neomycin phosphotransferase II and β-glucuronidase activities in transgenic plants. Plant Mol Biol Rep 11: 38–47.

54. Baszczynski CL, Barbour E, Zeka BL, Maddock SE and Swenson JL (1997) Characterization of a genomic clone for a maize dnaJ-related gene, ZmdJ1, and expression analysis of its promoter in transgenic plants. Maydica 42: 189–201.

Plant Actin Isolation and Characterization

Christopher J. Staiger[1], Bryan C. Gibbon and Haiyun Ren[2]
Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA
[1]Author for correspondence: 1392 Lilly Hall of Life Sciences, Dept. of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA. Fax: 1-765-496-1496; E-mail: cstaiger@bilbo.bio.purdue.edu
[2]Current address: Department of Biology, Beijing Normal University, Beijing 100875, China.

Introduction

Monomeric actin (G-actin) is a small (42,000 Da) globular protein that polymerizes into higher order structures called microfilaments or filamentous actin (F-actin) (for a detailed review see [36]). These cytoskeletal polymers form a dynamic filament network in plant cells and support many fundamental processes, including cytoplasmic streaming, organelle positioning, cytokinesis, wound repair, responses to pathogen-attack, and cellular morphogenesis (for reviews see [23, 25, 26, 38]). Angiosperms contain large multigene families for actin and actin binding proteins (ABPs), and the biochemical and cell biological characterization of these components have received considerable attention in the past several years. A wealth of data is available for the motor molecule myosin and the small ABPs, profilin, and actin depolymerizing factor (reviewed in [3, 23, 26, 37]).

Because large amounts of vertebrate actins are easily purified, it is a common practice to analyze actin–plant ABP interactions through reconstitution experiments that use heterologous components. For example, plant profilins have been shown to bind to rabbit skeletal muscle and to bovine cardiac actin isoforms [11, 30, 34]. Similarly, maize and *Arabidopsis* actin depolymerizing factors modulate the polymerization properties of rabbit skeletal muscle actin [4, 14, 15, 21]. However, it is known from numerous studies in non-plant systems that many ABPs interact with homologous and non-homologous sources of actin in a dramatically different fashion (reviewed in [12, 33]). For example, yeast profilin can sequester yeast G-actin, thereby preventing its polymerization, but it does not inhibit the polymerization of rabbit muscle actin [28]. Given the apparent diversity of plant ABPs, it will be important to characterize their biochemical properties with a physiologically-relevant actin. Reports of the isolation and characterization of functional plant actin are quite limited (reviewed in [24, 26]). To provide a reagent for biochemical studies, we recently developed a strategy for the rapid isolation of large quantities of polymerization–competent actin from maize pollen [31].

Among plant tissues, pollen presents one of the richest sources of starting material for the isolation of cytoskeletal proteins. Estimates for actin in pollen range from 2–20% of total soluble protein [1, 20, 31, 42]. Maize pollen has

served as the starting material for several previous actin purification schemes [1, 20]. To isolate actin from plant materials, two general approaches have been used: DNase I–affinity chromatography [1, 2, 17, 18, 22, 39, 43]; and conventional chromatography on anion exchange and/or size exclusion columns [8, 20, 27, 40, 41]. DNase I forms a high affinity 1:1 complex with G-actin and, when covalently linked to a chromatography support, provides a convenient method for the isolation of actin from a variety of eukaryotic tissues [45]. The main advantage of this procedure is that formation of the strong ($K_d = 10^{-9}$ M) DNase I–actin complex allows a rapid, single-step isolation of actin from crude extracts. This convenience comes with a significant drawback, however. In order to elute actin from DNase I, both proteins must be partially denatured with chaotropes such as formamide or urea. Where polymerization of plant actin purified by the DNase method has been reported, phalloidin or poly-L-lysine are required to drive polymerization or to stabilize the F-actin [1, 2, 39]. This is perhaps an indication that plant actin is extremely sensitive to mild denaturing conditions.

Liu and Yen [20] developed a procedure for the isolation of actin from maize pollen that provides up to 2 mg of actin from 10 g of pollen at an apparent purity of 92% [20]. Their protocol involves the preparation of a pollen acetone powder, low ionic strength extraction, ammonium sulfate precipitation, anion exchange chromatography, polymerization and depolymerization, and size exclusion chromatography. These multiple steps require at least a week to perform, and our experience demonstrates that polymerizability of the actin varies considerably between different batches. One potential source of variation seems to arise during the elution of actin from DEAE–cellulose with a salt gradient; actin–enriched fractions often contain considerable amounts of the G-actin binding protein profilin.

Our new method represents a substantial departure from earlier methods for plant actin isolation. The procedure uses another actin monomer–binding protein, profilin, for the indirect isolation of native actin through immobilization of the profilin–actin complex on a poly-L-proline (PLP)–Sepharose column. A similar strategy was used by Grolig and coworkers to reconstitute the profilin–actin complex *in vitro* and to isolate small amounts of actin from the green alga *Chara corallina* [34]. Our procedure includes preparation of a soluble protein extract from frozen maize pollen that is supplemented with recombinant profilin, isolation of the profilin–actin complex by affinity chromatography on PLP–Sepharose, high ionic strength elution of the actin, and a cycle of assembly and disassembly. The methods outlined below are a refinement of the original procedures described by Ren *et al.* [31].

The successful isolation of maize pollen actin depends on two key factors; (1) addition of large amounts of recombinant profilin to pollen extracts, and (2) the choice of human platelet profilin. The first step likely assists the depolymerization of endogenous F-actin in the pollen extracts and thereby enhances the ultimate yield of G-actin. The decision to use vertebrate profilin is motivated by the finding that human profilin binds with higher affinity to pollen actin than do several recombinant maize pollen profilins [9] and because it is the most potent

disrupter of actin–dependent cytoplasmic architecture following introduction into living plant cells [9, 31]. Additional factors that are integral to the success of this method include the use of affinity chromatography and the choice of an extraction buffer. As described for DNase I–affinity chromatography, the rapid separation of actin from the crude cytoplasmic extract may help to minimize proteolysis. For extraction of pollen, we use a buffer that was developed for the isolation of a high affinity profilin–actin complex from vertebrate cells [16]. The basis for the efficacy of this buffer is not fully understood; however, the inclusion of phosphatase inhibitors (NaPPi and NaF) is suggested to function in maintaining the phosphorylation of one of the components of the profilin–actin complex or a minor accessory factor necessary for the stabilization of the interaction [16]. Alternatively, as suggested by Andersland *et al.* [1] based on studies of DNase I–actin isolation, it may be necessary to inhibit endogenous ATPase activity in plant extracts to maintain high levels of ATP required for actin–ABP interactions.

The recovery of functional actin by this rapid and convenient procedure is substantial; our current average yield is ~4 mg of actin from 10 g of pollen. This new procedure provides a source of high-quality, native plant actin that is readily polymerizable, >98% pure, and abundant. The average yield is at least two-fold higher than the previous method for isolating actin from maize pollen [20] and requires less than half the time to perform. This source of pollen actin has been used in biochemical studies to characterize the assembly properties of plant actin [9, 31], to examine the interactions with plant and non-plant ABPs [5, 9, 10, 31], and to titrate the effects of excess profilin in living plant cells [31].

Procedures

Purification of recombinant human platelet profilin

Recombinant vertebrate profilin is used as a supplement to pollen cytosolic extracts for the isolation of native actin. The original cDNA for human platelet profilin (profilin I) was isolated by Kwiatkowski and Bruns [19]. The construction for expression in *Escherichia coli*, using the T7 promoter vector pMW172, was prepared by Fedorov *et al.* [7]. Recombinant profilin is purified from a soluble extract of bacterial cells by affinity chromatography using PLP-Sepharose, eluted with urea, dialyzed to allow renaturation, and stored at –80 °C.

Steps in the procedure
1. The affinity resin is prepared from poly-L-proline (M_r 12,000; Sigma, St. Louis) and CNBr–activated Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) according to the coupling procedures of Janmey [13]. Typically, 100 ml of resin is prepared and this can be stored for up to a year at 4 °C.
2. A column (2.5 cm $\times$ 10 cm) is packed with $\sim$ 7–9 ml of PLP–Sepharose and equilibrated with buffer I.
3. A flask containing 200 ml of LB medium + 100 μg/ml ampicillin is inoculated from a single colony or glycerol stock of *E. coli* strain BL21(DE3) containing the plasmid pMW172 with human profilin I cDNA insert. The culture is allowed to grow to saturation overnight at 37 °C with constant shaking.
4. The overnight culture is used to inoculate 2 liters of LB/AMP medium (50 ml into four flasks, each containing 500 ml of medium), and incubated for 2 h at 37 °C. Protein overexpression is induced by the addition of IPTG to a final concentration of 0.4 mM, and the cultures incubated for a further 4 h at 37 °C.
5. Bacterial cells are collected by centrifugation at 5500 X g (6000 rpm in Beckman J2-HS with JA-14 rotor) for 10 min at 4 °C. After decanting the supernatant solution; cell pellets are resuspended in ice-cold PBS and then centrifuged at 12,100 X g (10000 rpm in Beckman JA-20 rotor) for 10 min at 4 °C. The supernatants are again decanted, the cell pellets frozen in liquid nitrogen, and stored at –80 °C or used immediately.
6. Bacterial pellets are resuspended in buffer I plus 1 mM PMSF with a volume of 100 ml per liter of original cell culture. Cells are disrupted by sonication (Sonic Dismembranator, Model 300, Fisher Scientific) using three, 1 min bursts. The sonicate is placed on ice for at least 1 min between bursts to prevent overheating.
7. The sonicated bacterial extract is clarified by two successive centrifugations at 37,000 X g, 30 min, 4 °C (17,500 rpm in Beckman

JA-20 rotor), and the supernatant solution transferred to a fresh container.

8. Clarified bacterial extract is passed over PLP–Sepharose at a flow rate of $\sim$ 1 ml/min. Profilin is depleted from the flow-through fraction which can be discarded.

9. The PLP–Sepharose with bound profilin is washed by washing the column with 3–5 volumes ($\sim$ 30 ml) of buffer I.

10. Additional contaminants are removed by washing the column with 30 ml of buffer I containing 2 M urea.

11. Recombinant human profilin I is eluted by washing the column with buffer I containing 7 M urea. The eluate is collected in 1 ml fractions. A total of 50 ml of the 7 M urea solution is passed over the column before re-equilibrating with buffer I.

