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*Communication*

# Construction and Homologous Expression of a Maize *Adh1* Based *Nco*I Cassette Vector

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## ABSTRACT

**The alcohol dehydrogenase I (*Adh1*) gene of maize (*Zea mays* L.) was employed as a source of transcriptional, posttranscriptional, and translational regulatory sequences in the construction of an expression vector. By transforming the translation-initiating ATG and an ATG three triplets upstream from the translational termination triplet into *Nco*I sites (5′-CCATGG-3′), the maize *Adh1* gene was converted into a cassette vector allowing one-step placement of any structural gene under *Adh1* regulatory control. We inserted the structural gene for chloramphenicol acetyl transferase (CAT) into this cassette vector and found that this construct expressed the *cat* gene when transfected into maize protoplasts. Significant expression was observed with a construct that contained only 146 base pairs of *Adh1* sequence upstream of the transcription-initiation site. Derivatives with a further 266 or 955 base pairs of contiguous *Adh1* upstream sequences increased CAT expression approximately 5-fold or 8-fold, respectively.**

Genetic engineering in plants requires the regulated expression of altered or added genetic traits in a targeted plant species. The most obvious and dependable sources of gene regulatory sequences for a particular plant are those associated with an appropriately regulated gene from that same plant species. In maize, the alcohol dehydrogenase I (*Adh1*) gene is expressed in a number of tissues of interest, including embryo, pollen, root, and tissue culture cells (6, 17). Although not essential under standard growth conditions, *Adhl* can be expressed at very high levels (10% of total protein synthesis) in some tissues and under anaerobic stress induction (6, 9). For these reasons of tissue specificity, inducibility, and high level expression, the maize *Adhl* gene is particularly well suited to serve as a source of regulatory sequences for maize expression vectors.

In eukaryotes, sequences in various regions both external and internal to a structural gene may significantly influence expression of that gene. In order to maximize the appropriate expression of a gene under *Adh1* regulatory sequence control, one would like to employ as much of the *Adh1* gene as is feasible and convenient. In addition, the vector should be designed to allow simple and reproducible use of the *Adh1* regulatory sequences in many different chimeric gene constructions. One approach for the assembly of a reusable, or cassette, expression vector would involve the conversion of the translation-initiation ATG of maize *Adh1* into an *Nco*I restriction site (5′-CCATGG-3′). The 5′-CCATGG-3′ sequence is a preferred translation-initiation consensus in animal cells (12) and is also fairly common in plants (10). After a similar *Nco*I conversion at or near the translation termination signal, any structural gene may be placed under maize *Adh1* regulatory control by a single ligation step.

This report describes the construction of a maize *Adh1* based *Nco*I cassette vector, pMANC1, and the one-step insertion of the chloramphenicol acetyl transferase (CAT) gene into this vector. This study also details the use of this *Adh1*-CAT chimeric construct, pMACAT, to broadly define the extent of the maize *Adh1* promoter.

## MATERIALS AND METHODS

**Biological Materials.** The bacterial strains JM83, JM103, JM105, and JM109 were used for most routine manipulations and were kindly provided by J. Messing (16). The *dam*$^-$ F$^+$ *Escherichia coli* line LL33 was obtained from the conjugation of *dam*$^-$ *E. coli* line GM33 (15) with *Salmonella typhimurium* line TL243 (14). Cell suspension cultures of Black Mexican Sweet maize line BMS No. 1 (3) were used for all protoplast transformation experiments.

**DNA Sources.** The 5′ and 3′ *Adh1* regulatory sequences used in the construction of pMANC1 were derived from the *Adh1-S* allele-containing clone, pB428 (1). The 5.8 kb of 5′ distal sequences from the *Adh1-F* allele (18) were provided by S. Hake. The CAT structural gene used in these experiments was derived from pBR325 (2). Phage vector mp10w was obtained from J. Messing (16). The *Nco*I converted pUC19 derivative pCEL80 was provided by C. Waldron. A 17-mer oligodeoxynucleotide used in converting the translation initiating ATG of *Adh1* to an *Nco*I site was obtained from G. Gustafson.

**DNA Manipulations.** Restrictions enzymes *Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II, *Sph*I, *Xba*I (Bethesda Research Laboratories [BRL]), *Nco*I, *Sfa*NI (New England BioLabs); T4 DNA ligase (BRL); S1 nuclease (BRL); and Klenow DNA polymerase (Boehringer Mannheim Biochemicals) were employed approximately as specified by the manufacturer. Conversion of the translation initiating ATG of *Adh1* to an *Nco*I site was performed as described by Hutchison *et al.* (11).

