BIOLOGIA PLANTARUM 39 (3): 361-367, 1997

Molecular analysis of genomic DNA-mediated transformation in *Zea mays*

W. SAWAHEL*

Genetic Engineering and Biotechnology Division, National Research Centre, Cairo, Egypt

Abstract

Total genomic DNA isolated from maize hygromycin B resistant cell line (hyg^r-G204) was used to transform the maize hygromycin B sensitive cell line (hyg^s-G204) to the hyg^r-phenotype using polyethyleneglycol treatment and the transformed calli were selected using hygromycin B. The primary transformant maize plants were regenerated and analysed at the molecular level using DNA hybridization, transgenome rescue and histochemical β-glucuronidase assay. The results indicated that genomic DNA-mediated transformation can lead to transfer, expression and stable integration of a DNA fragment into the host genome.

Additional key words: marker genes expression, protoplast transformation, segregation pattern, transgenome rescue.

Introduction

Plant genetic transformation technology provides a means of transferring economically-important genetic traits from any organism into a plant (Sawahel 1994). This is important particularly for crop plants in which the genomes are already fine tuned by centuries of breading. However, the application of such technology to rational plant improvement is currently limited by a shortage of cloned genes for important traits (Sawahel and Fukui 1995).

In the last years, several attempts have been performed to elicit plant genetic transformation using donor genomic DNA with the aim to transfer uncloned genes encoding molecularly-uncharacterized phenotypes (Sawahel and Cove 1992). Some

Received 30 May 1996, *accepted* 4 September 1996.

Abbreviations: amp^r - ampicillin resistant; CaMV - cauliflower mosaic virus; GUS - β-glucuronidase; hyg^r - hygromycin B resistant; hyg^s - hygromycin B sensitive; *hpt* - hygromycin phosphotransferase gene; PEG - polyethyleneglycol; R0 - primary transformant plant.

Acknowledgements: The author would like to thank Prof. M.Bevan (PBI, Cambridge, U.K.) for providing pBI221.23, the Agricultural Research Centre (Cairo, Egypt) for providing maize varieties, and Dr. G. Sastry (Leeds University, U.K.) for providing *E. coli* DH10B strain.

* Recent address: International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi - 110 067, India. Fax: (+91-11)-6162316.

of these attempts proved to be successful (Ohta 1986) and the others were unclear and contentious because of the lack of an effective molecular marker, the poor reproducibility (Ledoux 1977, Ledoux *et al.* 1974, Wigler *et al.* 1978, Soyfer 1980, Sanford *et al.* 1985) as well as the doubt on the possibility of gene-specific, directed, and inheritable changes in higher plant in the course of foreign-genomic DNA treatment.

This paper describes a well-defined strategy for genomic DNA-mediated transformation and rescue of the transgenome. In addition, it demonstrates that donor genomic DNA can be expressed and stably integrated into the host genome.

Materials and methods

Plants: A stable transgenic line of *Zea mays* L. (hyg^r-G204), which contains the hygromycin phosphotransferase gene (*hpt*) and the β-glucuronidase reporter gene (*GUS*) under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (pBI 221.23) in its genome (Fig. 1), was used as a donor for genomic DNA. The hyg^s-G204 was used as a genomic DNA-recipient cell line.

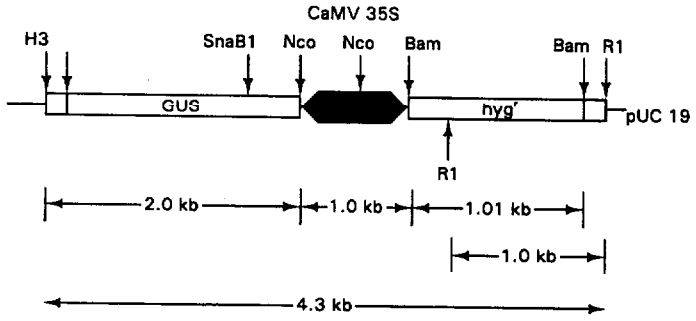

Fig. 1. Schematic representation of pBI221.23 plasmid.

Isolation of genomic DNA: Genomic DNA was prepared by a miniprep method (Dellaporta *et al.* 1983) and sheared through an 18-gauge needle. Pulsed gel electrophoresis (Schwartz and Cantor 1984) showed that most of the resulting DNA fragments were in the 25-45 kbp range.

Genomic DNA-mediated transformation: Plant genomic DNA transfer experiments were performed using PEG protocol as essentially described by Negruitu *et al.* (1987). After PEG treatment, the protoplasts were washed and resuspended in culture medium.

Protoplast culture, selection and regeneration: The culture media and procedures used were according to Shillito *et al.* (1989). After 24 d culture, transformed calli were selected on medium supplemented with 30 μg cm^{-3} hygromycin B as a selective agent.