12. Fractions that contain protein are detected by placing 10 μl samples into a 96-well microtiter plate and adding 200 μl of diluted (1:4 with dH$_2$0) Bradford dye reagent (Bio-Rad, Hercules, CA) into each well.

13. Protein-containing fractions are pooled into 6000–8,000 Da molecular weight cutoff membrane tubing (Spectrum Spectra/Por® 1, Fisher Scientific) and are dialyzed against 3 changes, 1 l each, of buffer I over a 24 h period.

14. Renatured profilin is partitioned into 100 μl aliquots, frozen in liquid nitrogen, and stored at –80 °C.

Notes

1. The cDNA clone for expressing human profilin I can be obtained from Dr. Steven Almo, Albert Einstein University, College of Medicine, 1300 Morris Park Avenue, The Bronx, NY 10461 U.S.A. (almo@zugbug.bioc.aecom.yu.edu).

2. For routine use, the PLP–Sepharose is regenerated by extensive washes with buffer I, and stored under buffer I plus 0.1% [w/v] NaN$_3$. If flow rates over the matrix become noticeably slower, the beads can be washed according to a method described by Rozycki *et al*. [32]. The PLP–Sepharose is removed from the column and washed three times, 50 ml each, in a 2% [v/v] FL-70™ detergent (Fisher Scientific) solution prepared in dH$_2$O. The resin is collected after each wash by centrifugation at 200 X g (1000 rpm in Beckman GS-6R with GH 3.8 rotor) for 5 min. Beads are rinsed 3–5 times, each with 50 ml of buffer I, before repacking the column.

Solutions

All solutions are stored at 4 °C. DTT and PMSF are added just prior to use.

– LB medium, or Luria-Bertani Medium, is according to Sambrook *et al*. [35]:
  – 10 g/L bacto-tryptone
  – 5 g/L yeast extract

- 10 g/L NaCl
- pH to 7.0 with NaOH

- Phenylmethyl sulfoxide (PMSF) solution:
  - 100 mM in isopropanol

- DTT solution:
  - 1 M dithiothreitol
     dissolve in dH_2O and store at $-20\ °C$

- Buffer I:
  - 20 mM Tris
  - 150 mM KCl
  - 0.2 mM DTT
  - pH 7.5

Isolation of actin from maize pollen (*modified from* [31])

Preparation of cytosolic extract from frozen pollen

Steps in the procedure
1. Maize pollen is harvested from field-grown plants according to methods described by Neuffer [29]. Anthers and debris are removed by filtration through a single layer of cheesecloth. Pollen is frozen in the field by placing a 5–10 g aliquot into a plastic scintillation vial top centrifuge tube and then onto dry ice. Pollen is stored at −80° C in a container with dessicant.
2. To prepare the cytosolic extract, cool a mortar and pestle on ice and prepare 50 ml each of buffer A and buffer B from stock solutions stored at 4 °C.
3. Place 5–10 g of frozen pollen into the mortar along with 1 g quartz sand, and grind in 20 ml buffer A for 5 min.
4. Add 20 mg of recombinant human profilin per 5 g of pollen and continue grinding for an additional 25 min. The slurry will begin to thicken and become more pale towards the end of this time.
5. Pour into 2 centrifuge tubes and add the remaining 30 ml of buffer A. The buffer can be used to wash pollen from the sides of the mortar and pestle.
6. Sonicate each tube 5 times for 20 s each repetition, alternating tubes and keeping on ice to prevent overheating.
7. Balance the tubes and centrifuge for 30 min at 27,000 X g (15,000 rpm in Beckman JA-20 rotor)
8. Transfer as much supernatant solution as possible to new tubes with a pipette. Most of the white lipid will stick to the sides of the tube. Centrifuge the supernatant solution for 30 min at 46,000 X g (19,500 rpm in Beckman JA-20 rotor).
9. Transfer the supernatant solution from both tubes to a small plastic beaker with a pipette, being careful not to take up loose material coming off the pellet.
10. Check the pH and adjust to 7.50–7.55 with 1 M KOH if necessary. Add an additional 0.4 mM ATP (200 μl of 0.1 M stock).
11. Pour extract into a single ultracentrifuge tube and balance with another tube containing water. Centrifuge for 1 h at 120,000 X g (32,000 rpm in Beckman 45-Ti rotor).

Notes
1. Human profilin should be added to extracts from a concentrated stock solution (e.g. 7–10 mg/ml). If necessary, profilin can be concentrated on a Centricon-10 microconcentrator (Amicon, Inc. Beverly, MA).
2. A change from the original procedure of Ren *et al.* [31] is the addition of recombinant profilin to the grindate rather than to the 27,000 X g supernatant solution. Also, note that the ATP concentrations used here are higher.
3. Use a minimal amount of sand for grinding (i.e., no more than 2 g for 10 g of pollen).

Poly-L-proline affinity chromatography

Steps in the procedure
1. Equilibrate a PLP–Sepharose column ($\sim$ 7–9 ml of resin in a 2.5 x 10 cm column) with buffer B
2. Transfer the clarified supernatant solution to the column, being careful to avoid the whitish lipid layer and loose material from the pellet (usually $\sim$ 5 ml cannot be used).
3. Pass the supernatant solution over the column at a flow rate of $\sim$ 0.6 ml/min; this is best accomplished using a peristaltic pump.
4. When most of the extract has passed over the column, carefully layer 40 ml of buffer B over the extract. The extract is dense enough that a sharp interface will form.
5. After most of the buffer B has passed over the column, begin collecting fractions of 1.5–2 ml each.
6. As soon as the meniscus of the wash buffer just touches the column matrix, carefully add $\sim$ 2 ml buffer G + 1 M KCl. Allow this to run down to the meniscus again, and add another $\sim$ 2 ml buffer G + 1 M KCl. When this reaches the matrix, place 50 ml of the same solution over the column and continue collecting fractions.
7. The protein peak can be detected by placing 10 μl samples into a 96-well microtiter plate and adding 200 μl of diluted Bradford reagent into each well. Continue to collect fractions until the protein concentration falls to less than 0.2 mg/ml; this is judged by eye in comparison to a well containing 2 μl of 1 mg/ml BSA protein standard.
8. Pool the protein-containing fractions (usually 15–45 ml total volume) and dialyze overnight against 1 liter of buffer G.
9. Remaining actin and the recombinant profilin are eluted from the column by washing with 50 ml of buffer G containing 7 M urea. The column is regenerated with buffer B or buffer I.

Notes
1. Best yields are recovered when using a column with a high surface area/volume ratio. Our current column is 2.5 cm diameter with a 7–9 ml PLP–Sepharose bed volume.
2. Use of a *slow* flow rate is important; faster rates dilute the actin too much and the loading of profilin–actin on the column seems to be affected as well.
3. The wash must be pH 7.5 or higher, otherwise actin elutes from the column.
4. Recombinant vertebrate profilin eluting from the column at the end of the procedure is not re-used, as it is contaminated with native pollen profilin.

Actin polymerization and depolymerization

Steps in the procedure
1. Pipette the solution from the dialysis bag(s) into an ultracentrifuge tube that has been tared on a balance to measure the volume of actin solution.
2. Polymerize the actin by adding KCl to 100 mM from a 3 M stock, $MgCl_2$ to 5 mM from a 1 M stock solution, and Tris pH 7.5 to 10 mM from a 1 M stock solution. Incubate overnight at 4 °C.
3. Collect F-actin by centrifuging for 3 h at 120,000 X g (32,000 rpm in Beckman L3-50 with 45-Ti rotor).
4. Decant the supernatant fluid and a translucent pellet should be present. The pellet is sometimes ringed by cloudy material or slightly yellowish material.
5. Cover the pellet with ∼ 0.5 ml of buffer G and allow it to loosen on ice for 1 h.
6. Use a plastic pipette to gently resuspend the pellet into the buffer. Transfer the solution to dialysis tubing. Wash the sides of the centrifuge tube with an additional 0.5–1 ml buffer G, and add this to the dialysis tube.
7. Dialyze the actin for 4–5 d against daily changes of 1 l of buffer G.

Yield determination and storage

Steps in the procedure

1. Remove precipitates from the G-actin by centrifuging the dialyzed actin solution for 1 h at 120,000 X g (35,000 rpm in Beckman 70.1-Ti rotor).
2. Carefully remove the supernatant solution to a clean tube. The pellet, which can be discarded, will be composed of the whitish and yellowish material.
3. Actin can be stored on ice for not more than 16 h before use in experiments. For storage up to a week, place the clarified solution into dialysis tubing and exchange the dialysis solution daily with fresh buffer G.
4. Protein yields are determined by the Bradford method (BioRad protein assay dye) using BSA as a standard.
5. Purity is determined by separating ~ 10 μg of protein on a 10% SDS–PAGE gel and Coomassie Brilliant Blue staining. Pollen actin migrates at a M_r of 42,000.

Notes

1. The current average yield using this procedure is 3.9 mg from 10 g of pollen ($n = 14$). This value is somewhat lower than the average of 6.0 mg ($n = 10$) reported previously [31].
2. Yields of less than 2 mg are considered to be unsatisfactory. In this event, it is useful to check the pH of all buffers, to remake buffer A and buffer B, and to prepare fresh ATP solution.
3. The presence of lower molecular weight proteins (especially a 37,000 M_r polypeptide) in the final product most likely represents protease activity. PI cocktail and PMSF should be added immediately prior to use of solutions.

Solutions

All solutions are stored at 4 °C. DTT, PI cocktail, PMSF and ATP are added just prior to use.

– ATP solution:
  – 0.1 M adenosine–5'–trisphosphate, disodium salt
    dissolve in dH_2O, pH to 7.0 with 1N NaOH, and store at –20 °C

– Protease inhibitor (PI) cocktail:
  – 1.6 mg/ml Benzamidine HCl
  – 1 mg/ml Aprotinin
  – 1 mg/ml Leupeptin
  – 1 mg/ml Pepstatin A
  – 0.12 mg/ml Phenanthroline
    dissolve in ethanol and store at –20 °C
    some material will remain insoluble, solution should be mixed
    before use

– Buffer A (modified from [16]):
  – 10 mM Tris
  – 50 mM NaF
  – 30 mM Na_4PPi
  – 0.5 mM $CaCl_2$,
  – 0.01% [w/v] NaN_3,
  – 0.8 mM ATP
  – 0.5 mM DTT
  – 1/200 dilution of PI cocktail
  – 1/200 dilution of PMSF
  – pH 8.5

– Buffer B:
  – same as Buffer A except: $CaCl_2$, is 0.2 mM; ATP is 0.4 mM; pH is
    7.5; and PMSF or PI cocktail are *not* added

– Buffer G:
  – 5 mM Tris, pH 8.0
  – 0.2 mM $CaCl_2$
  – 0.01% [w/v] NaN_3
  – 0.5 mM DTT
  – 0.2 mM ATP

Determining a critical concentration (C$_c$) for actin assembly

Actin polymerization and steady state polymer levels can be measured by 90° light scattering as described by Cooper and Pollard [6]. The light scattering method is extremely useful for determining polymer concentration because the amount of scattered light is proportional to the total polymer mass [44]. Moreover, the assay is not sensitive to filament length when the average polymer is longer than the wavelength of light used for the analysis [44]. The amount of polymeric actin found in an assembly reaction at steady-state equilbrium depends upon the initial actin concentration. A key measure of actin quality is the critical concentration (C$_c$) for polymerization, or the actin concentration below which no polymer formation occurs. For the vertebrate skeletal muscle α-actin isoform this value is 0.1 μM [36].