**Transient Expression Assays.** For each experiment equal numbers of protoplasts isolated from cell suspension cultures (3) were divided into aliquots and transfected with 50 μg of each plasmid DNA plus 100 μg of carrier (calf thymus) DNA by a polyethylene glycol procedure (13). Transfected protoplasts were

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harvested after 24 h and CAT activity was assayed essentially as described (8). Quantification was performed by liquid scintillation counting of the acetylated [$^{14}$C]chloramphenicol derivatives, after separation by TLC.

## RESULTS AND DISCUSSION

**Vector Construction.** An *Xba*I/*Hin*dIII restriction fragment containing the maize *Adh1-S* translation initiation sequence was subcloned from pB428 (1) into m13 vector mp10w (16) to create mpAXH10 (Fig. 1). Our first attempts to replace the *Adh1-S* 5′-CAATGG-3′ sequence with 5′-CCATGG-3′ through oligonucleotide-directed, site-specific mutagenesis (11) were unsuccessful. Since there are four repeats of the *dam* methylase recognition sequence 5′-GATC-3′ within 100 bp[3] on either side of the initiating ATG in *Adh1-S* (4), we suspected that the extensive *dam* modification on the parental mpAXH10 strand in this region would strongly bias repair of the mismatched, *in vitro* replicated mpAXH10 back to the unchanged mpAXH10 sequence. Oligonucleotide-directed, site-specific mutagenesis on mpAXH10 single-stranded DNA prepared from *dam*⁻ *E. coli* line LL33 yielded three *Nco*I-converted transformants (mpAXH11) out of 23 transformants analyzed.

The 3′ end of the maize *Adh1-S* gene contains an *Sfa*NI restriction site 20 bp upstream from the translation terminating TAG (4). Restriction enzyme *Sfa*NI digests DNA 10 bp 3′ to its asymmetric recognition sequence. At the 3′ end of *Adh1-S*, the 4 bp 5′ extensions generated by *Sfa*NI digestion are homologous to the 4 bp 'sticky ends' generated by *Nco*I (Fig. 1). Hence, the 3′ *Sfa*NI/*Sal*I fragment of *Adh1-S* was ligated into *Nco*I/*Sal*I digested pCEL80 to create pLB522. The *Adh1-S* 5′ *Eco*RI/*Nco*I fragment from mpAXH11 was then inserted into *Eco*RI/*Nco*I digested pLB522 to produce pMANC1 (Fig. 1).

[3] Abbreviation: bp, base pairs.