Histochemical *GUS* assay: Expression of the *GUS* gene was detected according to Jefferson (1989) by supplying transformed calli with the chronogenic substrate of the glucuronidase enzyme, 5-bromo-4-chloro-3-indolyl-β-glucuronic acid. For pollen *GUS* assay, free pollen was shaken into a Petri-dish containing the *GUS* buffer and substrate. Great care was taken to prevent any extraneous tissue, *i.e.* anther, petal, *etc.*, from contaminating the pollen assay. Any such contamination could yield false positive results under certain conditions. Pollen was incubated at 37 °C for up to 4 h, then scored for the presence of *GUS* activity.

Southern blot analysis: After genomic DNA digestion with restriction enzymes, the DNA was separated by electrophoresis in a 0.7 % (m/v) agarose gel, denatured and transferred to nitrocellulose memberane according to standard procedures (Sambrook *et al.* 1989) and hybridized with the gene probe labelled with ^{32}P by nick-translation.

Transgenome rescue: Maize genomic DNA from untransformed plants, transformed plants and plasmid DNA pBI221.23 were used to transform electro-competent *E. coli* DHI0B cells with a Bio-Rad gene pulser (Sawahel *et al.* 1993). The DNA treatments (*Hind III* or *BamHI* cut only or after religation) were used. The electroporated *E. coli* cells were plated on L-broth medium using a selection by ampicillin (100 μg cm^{-3}). The rescued transgenome was analysed by restriction analysis.

Results

Genomic DNA-mediated transformation: Maize (hyg^r-G204) genomic DNA used in the transformation experiments was previously marked with the bacterial hygromycin phosphotransferase (*hpt*) after PEG-mediated transformation of (hyg^s-G204) protoplast with pBI221 23.

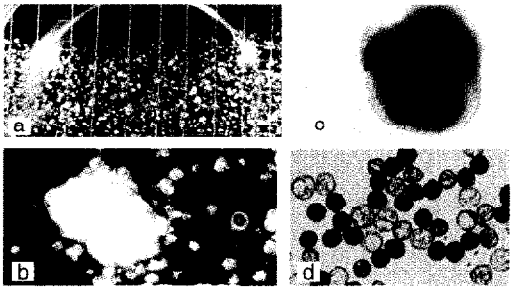

Fig. 2. Expression of the selectable and screanable marker genes: *a*) growth of PEG-treated protoplasts in non-selective medium, *b*) growth of protoplast-derived colony in selective medium, *c*) transformed callus expressing *GUS* activity, *d*) pollen, from R0 transformant, expressing *GUS* activity.

PEG treatment mediated genomic DNA transfer (Table 1) and good level of *GUS* gene activity was detected (Fig. 2*c*). Recovery of stable hyg^r-transformants, after both genomic and plasmid DNA transformation, was carried out by allowing treated

protoplasts to grow in non-selective medium for 24 d (Fig. 2*a*) before small 4-mm microcalli were randomly picked to selective medium containing 30 μg cm^{-3} hygromycin B (Fig. 2*b*). The conditions under which the primary transformants (R0) were regenerated (from hyg^r protoplast-derived colonies), transferred to the greenhouse and maintained, were the same as those reported by Shillito *et al.* (1989).

Table 1. Genomic DNA-mediated transformation frequency (number of tranformants per number of protoplasts) and efficiency (number of tranformants per mg DNA).

Treatment	Transformation frequency	Transformation efficiency
PEG	0	0
Genomic DNA	0	0
PEG + pBI221.23	0.1	1.0
PEG + genomic DNA	0.3	0.5

Marker genes expression in primary transformant: At this point, pollen and seeds of R0 plants were used to examine the expression of the screanable *GUS* and the selectable *hpt* marker genes, respectively. It was found that pollen expressed *GUS* activity and a segregation pattern of approximately 1:1 was detected (Fig. 2*d*). In addition, seeds harvested from one R0 plant and germinated on hormone-free Murashige and Skoog (1962) medium (MS) solidified with 0.6 % (m/v) agar and containing 30 μg cm^{-3} hygromycin B, produced seedlings which remained green after 21 d, *i.e.* seeds expressed *hpt* marker gene.

Molecular confirmation of the transformation event: The foreign transgenome can only be rescued in *E. coli* after *HindIII* or *BamHI* digestion followed by re-ligation,

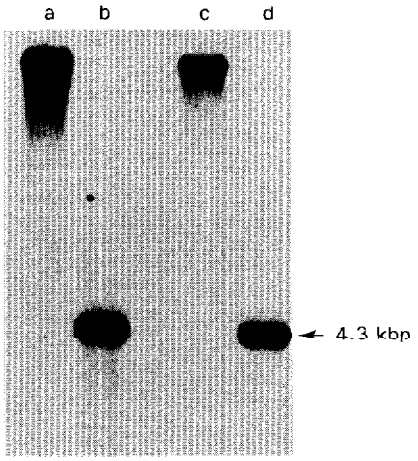

Fig. 3. Southern blot analysis of DNA from stable transformants probed with linerized pBI221.23. Lanes *a, c* - uncut DNA from original and produced transformed clones, respectively, lanes *b, d* - *HindIII*-digested DNA from original and produced transformed clones, respectively.