Steps in the procedure
1. G-actin should be clarified prior to use by centrifugation at 120,000 X g (35,000 rpm in Beckman 70.1 Ti rotor) for 1 h at 4 °C.
2. Prepare 1.8 ml samples of G-actin at final concentrations of 1.0, 1.5, 2.0, 2.5 and 3.0 μM in LSF buffer, keeping the total volume of additional Buffer G constant.
3. Set up a spectrofluorimeter (SLM 8000, SLM Instruments Inc., Urbana, IL) for detection of light scattered at 90° with an excitation wavelength of 450 nm and detecting scattered light with the emission monochromator set to 450 nm.
4. Place each sample into the chamber and record an initial value for the light scattered by the G-actin solution and cuvette.
5. To initiate the polymerization of each sample, add KCl to 100 mM from a 3 M stock solution, and MgCl$_2$ to 5 mM from a 1 M stock solution. Allow the actin to polymerize overnight at room temperature.
6. Set the spectrofluorimeter with parameters identical to those used to measure initial light scattering and read the scattering from each sample.
7. Plot the difference of initial and final light scattering values *vs.* the actin concentration in each sample. The C$_c$ for actin assembly is the intercept of the regression line on the ordinate (see Figure 1).
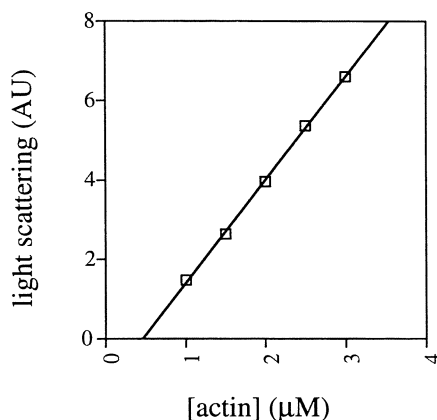
Fig. 1. Determination of critical concentration C_c for actin polymerization.
Data from an experiment to determine C_c were plotted as the original concentration of actin in each sample (x-axis)*versus* the change in light scattering following overnight polymerization (y-axis). The intercept of the regression line on the ordinate is the C_c. For this representative experiment, the C_c was calculated to be 0.46 μM. AU = arbitrary light scattering units.

Notes
1. Centrifuge all solutions before adding to cuvettes to prevent excess scattering; this is especially critical for protein-containing solutions.
2. It is important not to allow G-actin to remain in LSF buffer for longer than 1 h prior to addition of polymerizing salts as the low concentration of divalent cation is detrimental to activity.
3. Typical values for C_c for our batches of pollen actin range from 0.27–0.79 μM, with an average value of 0.48 $\pm$ 0.13 μM ($\pm$ SD, n = 13). If values above 0.6 μM are observed, the actin should be checked for contaminants by SDS-PAGE. Further, if material with a high C_c is to be used for quantitative measurement of interactions with actin binding proteins it should be recycled by polymerization/depolymerization prior to use.

Solutions
– Light scattering F-buffer (LSF buffer):
  – 5 mM Hepes, pH 7.0
  – 0.5 mM DTT
  – 0.2 mM ATP
  – 0.01% [w/v] NaN_3

Acknowledgements

References

1. Andersland JM, Jagendorf AT and Parthasarathy MV (1992) The isolation of actin from pea roots by DNase I affinity chromatography. Plant Physiol 100: 1716–1723.
2. Andersland JM and Parthasarathy MV (1993) Conditions affecting depolymerization of actin in plant homogenates. J Cell Sci 104: 1273–1279.
3. Asada T and Collings D (1997) Molecular motors in higher plants. Trends Plant Sci 2: 29–37.
4. Carlier M-F, Laurent V, Santolini J, Melki R, Didry D, Xia G-X, Hong Y, Chua N-H and Pantaloni D (1997) Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility. J Cell Biol 136: 1307–1322.
5. Clarke SR, Staiger CJ, Gibbon BC and Franklin-Tong VE (1998) A potential signaling role for profilin in pollen of *Papaver rhoeas*. Plant Cell 10: 967–979.
6. Cooper JA and Pollard TD (1982) Methods to measure actin polymerization. Methods Enzymol 85: 182–210.
7. Fedorov AA, Pollard TD and Almo SC (1994) Purification, characterization and crystallization of human platelet profilin expressed in *Escherichia coli*. J Mol Biol 241: 480–482.
8. Ghosh G and Biswas S (1988) An actin of higher plant: Purification and characterization. Pl Physiol Biochem 15: 153–160.
9. Gibbon BC, Ren H and Staiger CJ (1997) Characterization of maize (*Zea mays*) pollen profilin function *in vitro* and in live cells. Biochem J 327: 909–915.
10. Gibbon BC, Zonia LE, Kovar DR, Hussey PJ and Staiger CJ (1998) Pollen profilin function depends on interaction with proline-rich motifs. Plant Cell 10: 981–993. [A printed Correction appeared in Aug. (1999) 11: 1603.]
11. Giehl K, Valenta R, Rothkegel M, Ronsiek M, Mannherz H-G and Jockusch BM (1994) Interaction of plant profilin with mammalian actin. Eur J Biochem 226: 681–689.
12. Hennessey ES, Drummond DR and Sparrow JC (1993) Molecular genetics of actin function. Biochem J 282: 657–671.
13. Janmey PA (1991) Polyproline affinity method for purification of platelet profilin and modification with pyrene–maleimide. Methods Enzymol 196: 92–99.
14. Jiang C-J, Weeds AG and Hussey PJ (1997) The maize actin-depolymerizing factor, ZmADF3, redistributes to the growing tip of elongating root hairs and can be induced to translocate into the nucleus with actin. Plant J 12: 1035–1043.
15. Jiang C-J, Weeds AG, Khan S and Hussey PJ (1997) F-actin and G-actin binding are uncoupled by mutation of conserved tyrosine residues in maize actin depolymerizing factor (ZmADF). Proc Natl Acad Sci USA 94: 9973–9978.
16. Katakami Y, Katakami N, Janmey PA, Hartwig JH and Stossel TP (1992) Isolation of the phosphatidylinositol 4-monophosphate dissociable high-affinity profilin-actin complex. Biochim Biophys Acta 1122: 123–135.
17. Kulikova AL (1986) Quantity and forms of actin in conducting tissues of *Heracleum sosnowskyi*. Fiziologiya Rastenii 33: 481–487.
18. Kursanov AL, Kulikova AL and Turkina MV (1983) Actin-like protein from the phloem of *Heracleum sosnowskyi*. Physiol Vég 21: 353–360.
19. Kwiatkowski DJ and Bruns GAP (1988) Human profilin. J Biol Chem 263: 5910–5915.
20. Liu X and Yen L-F (1992) Purification and characterization of actin from maize pollen. Plant Physiol 99: 1151–1155.
21. Lopez I, Anthony RG, Maciver SK, Jiang C-J, Khan S, Weeds AG and Hussey PJ (1996) Pollen specific expression of maize genes encoding actin depolymerizing factor-like proteins. Proc Natl Acad Sci 93: 7415–7420.
22. McCurdy DW and Williamson RE (1987) An actin-related protein inside pea chloroplasts. J Cell Sci 87: 449–456.
23. McCurdy DW and Williamson RE (1991) Actin and actin-associated proteins. In: Lloyd CW (ed.), The Cytoskeletal Basis of Plant Growth and Form. Academic Press Ltd, London, pp. 3–14.

24. Meagher RB (1991) Divergence and differential expression of actin gene families in higher plants. Int Rev Cytol 125: 139–163.
25. Meagher RB and McLean BG (1990) Diversity of plant actins. Cell Motil Cytoskel 16: 164–166.
26. Meagher RB and Williamson RE (1994) The plant cytoskeleton. In: Meyerowitz E, Somerville C (eds) Arabidopsis. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 1049–1084.
27. Metcalf III TN, Szabo LJ, Schubert KR and Wang JL (1980) Immunochemical identification of an actin-like protein from soybean seedlings. Nature 285: 171–172.
28. Nefsky B and Bretscher A (1992) Yeast actin is relatively well behaved. Eur J Biochem. 206: 949–955.
29. Neuffer MG (1994) Growing maize for genetic studies. In: Freeling M, Walbot V (eds) The Maize Handbook. Springer-Verlag, New York, pp. 197–209.
30. Perelroizen I, Didry D, Christensen H, Chua N-H and Carlier M-F (1996) Role of nucleotide exchange and hydrolysis in the function of profilin in actin assembly. J Biol Chem 271: 12302–12309.
31. Ren H, Gibbon BC, Ashworth SL, Sherman DM, Yuan M and Staiger CJ (1997) Actin purified from maize pollen functions in living plant cells. Plant Cell 9: 1445–1457
32. Rozycki M, Schutt CE and Lindberg U (1991) Affinity chromatography-based purification of profilin:actin. Methods Enzymol 196: 100–118.
33. Rubenstein PA (1990) The functional importance of multiple actin isoforms. Bioessays 12: 309–315.
34. Ruhlandt G, Lange U and Grolig F (1994) Profilins purified from higher plants bind to actin from cardiac muscle and to actin from a green alga. Plant Cell Physiol 35: 849–854.
35. Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36. Sheterline P, Clayton J and Sparrow JC (1998) Actin. Protein Profile 4: 1–272.
37. Staiger CJ, Gibbon BC, Kovar DR and Zonia LE (1997) Profilin and actin depolymerizing factor: modulators of actin organization in plants. Trends Plant Sci 2: 275–281.
38. Staiger CJ, Schliwa M (1987) Actin localization and function in higher plant cells. Protoplasma 141: 1–12.
39. Turkina MV, Kulikova AL, Sokolov OI, Bogatyrev VA and Kursanov AL (1987) Actin and myosin filaments from the conducting tissues of *Heracleum sosnowskyi*. Plant Physiol Biochem 25: 689–696.
40. Vahey M and Scordilis SP (1980) Contractile proteins from tomato. Can J Bot 58: 797–801.
41. Vahey M, Titus M, Trautwein R and Scordilis S (1982) Tomato actin and myosin: Contractile proteins from a higher land plant. Cell Motil 2: 131–147.
42. Vidali L and Hepler PK (1997) Characterization and localization of profilin in pollen grains and tubes of *Lilium longiflorum*. Cell Motil Cytoskel 36: 323–338.
43. Villanueva MA, Ho S-C and Wang JL (1990) Isolation and characterization of one isoform of actin from cultured soybean cells. Arch Biochem Biophys 277: 35–41.
44. Wegner A and Engel J (1975) Kinetics of the cooperative association of actin to actin filaments. Biophys Chem 3: 215–225.
45. Zechel K, Weber K (1978) Actins from mammals, bird, fish and slime mold characterized by isoelectric focusing in polyacrylamide gels. Eur J Biochem. 89: 105–112.