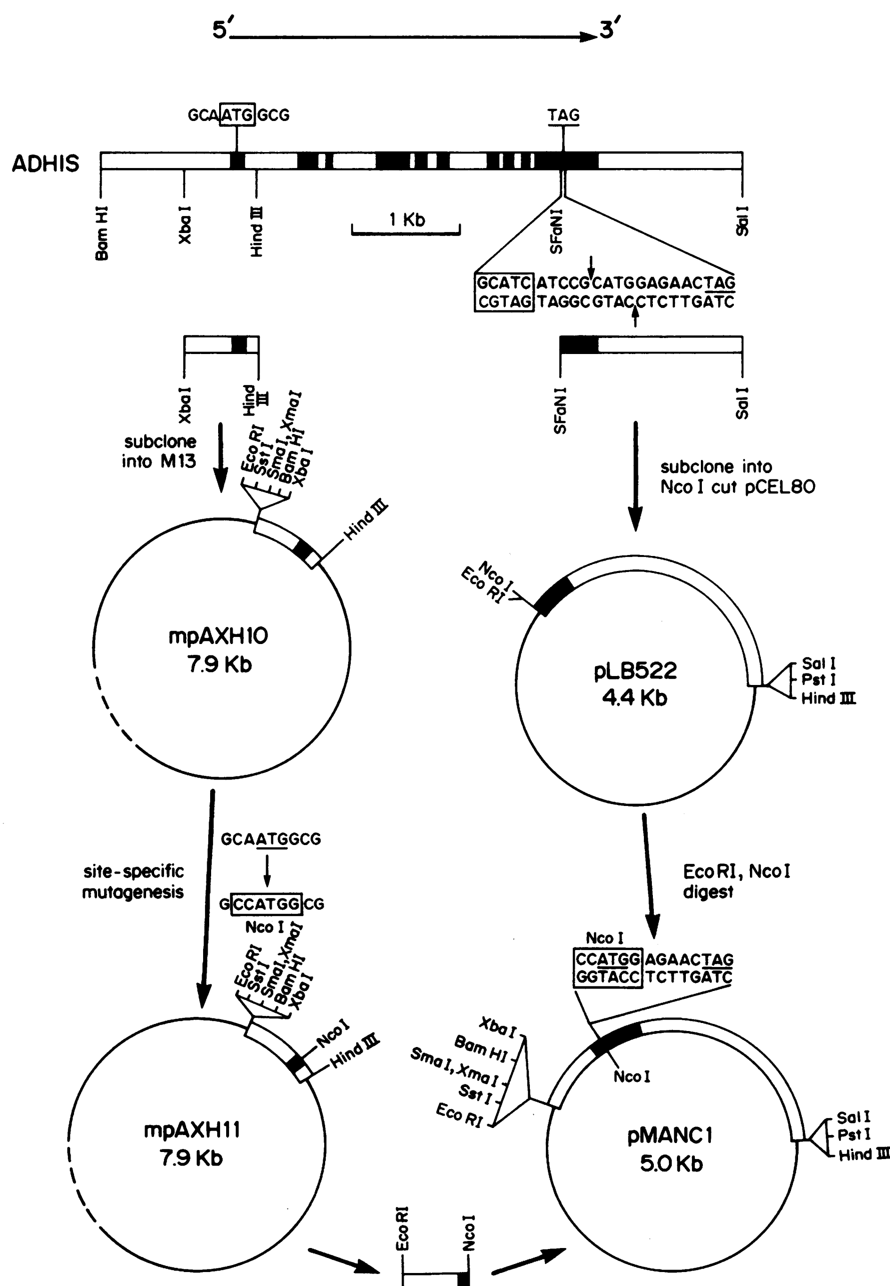


FIG. 1. Steps in the construction of the *Adh1*/*Nco*I cassette vector pMANC1. The top of the figure presents a map of the *Adh1-S* gene, its exons (shaded regions), and the mapped RNA transcript (uppermost arrow). Translational initiator and terminator sequences in *Adh1-S* are shown, as are the steps in constructing pMANC1 (described in text).