Use of the R-*RS* Site-Specific Recombination System in Plants

Chiyoko Machida[1,2], Hitoshi Onouchi[1], Endang Semiarti[1], Takaaki Ishikawa[1] and Yasunori Machida[1]
[1]Laboratory of Developmental Biology, Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan (Tel:+81-52-789-2502; Fax: +81-52-789-2966); [2] On leave from Kirin Brewery Co., Ltd. Central Laboratories for Key Technology, 1-13-5 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan

Introduction

The circular plasmid pSR1 from *Zygosaccharomyces rouxii* includes a pair of inverted repeat sequences of 959 bp that contain recombination sites (*RS*; 58 bp at most) for intramolecular recombination [1]. Experiments performed *in vitro* with this recombination system indicated that the system requires only the R protein, the recombinase, that is encoded by the *R* gene of pSR1 [2]. The pSR1 recombination system (R-*RS* system) is similar, in terms of its recombination mechanism, to the Cre-*loxP* system derived from bacteriophage P1 [40] and the FLP-*FRT* system of the 2-μm plasmid of *Saccharomyces cerevisiae* [7] Use of these site-specific recombination systems in heterologous organisms seems to offer several advantages: recombination takes place only between specific sequences, which are usually several dozen base pairs (bp) in length (high specificity); recombination is catalyzed by a single recombinase protein, and no other protein is required (simple mechanism); and the recombination frequency is remarkably high.

The excision, inversion, and translocation of a large segment of chromosomal DNA was first demonstrated in *S. cerevisiae* cells by use of the R-*RS* system from *Z. rouxii* [24,27]. When *RS* elements were inserted into both the *RAS1* and *HIS3* loci on chromosome XV of *S. cerevisiae*, respectively, which are separated by approximately 180 kb, the R protein efficiently catalyzed the excision or inversion of the chromosomal region between these loci, depending on the relative orientations of the *RS* elements. It also catalyzed chromosome translocation when two *RS* elements were present on two nonhomologous chromosomes [28]. These results suggest that the R-*RS* system is useful for generating chromosomal rearrangements in heterologous eukaryotes.

To test the feasibility of developing a DNA recombination technology with this system, we first examined whether this system was operable in tobacco suspension-cultured cells. Our results demonstrated that *R*-gene-mediated excision and inversion were efficiently induced [34]. We also introduced R-*RS* system into *Arabidopsis thaliana* and showed that *R*-gene-mediated recombination can be induced in both somatic and germ cells of this species [35]. This chapter details two methods. One permits visualization of R-*RS* recombination in tobacco cultured cells and *Arabidopsis* plants, and the other facilitates introduction of

two *RS* elements on the same chromosome by using a derivative of the *Ac* element that contains *RS* sites, followed by induction of a deletion between the two *RS* elements.

Visualization of R-*RS* Recombination in Plants

A system for visualization of R-RS recombination events

Recombinational events induced by the recombinase (*R*) gene in plants can be monitored by two types of cryptic reporter genes (Figure 1) that can serve as
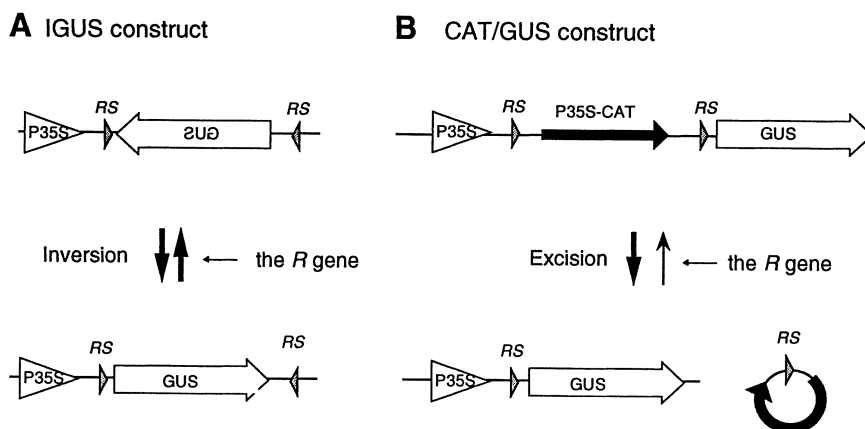
Fig. 1. Strategy for detection of site-specific recombination mediated by the *R* gene in tobacco cells and *Arabidopsis* plants. **(A)** Schematic diagram of the IGUS construction in pGI used for detection of inversion. pRGR has the same structural organization as pGI except for the orientation of GUS. **(B)** Schematic diagram of the CAT/GUS construction in pCATG used for detection of excision. The heavy line represents the CAT gene expression unit [13]. P35S, the promoter for 35S RNA from cauliflower mosaic virus 35S RNA; GUS, the coding sequence of β-glucuronidase; RS, recombination site recognized by R protein. The large open arrow represents the orientation of the GUS coding sequence. Arrowheads indicate the orientations of *RS* elements.

substrates for the R protein [35]. The IGUS construction, that is used for detecting inversions, contains the coding sequence for the GUS gene flanked by two copies of the recombination site (*RS*) in the inverted orientation (Figure 1A). This GUS construction with two *RS* elements is inserted, in the anti-sense orientation, downstream from the cauliflower mosaic virus 35S promoter (P35S). The GUS coding sequence is transcribed when an inversion between two *RS* elements is induced by the product of the *R* gene. CAT/GUS is used for monitoring *R* gene-mediated deletion (Figure 1B). This construction contains the chloramphenicol acetyltransferase (CAT) transcription unit between P35S and the GUS coding sequence. Because the CAT gene unit is bounded by two *RS* sequences in direct orientation, precise *R* gene-mediated recombination can re-

sult in the excision of the CAT cassette to generate an active GUS gene (Figure 1B). Upon excision, the CAT cassette is thought to be circularized [28]. Although the R protein can catalyze the backward (integration) reaction, the relative rate of this reaction is expected to be much lower than that of the excision reaction. To drive expression of the R gene, use of a modified 35S promoter, in which the enhancer region of P35S is duplicated, is recommended.

Using this system, recombination can easely be detected and visualized by staining activity for GUS in tobacco cultured cells. It is also possible that recombination can be detected as varied staining patterns of GUS-activity in plant tissues.

Plasmids

Plasmids used and their relevant characteristics are listed in Table 1.

Table 1. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference
pGAHGI	Derivative of pGAH (binary vector plasmid) containing the IGUS construction	[35]
pBIHCATG	Derivative of pBI121 (binary vector plasmid) containing the CAT/GUS construction	[35]
pGAHR	Derivative of pGAH containing the R gene under control of a modified 35S promoter	[35]
pGAHΔR	Derivative of pGAH containing a mutant R gene, with the C-terminal deletion, under control of a modified 35S promoter	[35]
pIG121HM	Derivative of pBI121 containing a GUS gene with an intron in the 5' proximal region of the GUS sequence	[38]
pGA492	Binary vector plasmid containing the neomycin phosphotransferase II gene and a multiple cloning site	[3]
pGAH	Derivative of pGA492 containing a multiple cloning site between the neomycin phosphotransferase II gene and the hygromycin phosphotransferase gene	[35]

Plant cells and transgenic plants

Tobacco cell lines used are listed below.
1. Suspension cultures of the cell line BY-2 of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2) [23].
2. The transformed cell line BY-2(IGUS).
3. The transformed cell lines BY-2(CAT/GUS).

Procedures

Recombination between two RS elements in tobacco chromosomes

Induction of recombination between two RS elements in tobacco chromosomes
1. Culture transformed BY-2 cells [BY-2(IGUS) and BY-2(CAT/GUS)] for 3 days in fresh Linsmaier and Skoog (LS) liquid medium [25] with 0.2 mg/l 2,4-D and 50 mg/l hygromycin B at 26 °C.
2. Culture *Agrobacterium* cells carrying pGAHR or pGAHΔR (see Table 1) until the absorbance at 600 nm is 1.5 and concentrate the culture four-fold in LB medium.
3. Mix 100 μl of this *Agrobacterium* culture with 4 ml of a suspension culture of the transformed BY-2 cells from 2.4.1.1.
4. Incubate the mixture at 26 °C in darkness for 48 hr, 72 hr, or 96 hr.

Assays for GUS activity

Fluorometric assay
1. Collect tobacco cells after co-culturing.
2. Disrupt the cells by sonication (three 30-sec pulses).
3. Remove cell debris by centrifugation.
4. Recover the supernatant fluids for determination of protein concentration and GUS activity using a fluorometric assay [21].

Histochemical assay
To measure the proportion of tobacco cells in which DNA excision, mediated by the R protein, takes place under these conditions, tobacco cells that express GUS can be detected as follows.
1. Co-culture tobacco transformed cells BY2(CAT/GUS) with *Agrobacterium* cells that harbor pGAHΔR, pGAHR or pIG121HM for 4 days as described in 2.4.1.
2. Collect the tobacco cells by centrifugation at 1000 rpm for 5 min.
3. To prepare protoplasts, suspend the co-cultured cells in protoplast isolation solution that contains LS liquid medium [25] with 0.5 M mannitol, 1% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Nishinomiya, Japan), and 0.2% Pectolyase Y23 (Seishin Pharmaceutical Industry Co., Ltd., Tokyo, Japan) and adjust pH to 5.8 with KOH.
4. Incubate at 26 °C with gentle shaking for 4 hr.
5. Filter the suspension through a nylon mesh to remove undigested calli.
6. Incubate protoplasts overnight at 37 °C in a solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), 50 mM sodium phosphate (pH 7.0), and 0.35 M mannitol.