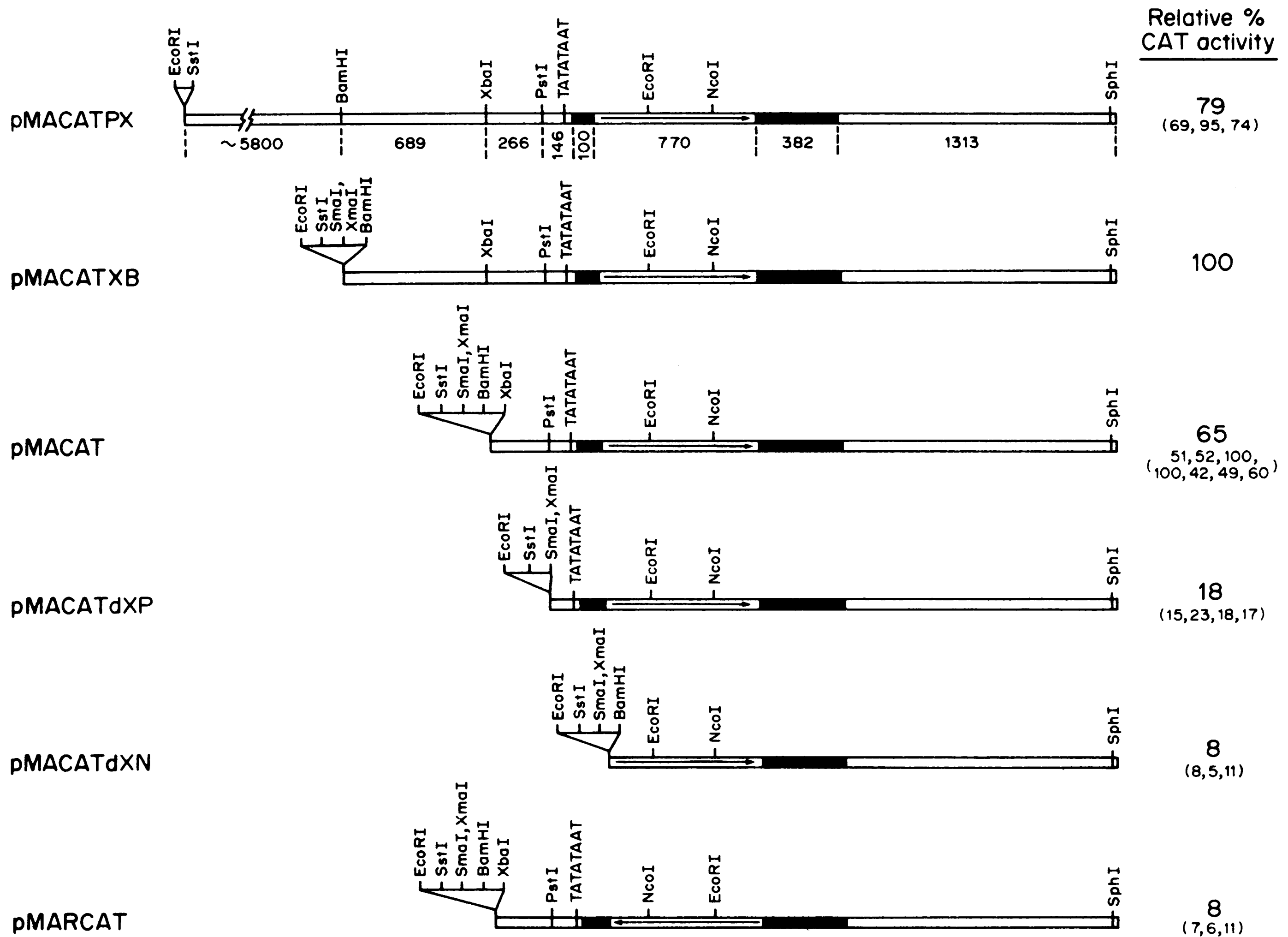


FIG. 2. Structure and activity of the pMACAT vector series. Maps of each of the pMACAT vectors are shown. These differ only in the length of *Adh1* 5′ proximal sequence included, as described in the text. The uppermost drawing of pMACATPX presents the presumptive *Adh1-S* TATA box sequence, transcribed portions of the *Adh1* gene (shaded regions), the position and orientation of the CAT structural gene (arrow), and the size in base pairs of relevant restriction fragments (numbers below the map). The construction of each of these vectors is described in the text. pMARCAT is similar to pMACAT, but with the *cat* gene inserted in the opposite orientation. CAT activities are expressed as average percent activity (relative to 100% for pMACATXB) of each vector, with the results of independent experiments in parentheses. Background incorporation averaged 6% (6%, 8%, 6%, and 4%, in separate experiments) of pMACATXB expression when extracts from mock transformed cells were assayed for CAT activity. An assay performed without any cell extract indicated apparent conversion or contamination of [$^{14}$C]chloramphenicol with a labeled compound that gave a background of 5% of pMACATXB expression.

The maize *Adh1* cassette vector pMANC1 contains 412 bp of sequence 5′ to the transcription start site in *Adh1-S*, 100 bp of mRNA leader sequence up to the translation initiating ATG, the two terminal amino acids and translational terminator TAG (all in frame), and 382 bp of mRNA trailer sequence including the polyA addition sites (4, 18). We have since further simplified pMANC1 by deletion of about 500 bp of 3′ distal sequences to create pMANC2, removal of some extraneous 5′ polylinker restriction sites to produce pMANC3, and conversion of the *Nco*I site to an *Nco*I/*Bgl*II/*Pvu*II linker to construct pMANC4 (not shown).

The best way to insert a structural gene into the pMANC derivatives would be to convert its translation initiating ATG into an *Nco*I site. The *cat* gene of pBR325 contains an internal *Nco*I site, complicating this approach. Therefore, the *Taq*I fragment of pBR325 containing the CAT gene was treated with Klenow DNA polymerase and blunt end ligated into *Nco*I digested, S1 nuclease treated pMANC2 to create pMACAT (Fig. 2). Subsequent DNA sequencing confirmed that the initiating ATG of pMANC2 had been deleted and that the first ATG downstream from the *Adh1-S* promoter and mRNA leader sequences was the translation initiating ATG of the CAT gene. An insertion of the CAT gene in the opposite orientation, pMARCAT, was also identified.