Results of introduction of the R-RS system in tobacco cultured cells

Table 2. GUS activity of BY-2 cells transformed with the cryptic reporter constructions after co-culture with *Agrobacterium* cells that carried the *R* gene.

Cryptic GUS in transformed line	Binary vector	GUS activity (pmoles/min/mg protein) after co-culturing for		
		48 h	72 h	96 h
CAT/GUS	pGAHΔR	14.2±2.3	9.1±0.7	11.5±1.4
(cell line 1)[a]	pGAHR	81.5±17.6	163±2.7	252±4.4
CAT/GUS	pGAHΔR	23.3±0.7	23.9±0.8	19.3±0.7
(cell line 2)[a]	pGAHR	36.4±4.0	94.4±2.3	124±5
IGUS	pGAHΔR	16.2±2.6	15.0±1.1	15.6±1.4
	pGAHR	18.8±3.1	52.9±7.5	90.5±12.4

[a] Independently-isolated cell lines

Table 2 shows GUS activity of BY-2 cells transformed with the cryptic reporter constructions after co-culture with *Agrobacterium* cells that carried the *R* gene. Significant levels of activity were detected in both BY-2(CAT/GUS) and BY-2(IGUS) cell lines 48 hr after co-culturing; activity increased as co-culturing was prolonged. When cells were co-cultured with *Agrobacterium* that harbored pGAHΔR with the mutant *R* gene, GUS activities remained at background levels. These results indicate that the R protein mediates the site-specific recombination between *RS* elements that are resident on the tobacco chromosomes.

Table 3. Histochemical staining of protoplasts of CAT/GUS-transformed BY-2 cells co-cultured with *Agrobacterium* cells that carried the *R* gene in the binary vector.

Binary vector	Number of blue cells	Total cells examined	Percentage of total cells that stained
pGAHΔR	0	1542	0
pGAHR	43	2153	2.0
pIG121HM[a]	70	1525	4.6

[a] pIG121HM contains a GUS gene with an intron in the 5' non-coding region in such a way that it can be expressed only in plant cells.

Table 3 shows the results of an experiment in wich histochemical staining was measured Protoplasts of CAT/GUS-transformed BY-2 cells co-cultured with *Agrobacterium* cells that carried the *R* gene in the binary vector. Two percent of the co-cultured cells showed blue staining. When we co-cultured BY-2(CAT/GUS) cells with *Agrobacterium* cells that carried a GUS reporter gene instead of the *R* gene in

the binary vector (pIG121HM in Table 1), 4.6% of the cells were stained. Therefore, it is likely that excision of the CAT cassette took place in approximately 40% of the plant cells into which the *R* gene had been incorporated.

When we analyzed the excision product from the CAT/GUS construction and the inversion product from the IGUS by PCR, the expected sizes of DNA fragments were amplified, respectively. Analyses of the nucleotide sequences of the amplified DNA fragments showed that recombination took place precisely within the two *RS* elements of each construction.

Induction of R-RS recombination in Arabidopsis *plants*

Transgenic plants
1. The transgenic *Arabidopsis thaliana* line R-21 (hygromycin resistant) carries the *R* gene under the control of a modified 35S promoter. R-21 provided the highest efficiency of R-*RS* recombination among the three lines we tested.
2. The transgenic *Arabidopsis thaliana* line CATGUS-12 (hygromycin and kanamycin resistant) carries the CAT/GUS cryptic reporter gene.

Cross-pollination between Arabidopsis *plants that carry the* R *gene and plants that carry two* RS *elements.*
1. Plant the transgenic *Arabidopsis thaliana* line that carries the *R* gene under the control of the modified 35S promoter (R-21) and the line that carries two *RS* elements and the cryptic GUS reporter gene (CATGUS-12) on soil.
2. Cross R-21 with CATGUS-12, using the latter plant as the pollen parent.
3. Grow transgenic plants under continuous light (3000-6000 lux) at 22 °C.
4. Harvest seeds.
5. Sow F_1 seeds on MS plates containing 15 mg/liter hygromycin B.
6. Incubate under continuous light (3000–6000 lux) at 22 °C.
7. To examine germinal recombination, self-pollinate the F_1 progeny and harvest F_2 seeds.

Histochemical assay for GUS activity
Histochemical detection of GUS expression can be performed essentially as described [6,21]
1. Obtain twelve-day-old *in vitro* plantlets.
2. Place plantlets in a solution of 1 mg/ml X-Gluc, 50 mM NaH_2PO_4 (pH 7.0), and 0.5% Triton X-100.
3. Introduce the staining solution into the plantlets by vacuum infiltration for 30 min.
4. Incubate at 22 °C for 16 h.
5. To remove chlorophyll, transfer the plantlets to a solution of 5% formaldehyde, 5% acetic acid, and 20% ethanol and incubate for 10 min.
6. Transfer to 50% ethanol and incubate for 2 min.
7. Transfer to 100% ethanol.

Results of R-RS recombination in Arabidopsis plants

Figures 2A, B, and C show typical staining patterns of F_1 progeny plantlets. On the basis of these staining patterns, F_1 progeny were classified into three basic types (A, B and C). (1) Type A exhibited distinct, patch-like blue spots on cotyledons and a few very small spots on leaves, hypocotyls, and roots (Figure 2A). (2) Type B exhibited staining of whole cotyledons, hypocotyls, and some regions of roots (Figure 2B). (3) Type C also showed staining of whole cotyledons, hypocotyls, and some regions of roots, as did type B. In addition, type C exhibited sectorial chimerism in the first, second, and third leaves (Figure 2C). Even in type C, however, only a few very small spots were visible on the later leaves (the fourth and fifth leaves); (Figure 2C). Three out of the twelve progeny of these parents exhibited sectorial chimerism. Note that the stained sectors were found in each of the first three leaves in these three progeny and that all of the sectors extended from the base of petioles almost as far as the tips of the leaves. The above results indicate that R-RS recombination can be induced in somatic cells, at least, of Arabidopsis plants.
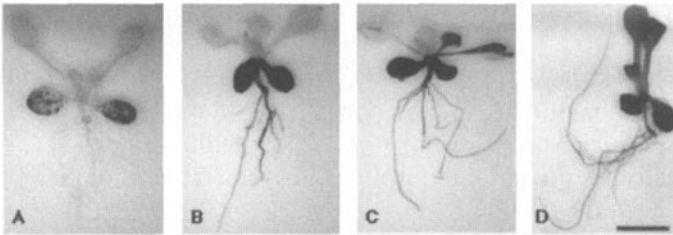
Fig. 2. Analysis of somatic recombination in F_1 progeny and germinal recombination in F_2 progeny. Plants that were generated by cross pollination of a CATGUS transformant line with the R gene transformant line. Twelve-day-old plantlets from among the F_1 progeny of CATGUS-12 x R-21 (A, B, C) and the F_2 progeny (D) were incubated with X-Gluc. Bar = 5 mm.

Figure 2D shows F_2 progeny plantlets that exhibited staining of the entire plantlet. To examine the frequency of R-RS recombination in germ cells of F_1 progeny (germinal recombination), F_1 plants were self-pollinated, and the resulting F_2 plants were stained with X-Gluc. Among the progeny from these parents, 2.4% exhibited staining of the entire plantlet (Fig. 2D). These plants must have been derived from germ cells of the F_1 plant in which the cryptic GUS gene had been activated by R-RS recombination (germinal R-RS recombination). We further confirmed the germinal R-RS recombination event at the DNA level by Southern hybridization and PCR [3].

*Prospects for introduction of the R-RS system in plants:
The R-RS system may be useful for clonal analysis of leaf
development*

One of the potential applications of site-specific recombination systems is use in the clonal analysis of cell lineage [17,33,34,41]. If expression of the *R* gene could be controlled by a promoter that was transiently activated at a developmental state that substantially precedes overt differentiation, the cryptic GUS reporter gene could be activated at that developmental stage. Then, the gain of function of the reporter gene would be heritable in progeny cells throughout later developmental stages and would be easily detectable by simple histochemical staining. It would thus be possible to construct a fate map of embryonic cells during organ development that might allow us to correlate embryonic patterns of gene expression with the organization of cells in mature organs.

The results in Figure 2 show that the cryptic reporter/R gene system is, in fact, applicable to such an analysis. Although we used the 35S promoter, which is thought to be active in all organs at all stages of development, to drive the *R* gene, sectorial chimerism of GUS activity was found in the first three leaves of three progeny from a particular combination of parents, namely, CATGUS-12 × R-21 (Figures 2B and 2C). Because each sector extended from the base to the tip of each leaf, the recombination event that gave rise to the sector might have taken place prior to germination in a single cell or in a discrete group(s) of cells in the immature shoot apical meristem of an embryo, which might have generated the first three leaves. Alternatively, it might have occurred at a very early stage of leaf development after germination. Shapes of the sectors observed in our study were similar to those of the sectors found during clonal analysis of leaf development by X-ray irradiation of dry seeds [14,19]. This correlation supports the idea that activation of the cryptic reporter gene by the R-*RS* recombination might have occurred prior to germination. However, for the systematic and detailed analysis of cell lineage, it is necessarily to use a promoter that can be activated at a specific developmental stage of interest.

Deletion of a Chromosomal Region Flanked by Two *RS* Elements in *Arabidopsis* Plants

We and other groups have previously proposed that site-specific recombination systems may be useful for introducing deletions and inversions of large regions of plant chromosomes if two recombination sites could be placed in a single chromosome [29,35,37]. To this end, we took advantage of the maize transposable element *Ac* because it transposes efficiently to regions within the same chromosome (intrachromosomal transposition) of maize [11,15,16,30,43], tobacco [12,22], *Arabidopsis thaliana* [5,26], and tomato [8,36].

To develop this system, a plant in which the distance between two *RS* elements has been determined must be used because the longer the distance is, the lower the frequency of recombination is expected to be. Therefore, we first prepared such plants.

Generation of Arabidopsis plants which contain two *RS* elements
separated by determined distances in a single chromosome.