In order to define an optimum promoter fragment for our expression vector, further derivatives of pMACAT were generated by adding 689 bp of contiguous upstream *Adh1-S* sequence (Fig. 1) to create pMACATXB, adding an additional approximately 5.8 kbp of contiguous 5′ *Adh1-F* upstream sequence to pMACATXB to construct pMACATPX, removing the 266 bp *Xba*I/*Pst*I promoter fragment of pMACAT to produce pMACATdXP, and removing all 5′ *Adh1* sequences from pMACAT to create pMACATdXN (Fig. 2).

**Expression of pMACAT Vectors in Maize.** The ability of the different *Adh1* promoter constructs to drive the expression of the *cat* gene was tested in a transient expression system using maize protoplasts derived from cell suspension cultures. The relative CAT activities present in crude extracts of protoplasts transfected

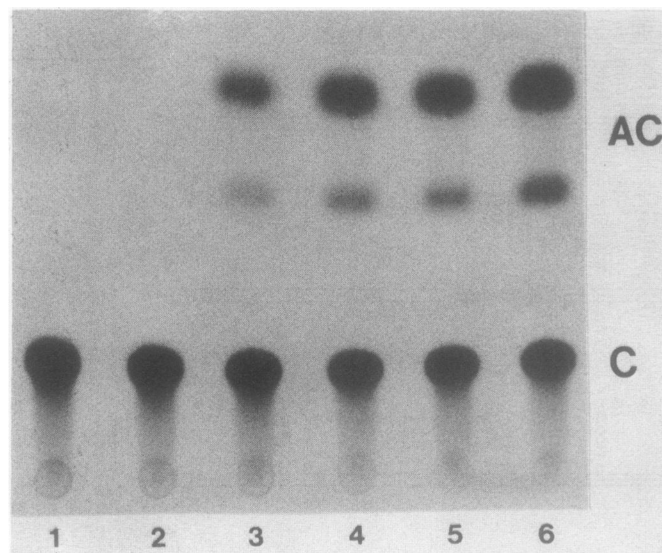


FIG. 3. Chloramphenicol acetyl transferase activity of maize protoplasts transfected with pMACAT constructs. AC, Acetylated [$^{14}$C]chloramphenicol; C, [$^{14}$C]chloramphenicol. Lane 1, pMARCAT; lane 2, pMACATdXN; lane 3, pMACATdXP; lane 4, pMACAT; lane 5, pMACAT; lane 6, pMACATXB.

with the different vectors are shown in Figure 2. In order to control for variation between protoplast preparations in transformation and construct expression efficiency, the activity of the most highly expressed vector (pMACATXB) was arbitrarily defined as 100% expression and all other vectors (in parallel samples) quantitated as a percent of pMACATXB activity (Fig. 2). The results were found to be highly reproducible between the four experiments performed (Fig. 2).

The *Adh1-S* promoter deleted vector pMACATdXN and the vector with the *cat* gene in the inverted orientation relative to the *Adh1-S* regulatory sequences, pMARCAT, did not yield CAT activity significantly above background (Figs. 2, 3). This indicates that neither the *cat* gene fragment nor the pCEL80 vector sequences employed exhibit fortuitous promoter activity in maize protoplasts.

Three separable regions of *Adh1-S* upstream from the structural gene were found to have positive effects on *cat* gene expression in these chimaeric constructs. The 246 bp region 5′ proximal to the translation initiating ATG of *Adh1-S*, containing 100 bp of mRNA leader encoding sequence, was sufficient to promote expression at a level 3-fold above background (Fig. 2). Ellis *et al.* (5) have reported that this region is adequate for anaerobic regulation of the maize *Adh1* promoter in tobacco. If one subtracts background incorporation, the 266 bp, 5′ contiguous *Xba*I/*Pst*I fragment of *Adh1-S* increased CAT activity another 5-fold. A slight increase in CAT activity, about 1.5-fold, was supplied by the 689 bp *Bam*HI/*Xba*I fragment from the 5′ end of *Adh1-S*. No further enhancement of CAT activity was generated by addition of an approximately 5.8 kbp fragment of primarily repetitive DNA (JL Bennetzen, K Schrick, S Hake, unpublished observations) found in the analogous position 5′ to the *Adh1-F* locus (18). Overall, the highest level of CAT activity observed due to *Adh1-S* promoter activity was 17-fold over background (Fig. 2).