A

B
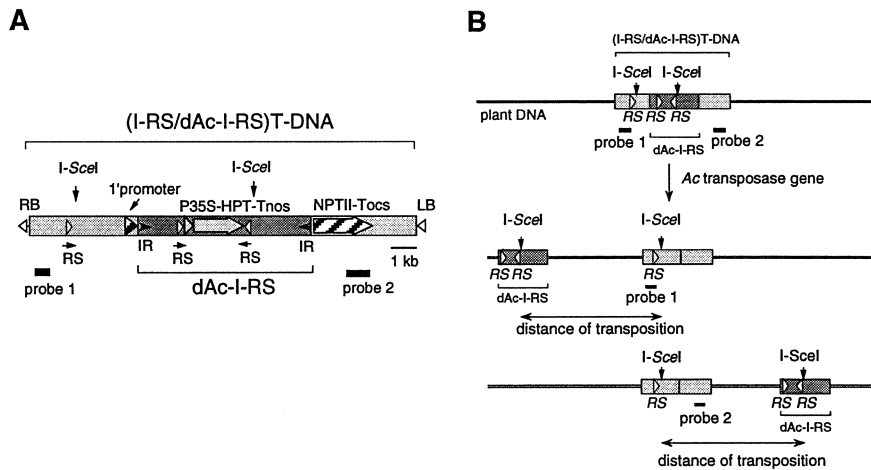
Fig. 3. (A) Schematic diagram of the (I-RS/dAc-I-RS)T-DNA. LB and RB indicate left and right
border sequences on the T-DNA, respectively. I-*Sce*I, the cleavage site for endonuclease I-*Sce*I; 1'
promoter, the 1' promoter of the octopine Ti plasmid TR-DNA; P35S, the promoter of the gene for
35S RNA from cauliflower mosaic virus; HPT, the coding sequence of the gene for hygromycin
phosphotransferase; NPTII, the coding sequence of the gene for neomycin phosphotransferase II;
Tnos, the terminator of the gene for nopaline synthase; Tocs, the terminator of the gene for octopine
synthase; IR, the terminal inverted repeat sequences of *Ac*; *RS*, the recombination site that is recog-
nized by the R protein from *Zygosaccharomyces rouxii*. Thick lines indicate the probes (probes 1
and 2) used for Southern blot analysis. (B) The strategy for measurement of the distance of trans-
position. I-*Sce*I indicates the site of cleavage by endonuclease I-*Sce*I. A dark gray box indicates
dAc-I-RS. Light gray boxes indicate regions of T-DNA other than dAc-I-RS in (I-RS/dAc-I-RS)T-
DNA.

Figure 3 shows the strategy for measurement of the distance of two
RS elements in the same chromosome [26]. The T-DNA construction
in Figure 3 contains three *RS* sequences: two are inside and the other
is outside the modified *Ac* transposable element. Furthermore, the T-
DNA construction has two recognition sites for I-*Sce*I: one is inside
and the other is outside the transposable element. We designated this
element dAc-I-RS because it had the *de*fective *Ac* element that con-
tained the site for *I-Sce*I and *RS* elements and this T-DNA as (I-RS/dAc-
I-RS)T-DNA as described in Machida et al. [26]. Because I-*Sce*I recog-
nizes a specific 18-base-pair sequence [9,10], the expected frequency
of occurrence of the cleavage site for this enzyme is less than one for
the chromosomes of *Arabidopsis thaliana*. Therefore, if such cleavage
sites are introduced into the *Ac* element and at the original chromo-
somal location of the *Ac* element, the physical distance of transposi-
tion can be directly determined by measuring the size of the DNA seg-

ment generated by digestion of the genomic DNA with I-*Sce*I . After transposition of dAc-I-RS on the same chromosome, digestion of genomic DNA with I-*Sce*I should give rise to a segment of chromosomal DNA flanked by part of the T-DNA at the original integration site and part of the transposed dAc-I-RS sequence (see Figure 3). Using two DNA fragments as probes (probes 1 and 2) for Southern hybridization (see Figure 3), we can distinguish the relative direction of each transposition.

For monitoring the excision of the dAc-I-RS element, it was inserted into a region between the 1' promoter [44] and the coding sequence of the gene for neomycin phosphotransferase II (NPTII), generating a cryptic kanamycin resistance gene [4], as shown in Figure 3A. Thus, excision of dAc-I-RS in transgenic *Arabidopsis* plants that contain (I-RS/dAc-I-RS)T-DNA should create an active NPTII gene.

We chose a transgenic *Arabidopsis* line that had a single copy of (I-RS/dAc-I-RS)T-DNA in chromosome 1 (#14) and induced transposition to measure the physical distance between two *RS* elements on the chromosome. Figure 4 shows distances of transposition, i.e., the distances between the two *RS* elements in transgenic *Arabidopsis* plants which we generated using line #14.

These *Arabidopsis* plants are useful for a systematic examination of chromosomal deletions mediated by the R-*RS* system. We present here the procedure for inducing transposition of dAc-I-RS and measuring the physical distance between two *RS* elements on the chromosome. We also describe the detection of a deletion of a chromosomal region flanked by two *RS*s in *Arabidopsis* somatic cells.

Plant materials

1. The transgenic *Arabidopsis* line I-RS/dAc-I-RS#14 that carries a single copy of (I-RS/dAc-I-RS)T-DNA.
2. The transgenic *Arabidopsis* line P35SAcTPase#9 that carries the gene for *Ac* transposase under the control of a modified 35S promoter.
3. The transgenic *Arabidopsis* line R-21 that carries the *R* gene under the control of a modified 35S promoter.

A

kb

1900 –
1100 –

915 –
815 –   ← 820 kb

680 –

450 –   ← 370 kb

225 –
97 –
49 –

probe 2

B
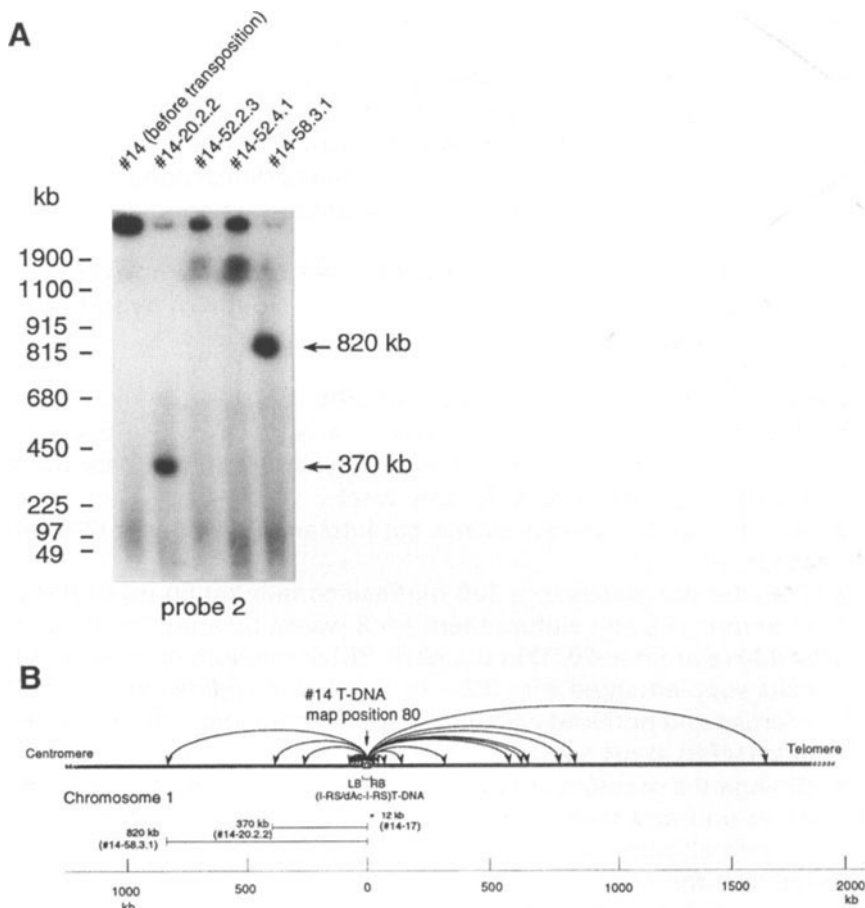
Fig. 4. (A) Southern blot analysis for measurement of the distance of transposition of dAc-I-RS determined by pulse-field gel electrophoresis. High molecular-weight genomic DNA was prepared from *Arabidopsis* plants that had a transposed dAc-I-RS which originated from line #14. Isolated DNA was digested with I-*Sce*I and then fractionated by PFGE through a 1% agarose gel for 18 h at 170 V with a switch interval of 80 s and then for 3 h at 170 V with a switch interval of 110 s. (B) Summary of distances of transposition in the I-RS/dAc-I-RS#14 line. LB and RB indicate left and right border sequences on the T-DNA, respectively. Some of the plant names are indicated in parentheses.

Measurement of distances of transposition of dAc-I-RS

Induction of transposition of dAc-I-RS and selection of plants with a transposed dAc-I-RS.

1. Cross plants of transgenic line #14 with line P35SAcTPase#9, using the latter plant as the pollen parent.
2. Self-pollinate F$_1$ progeny and harvest F$_2$ seeds.
3. Sow the F$_2$ seeds on MS basic medium [Murashige and Skoog

Plant Salt Mixture (Wako Pure Chemical Industries Ltd.) with 20 g/l sucrose, 3 mg/l thiamine, 0.5 mg/l pyridoxine, 5 mg/l nicotinic acid, and 0.2 % Gellan Gum (Wako Pure Chemical Industries Ltd.), pH 6.3] with 35 mg/l kanamycin sulfate to select plants in which dAc-I-RS has been excised to create the active kanamycin resistance gene (see Generation of arabidopsis plants which contain two RS elements separated by determined distances in a single chromosome).

4. Select plants that have reinserted dAc-I-RS elements by examining the presence of the dAc-I-RS sequence by Southern hybridization or polymerase chain reaction (PCR).

Preparation of high-molecular-weight DNA [18,20].

1. Sow seeds from plants that have transposed dAc-I-RS on MS plates containing 15 mg/liter hygromycin B and incubate under continuous light at 22 °C for one week.

2. Collect 100–150 seedlings and cut into small segments (2–5 mm long).

3. Transfer the pieces to a 300 ml flask containing 50 ml of RM 28 medium [18], and culture them for 3 weeks by agitating the flask at 130 rev/min at 26 °C in the dark. RM28 medium consists of MS salts supplemented with 0.2 mg/l 2,4-D, 0.05 mg/ml kinetin, 0.1 M sucrose and buffered with 3 mM 2[N-morpholino] ethanosulfonic acid (MES) at pH 5.8.