The *Adh1* gene of maize is expressed at a particularly high level in tissue culture cells, probably due to the anaerobic nature inherent to cultured cells and the inducibility of *Adh1* by anaerobic stress (6, 9, 17). It is not clear whether the three separable regions of the *Adh1-S* promoter respond to distinct signals, for instance tissue specificity *versus* anaerobic induction, or are all components of one promoter activity. Further analysis will require more detailed deletion and point mutagenesis of pMACAT vectors followed by transformation into a maize cell system that allows accurate manipulation of the aerobic and anaerobic environments.

Besides their usefulness for analysis of the *Adh1-S* promoter, the pMANC cassette vectors may be employed to express other genes in maize. An appropriate antibiotic resistance gene placed into pMANC should create a vector allowing selection of stably transformed maize tissue culture cells (7), pollen, or embryos. In the near future, genetic engineering of maize will be facilitated by an array of cassette vectors targeting expression of introduced chimaeric genes in specific tissues and at specific times and levels in the intact plant.

**Note Added in Proof.** Since submission of this manuscript, EA Howard *et al.* (1987 Planta 170: 535–540) have reported that the first 1096 bp upstream of the transcription initiation site in *Adh1-S* are sufficient to promote expression and anaerobic induction of a chimeric *cat* construct upon electroporation of maize protoplasts.

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LITERATURE CITED

1. Bennetzen JL, J Swanson, WC Taylor, M Freeling 1984 DNA insertion in the first intron of maize *Adh1* affects message levels: cloning of progenitor and mutant *Adh1* alleles. Proc Natl Acad Sci USA 81: 4125–4128
2. Bolivar F 1978 Construction and characterization of new cloning vehicles, III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene 4: 121–136
3. Chourey PS, DB Zurawski 1981 Callus formation from protoplasts of a maize cell culture. Theor Appl Genet 59: 341–344
4. Dennis ES, WL Gerlach, AJ Pryor, JL Bennetzen, A Inglis, D Llewellyn, MM Sachs, RJ Ferl, WJ Peacock 1984 Molecular analysis of the alcohol dehydrogenase (*Adh1*) gene of maize. Nucleic Acids Res 12: 3983–4000
5. Ellis JG, DJ Llewellyn, ES Dennis, WJ Peacock 1987 Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. EMBO J 6:11–16
6. Freeling M, DC Bennett 1985 Maize *Adh1*. Ann Rev Genet 19: 297–323
7. Fromm ME, LP Taylor, V Walbot 1986 Stable transformation of maize after gene transfer by electroporation. Nature 319: 791–793
8. Gorman CM, LF Moffat, BH Howard 1982 Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2:1044–1051
9. Hageman RH, D Flesher 1960 The effect of an anaerobic environment on the activity of alcohol dehydrogenase and other enzymes of corn seedlings. Arch Biochem Biophys 87: 203–209
10. Heidecker G, J Messing 1986 Structural analysis of plant genes. Ann Rev Plant Pathol 37: 439–466
11. Hutchison CA IV, S Phillips, MH Edgel, S Gillam, P Jahnke, M Smith 1978 Mutagenesis at a specific position in a DNA sequence. J Biol Chem 253: 6551–6560
12. Kozak M 1986 Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44: 283–292
13. Krens FA, L Molendijk, GJ Wullems, RA Schilperoort 1982 *In vitro* transformation of plant protoplasts with Ti plasmid DNA. Nature 296: 72–74
14. Mahan MJ, LN Csonka 1983 Genetic analysis of the proBA genes of *Salmonella typhimurium*: Physical and genetic analyses of the cloned proB$^+$A$^+$ genes of *Escherichia coli* and of a mutant allele that confers proline overproduction and enhanced osmotolerance. J Bacteriol 156: 1249–1262
15. Marinus MG, NR Morris 1974 Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K-12. J Mol Biol 85: 309–322
16. Messing J 1983 New m13 vectors for cloning. Methods Enzymol 101: 20–79
17. Peacock WJ, ES Dennis, WL Gerlach, D Llewellyn, H Lorz, AJ Pryor, MM Sachs, D Schwartz, WD Sutton 1983 Gene transfer in maize: controlling elements and the alcohol dehydrogenase genes. *In* K Downry, RW Vollmy, F Ahmad, J Schultz, eds, Proc 15th Miami Winter Symp, Vol 20. Academic Press, New York, pp 311–326
18. Sachs MM, ES Dennis, WL Gerlach, WJ Peacock 1986 Two alleles of maize *alcohol dehydrogenase 1* have 3′ structural and poly(A) addition polymorphisms. Genetics 113: 449–467