4. Change the medium every 5–7 days by pouring off the spent medium and any floating cells. Cultures should consist mainly of very small clumps.

5. Replace the medium with 50 ml of protoplast isolation medium (PIM), which contains RM 28 medium, 5 mM $CaCl_2$, 0.5 M mannitol, 1 % Cellulase Onozuka RS, and 0.2 % Pectolyase, and adjust its pH to 5.8 with KOH.

6. Incubate at 25 °C with gentle shaking for 4 h.

7. Filter the cell suspension, which has become turbid with protoplasts, through a nylon mesh (60 mm) to remove undigested calli.

8. Centrifuge the filtrate at 1000 rpm for 2 min.

9. Suspend protoplasts in 0.6 M mannitol at a final concentration of approximately 2×10^8 protoplasts/ml.

10. Mix protoplasts in 0.6 M mannitol with the same volume of molten 2% low-melting temperature agarose (InCertTM; FMC, Rockland, ME, USA) and pour into plastic molds ($2 \times 5 \times 10$ mm^3).

11. Place plastic molds on ice for 10 min until the agarose solidifies.

12. Place the plugs (100 μl /one plug) in a NDS solution that contains 0.5 M EDTA, pH 8.0, 1% sodium N -lauroylsarcosine, and 2 mg/ml proteinase K (Sigma, St. Louis, MO, USA).

12. Incubate for 48 h at 50 °C.

13. Transfer the plugs to fresh NDS and incubate for an additional 48 h.
14. Store at 4 °C.

Digestion of high-molecular-weight DNA in plugs with endonuclease I-SceI

1. For inactivation of proteinase K, place the plugs in a solution of 50 mM EDTA (pH 8.0) and incubate for 2 h at 25 °C.
2. Transfer the plugs to a solution of 50 mM EDTA (pH 8.0) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and store overnight at 4 °C.
3. Transfer the plugs to a solution of 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) and incubate for 2 h at 25 °C.
4. Transfer the plugs to fresh solution containing the same components and store at 4 °C.
5. Cut the plug and transfer one fourth of it into a tube.
6. Equilibrate the piece of plug (25 μl) overnight at 4 °C in 100 μl of a reaction buffer containing 0.1 M diethanolamine-Cl and 1 mM dithiothreitol, pH9.5, and then incubate it for two hours at 4 °C in 200 μl of the same buffer containing 200 μg/ml bovine serum albumin.
7. Transfer the plug into 50 μl of the same buffer containing enhancer (Boehringer Mannheim Biochemica, Mannheim, Germany) and 40 units of I- *Sce*I (Boehringer Mannheim Biochemica) for 2 h at 4 C. Because I-*Sce*I is known to be unstable in the presence of Mg^{2+}, diffusion of the enzyme into the plugs is needed before starting the reaction [42].
8. Start the reaction by addition of 1.25 μl of 0.2 M MgCl$_2$ (final conc. 5 mM) and incubate the tube for 40 min at 37 °C.
9. Analyze the DNA segment in the plugs directly by pulse-field gel electrophoresis (PFGE).

Conditions for Pulse-Field Gel Electrophoresis

PFGE is performed as essentially described by Guzman and Ecker [18].

1. Prepare agarose gels (1% SeaKem LE agarose FMC) in 0.5×TBE (0.045 M Tris-borate, 1 mM EDTA, pH 8.0) by pouring 120 ml of agarose into a 20×20 cm frame for separation of DNA fragments of 5-1,000 kb. For separating of DNA fragments of 1-5 mega-base pairs (Mb), prepare agarose gels (0.6% Fast Lane Agarose; FMC) in 0.5 x TAE (0.02 M Tris-acetate, 0.5 mM EDTA, pH 8.0) in the 20×20 cm frame.
2. Insert plugs containing digested DNA into gel wells.
3. Carry out electrophoresis in 0.5×TBE and 0.5×TAE running buffer for separation of DNA fragments of 5–1000 kb and 1–5 Mb, respec-

tively, and maintain a constant temperature of 10 °C by recirculation of the buffer through a heat exchanger.

4. Fractionate digested DNA by PFGE through a 1% agarose gel for 18 h at 170 V with a switch interval of 80 s and then for 3 h at 170 V with a switch interval of 110 s (for analysis of fragments of 5–1000 kb) or through a 0.6% agarose gel for 3 h at 50 V with a switch interval of 5 s and for 7 days at 50 V with a switch interval of 1800 s (for analysis of fragments of 1–5 Mb).

5. Carry out transfer of DNA to a filter and Southern blot by a standard protocol.

Results of measurement of distances of transposition

We analyzed a total of 60 transposition events from line #14 in this way. As summarized in Figure 3, 50% of all transposition events had occurred within 1700 kb on the same chromosome, with 35% within 200 kb. The elements transposed in both directions on the chromosome with roughly equal probability.

Induction of deletions mediated by R-RS recombination

Cross-pollination between Arabidopsis *plants that carry the R* gene
and plants that carried two *RS* elements separated by a determined
length in the same chromosome.

1. Plant the transgenic *Arabidopsis* line (R-21) that carries the *R* gene
   under the control of the modified 35S promoter and one of the lines
   that carry two RS elements on the same chromosome, as shown in
   Fig. 4 (I-RS/dAc-I-RS#14-17), on soil.
2. Cross R-21 with I-RS/dAc-I-RS#14–17, using the latter plant as the
   pollen parent.
3. Grow transgenic plants under continuous light (3000–6000 lux) at
   22 °C.
4. Harvest seeds.
5. Sow seeds of F_1 progeny on MS plates containing 15 mg/l hygro-
   mycin B and 35 mg/l kanamycin sulfate.
6. Incubate under continuous light (3000–6000 lux) at 22 °C for 20
   days.

Detection of somatic deletion by PCR
1. Extract DNA from the leaves of 20-day-old F_1 progeny by the
   method using cetyltrimethylammonium bromide described by
   Murray and Thompson [31].
2. Use the extracted DNA as a template for amplification by the poly-
   merase PCR during 30 cycles of 30 s denaturation at 94 °C, 1-min
   annealing at 60 °C, and 3-min elongation at 72 °C, using 10 ng of
   genomic DNA in a total volume of 100 μl.
3. Analyze the reaction mixture by electrophosesis through a 4%
   polyacrylamide gel.

Results of induction of somatic deletion

As shown in Figure 5, the 0.3 kb DNA fragment was amplified with genomic DNA of the F1 progenies, but not with parental lines. This result is to be expected if recombination takes place between two *RS* elements.
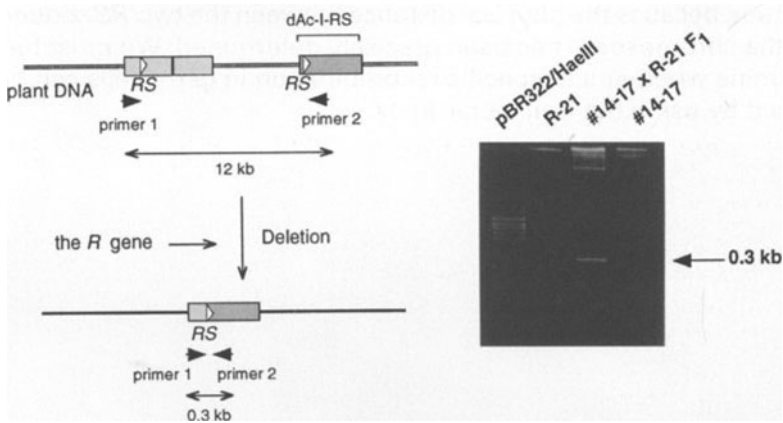
Fig. 5. PCR analysis of DNA produced by *R* gene-mediated recombination in somatic cells of F_1 plants. Template DNA was extracted from leaves of 20-day-old parents and F_1 plants. PCR was performed as described. PCR was carried out with the primers depicted.

Prospects for development of recombinant DNA technology

Development of recombinant DNA technology for manipulation of large segments of DNA is of importance for the structural and functional analysis of eukaryotic chromosomes [29,35,37,39]. The *Arabidopsis* plants that we generated are useful for a systematic examination of chromosomal deletions and inversions mediated by the R-*RS* system, because the physical distance between the two *RS* sequences on the chromosome has been precisely determined. We must further examine whether site-specific recombination in germ cells can be induced by using the transgenic lines.

Acknowledgements

References

1. Araki H, Jearnpipatkul A, Tatsumi H, Sakurai T, Ushio K, Muta T and Oshima Y. (1985) Molecular and functional organization of yeast plasmid pSR1. J Mol Biol 182: 191–203.
2. Araki H, Nakanishi N, Evans BR, Matsuzaki H, Jayaram M and Oshima Y (1992) Site-specific recombinase, R, encoded by yeast plasmid pSR1. J Mol Biol 225 25–37.
3. An G. (1987) Binary Ti vectors for plant transformation and promoter analysis. In: Wu and Grossman (eds), Methods in Enzymology. Recombinant DNA. Academic Press, Vol. 153, pp. 292–305.
4. Baker B, Coupland G, Fedoroff N, Starlinger P and Schell J (1987) Phenotypic assay for excision of the maize controlling element *Ac* in tobacco. EMBO J 6: 1547–1554.
5. Bancroft I and Dean C (1993) Transposition pattern of the maize element *Ds* in *Arabidopsis thaliana*. Genetics 134: 1221–1229.
6. Benfey PN, Ren L and Chua NM (1989) The CaMV 35S enhancer contains at least domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8: 2195–2202.
7. Broach JR (1981) The yeast plasmid 2 μm circle. In: Strathern JN, Jones EW and Broach JR (eds), The Molecular Biology of the Yeast *Saccharomyces:* life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 445–470.
8. Carroll BJ, Klimyuk VI, Thomas CM, Bishop GJ, Harrison K, Scofield R and Jones JDG (1995) Germinal transpositions of the maize element Dissociation from T-DNA loci in tomato. Genetics 139: 407–420.
9. Colleaux L, d'Auriol L, Betermier M, Cottarel G, Jacquier A, Galibert F and Dujon B (1986) Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E. coli* as a specific double strand endonuclease. Cell 44: 521–533.
10. Colleaux L, d'Auriol L, Galibert F and Dujon B (1988) Recognition and cleavage site of the intron-encoded omega transposase. Proc Natl Acad Sci USA 85: 6022–6026.
11. Dooner HK and Belachew A (1989) Transposition pattern of the maize element *Ac* from the *bz-m2* (*Ac*) allele. Genetics 122: 447–457.
12. Dooner HK, Keller J, Haper E and Ralston E (1991) Variable patterns of transposition of the maize element *Activator* in tobacco. Plant Cell 3: 473–482.
13. Fromm M, Taylor LP and Walbot V (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82: 5824–5828.
14. Furner IJ and Pumfrey JF (1992) Cell fate in the shoot apical meristem of *Arabidopsis thaliana*. Development 115: 755–764.
15. Greenblatt IM (1984) A chromosome replication pattern deduced from pericarp phenotypes resulting from movements of the transposable element Modulator in maize. Genetics 108: 471–485.
16. Greenblatt IM and Brink RA (1962) Twin mutations in medium variegated pericarp maize. Genetics 47: 489–501.
17. Golic KG and Lindquist S (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome. Cell 59: 499–509.
18. Guzman P and Ecker JR (1988) Development of large DNA methods for plants: molecular cloning of large segments of Arabidopsis and carrot DNA into yeast. Nucleic Acids Res 6: 11091–11105.
19. Irish VF and Sussex IM (1992) A fate map of the *Arabidopsis* embryonic shoot apical meristem. Development 115: 745–753.
20. Ishikawa M, Naito S and Ohno T (1993) Effects of the *tom1* mutation of *Arabidopsis thaliana* on the multiplication of tobacco mosaic virus RNA in protoplasts. J Virol 67: 5328–5338.
21. Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: β-glucuronide as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907.

22. Jones JDG, Carland F, Li E, Ralston E and Dooner HK (1990) Preferential transposition of the maize element Activator to linked chromosomal locations in tobacco. Plant Cell 2: 701–707.
23. Kato K, Matsumoto T, Koiwai A, Mizusaki S, Nishida K, Noguchi M and Tamaki E (1972) Liquid suspension culture of tobacco cells. In Terui G (ed.), Fermentation Technology Today. Soc. Ferment Technol. Japan, Osaka, pp. 689–695.
24. Kawasaki H, Matsuzaki H, Nakajima R and Oshima Y (1991) The *PHO80/TUP7* locus in *Saccharomyces cerevisiae* is on the left arm of chromosome XV: Mapping by chromosome engineering. Yeast 7: 859–865.
25. Linsmaier EM and Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100–127.
26. Machida C, Onouchi H, Koizumi J, Hamada S, Endang S, Torikai, S and Machida Y (1997) Characterization of the transposition pattern of the *Ac* element in *Arabidopsis thaliana* using endonuclease I-*SceI*. Proc Natl Acad Sci USA 85: 6022–6026.
27. Matsuzaki H, Araki H and Oshima Y (1988) Gene conversion associated with site-specific recombination in yeast plasmid pSR1. Mol Cell Biol 8: 955–962.
28. Matsuzaki H, Nakajima R, Nishiyama J, Araki H and Oshima Y (1990) Chromosome engineering in *Saccharomyces cerevisiae* by using a site-specific recombination system of a yeast plasmid. J Bacteriol 172: 610–618.
29. Medberry SL, Dale E, Qin M and Ow DW (1995) Intra-chromosomal rearrangements generated by Cre-lox site-specific recombination. Nucleic Acids Res 23: 485–490.
30. Moreno MA, Chen J, Green blatt I and Dellaporta SL (1992) Reconstitutional mutagenesis of the maize *P* gene by short-range *Ac* transpositions. Genetics 131: 939–956.
31. Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8: 4321–4325.
32. Odell J, Caimi P, Saue B and Russell S (1990) site-directed recombination in genome of transgenic tobacco. Mol Gen Genet 223: 369–378.
33. O'Gorman S, Fox DT and Wahl GM (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science 251: 1351–1355.
34. Onouchi H, Yokoi K, Machida C, Matsuzaki H, Oshima Y, Matsuoka K, Nakamura K and Machida Y (1991) Operation of efficient site-specific recombination system of *Zygosaccharomyces rouxii* in tobacco cells. Nucleic Acids Res 19: 6373–6378.
35. Onouchi H, Nishihama R, Kudo M, Machida Y and Machida C (1995) Visualization of site-specific recombination catalyzed by a recombinase from *Zygosaccharomyces rouxii* in *Arabidopsis thaliana*. Mol Gen Genet 247: 653–660.
36. Osborne BI, Corr CA, Prince JP, Hehl R, Tanksley SD, McCormick S, Baker B (1991) *Ac* transposition from a T-DNA can generate linked and unlinked clusters of insertions in the tomato genome. Genetics 129: 833–844.
37. Osborne BI, Wirtz U and Baker B (1995) A system for insertional mutagenesis and chromosomal rearrangement using the *Ds* transposon and Plant J 7: 687–701.
38. Ohta S, Mita S, Hattori T and Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol 31: 805–813.
39. Qin M, Bayley C, Stockton T and Ow AW (1994) Cre recombinase-mediated site-specific recombination between plant chromosones Proc Nat Acad Sai USA 91: 1706–1710.
40. Sauer B (1987) Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol 7: 2087–2096.
41. Scheres B, Wolkenfelt H, Willemsen V, Terlouw M, Lawson E and Dean C (1994) Embryonic origin of the Arabidopsis primary root and root meristem initials. Development 120: 2475–2487.
42. Thierry A, Perrin A, Boyer J, Fairhead C, Dujon B, Frey B and Schmitz G (1991) Cleavage of yeast and bacteriophage T7 genomes at a single site using the rare cutter endonuclease I-*SceI*. Nucleic Acids Res 19: 189–190.

43. van Schaik N and Brink RA (1959) Transposition of Modulator, a component of the variegated pericarp in maize. Genetics 44: 725–738.
44. Velten J, Velten L, Hain R and Schell J (1984) Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. EMBO J 3: 2723–2730.

Index

**Plant Molecular
Biology Manual**

Index

1

2

Buffers   F3/7, F3/8
  Buffer A   O1/11
  Buffer B   O1/11
  Buffer G   O1/11
  Buffer I   O1/6
  A Buffer   D6/6, E1/5, I1/4
  Alkaline Phosphate Buffer   F1/22
  Annealing Buffer   F4/15
  B Buffer   D6/6, I1/4
  BamHI buffer   H5/14
  Binding Buffer   F2/11
  Breaking Buffer   D6/8
  C Buffer   D6/6, I1/4
  GAT GT Buffer   C2/8
  Chromatography Buffer   I4/8
  CIAP Buffer   H1/17
  Compensation Buffer   J1/5
  CTAB Buffer   D1/8
  CTAB precipitation Buffer   D1/8
  D Buffer   D6/6, E2/8
  Dialysis Buffer   D6/8
  DMS Buffer   I2/12
  DNA Extraction Buffer   I1/6
  DNAse Buffer   D3/10
  DNase I Dilution Buffer   I3/17
  E Buffer   D6/6
  *Eco*RI Methylase Buffer   H1/17
  Electroporation Buffer   A1/13, J4/15
  Electrotransfer Buffer   F1/22
  Elution Buffer   F2/11
  EST Buffer   G5/9
  Firefly Luciferase Assay Buffer   C2/28
  Fluorescence Assay Buffer   B5/8
  Glycerol Buffer   F4/21
  Gradient Buffer   D5/11
  Grinding Buffer   I3/7, J1/18
  GUS Assay Buffer   A1/7
  GUS Extraction Buffer   A1/7, C2/17
  GUS Stop Buffer   A1/7
  HB Buffer   C2/8, H5/16
  HEPES Buffer   E1/8
  HIB Halo Isolation Buffer   D2/10
  *Hin*dIII buffer   H5/14
  Homogenization Buffer   B5/8, I2/4
  Import Buffer   J1/8
  Isoacorbate Buffer   D4/10
  Kinase Buffer   I2/5, I4/14
  Ligase Buffer   D6/16, F4/22, H1/18, I4/14
  Light scattering F-buffer (LSF buffer)   O1/13
  Loading Buffer   C1/9, F1/19
  Low-Ionic-Strength Electrophoresis Buffer I1/7
  Luciferase Assay Buffer   B2/14
  Luciferase Extraction Buffer   B2/14, C2/23
  Lysis Buffer   D6/8, J3/25
  Micrococcal Nuclease Buffer   I3/17
  Mitochondria Homogenizing Buffer   D3/11

3

10

13

16

18

Cell lysis Solution	M1/20
Coomassie Solution	D6/16
CTAB 10%	K3/15
DTT solution	O1/5
Denhardt's solution	G3/8, K3/15
Destaining Solution	D6/16
DM	L2/8
2,4-dichlorophenoxyacetic acid	J3/10, L2/6
DNA Wash Solution	H1/17
Ethidium Bromide Solution	F1/20
ESP	H5/18
FDA assay Solution	A3/12
Ficoll 5% Solution	F3/25
Ficoll 9% solution	N1/21
Ficoll 10% Solution	J3/26
5'-FOA medium	M1/25
H medium	A3/12
Gel Solution	D6/13
GTE Solution	H5/25
Hybridization Solution	E1/9, F3/17
Kanamycin	L2/13, L2/16
LCB Solution	D4/11
LCBF Solution	D4/11
Lithium acetate Solution	E3/21, M1/15
Lysis Mix Solution	D3/9
4M	G/14
Miniprep Solution I	B3/8
Miniprep Solution II	B3/8
MKC	L2/8
MKMC-B	L2/8
MKS	L2/8
MM Solution	A3/12
MMM Solution	A1/6
MSAR Stock Solutions	B2/10
MSV vitamines	L2/6
n-decanal Solution	C2/23
NAA	L2/16
Neutralization Solution	M1/20, M1/21
NIM Solution	E2/6
Nito Blue Tetrasolium Solution (NBT)	F1/22
NTP Mix	E1/8, G1/8
ONPG solution	E3/25
Paraformaldehide Fix Solution	C2/19
Pauly's Reagent Stock Solutions	C3/8
PBS Solution	G5/10
PCB Solution	D4/12
PEG Solution	A3/12, E3/21, M1/15, N3/10
Percoll Solutions	D6/8
Phenol-Chloroform Solution	B3/8, H5/15
Phenylmethyl sulfoxide (PMSF) solution	O1/6
Phosphate Saturation Solution	C1/11
Poly (dI-dC) Solution	I2/7
Ponceau S Solution	F1/22
Potassium acetate	H5/25
Prehybridization Solution	E1/9, F1/20, F3/16
Proteinase K Stock Solution	D5.8, E1/8

20