i.e. integrated plasmid molecule may be excised as linear fragments would result in circular molecules that would be transformed at a much higher efficiency than linear molecules. Digested and undigested genomic DNA from transformed and untransformed maize plants produced no *E. coli* transformants. This excludes the possibility that genomic DNA was contaminated with plasmid DNA. Although the restriction pattern of one of the rescued plasmids and pBI221.23 which had not been passed through maize was identical (data not shown), the possibility that some rearrangements and linkage between plasmid DNA and maize genomic sequences had occurred and that these plasmids were not rescued can not be discounted. This is because in plasmid rescue experiments, only those DNA molecules which retained a competent origin of replication and a functional antibioic (*amp*^{r}) gene through passage in maize cells would have been recovered, so the possibility exists that DNA rearangements and maize flanking sequences render a substantial proportion of the plasmid DNA incapable of transforming *E. coli*.

Hybridization pattern of DNA from hyg^r stable transformants probed with linerized pBI221.23 (Fig. 3). Lanes *a* and *c* migrate with the high molecular mass DNA and this is consistent with that predicted for uncut DNA. Lanes *b* and *d* show hybridization signals which are indicative of integration of plasmid DNA sequences into maize chromosomal DNA. No maize genomic sequences flanking the site of integration were detected. It is possible that the flanking sequences are of similar size and that 4.3 kb signal represents both or they are of high molecular mass.

Discussion

Genomic transformation approach described here is reminiscent of work carried out during the last thirty years. This work has been proved to be unclear especially at the molecular level because of the poorly-defined genetic systems used which were far from ideal. Soyfer and Titow (1981) have suggested that this approach might be explained in a number of ways: *In situ* DNA regulatory action, DNA mutagenic action, specific DNA action both at the chromosomal and cytoplasmic levels, transposon-like effect, action of extrachromosomal DNA elements (exosome, episome, plasmid, minichromosome) or unspecific DNA action.

However, the present study enabled us to evaluate the potential use of a well-defined genomic DNA as a source for uncloned genes and to understand further the molecular basis of genomic transformation. In addition, it demonstrated that the transfer of genomic DNA can result in stable transformation. Thus, there is no intrinsic feature of genomic DNA transformation process that precludes stable integration and expression of foreign transgenome. Furthermore, the foreign transgenome can be rescued in *E. coli*, *i.e.* the mobility of the transgenome in and out of the host genome can be controlled.

It is possible to transfer, identify and isolate a dominant plant gene by co-transferring the total genomic DNA and a plasmid carrying a selectable marker into a host plant cell using direct gene transfer, followed by gene rescue (Perucho *et al.* 1980, Gallois *et al.* 1992, Gems *et al.* 1994, Sawahel and Fukui 1995). The

advantage of the rescue approach rests largely in its simplicity; library construction and screening are not required. This approach requires the maintenance of a competent origin of replication and a functional antibiotic resistance gene through multiple passages in plant cell. Kuspa and Loomis (1992) have also demonstrated that the combination of gene rescue and insertional mutagenesis, by restriction enzyme mediated integration of the plasmid, has allowed the tagging of developmental genes as well as cloning of the disrupted gene.

Genetic transformation using genomic DNA which contains a defined molecular markers might have major applications for crop improvement. This is because it would be possible to use this approach for gene identification and cloning which is a major limitation of current plant improvement programmes *via* genetic engineering.

References

Dellaporta, S., Wood, J., Hicks, J.: A plant DNA minipreparation: Version II. - Plant mol. Biol. Rep. 1: 19-21, 1983.

Gallois, P., Lindsey, K., Malone, R., Krers, M., Jones, M.: Gene rescue in plants by direct gene transfer of total genomic DNA into protoplasts. - Nucl. Acids Res. **20**: 3977-3982, 1992.

Gems, D., Aleksenko, A., Belenky, L., Robertson, S., Ramsden, M.: An "instant gene bank" method for gene cloning by mutant complementation. - Mol. gen. Genet. **242**: 467-471, 1994.

Hess, D.: Cell modification by DNA uptake. - In: Bajaj, Y.P.S. (ed.): Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture. Pp. 506-577. Springer-Verlag, Berlin - Heidelberg - New York - Tokyo 1977.

Jefferson, R.: The *GUS* reporter gene system. - Nature **342**: 837-839, 1989.

Kuspa, A., Loomis, W.: Tagging developmental genes in *Diclyostelium* by restriction enzyme-mediated integration of plasmid DNA. - Proc. nat. Acad. Sci. USA **89**: 8803-8807, 1992.

Ledoux, L., Huart, R., Jacobs, M.: DNA-mediated genetic correction of thiamineless *Arabidopsis thaliana*. - Nature **249**: 17-21, 1974.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Negruitu, I., Shillito, D., Potrykus, I., Biasini, E., Sala, F.: Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. - Plant mol. Biol. **8**: 363-373, 1987.

Ohta, Y.: High-efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA. - Proc. nat. Acad. Sci. USA **83**: 715-719, 1986.

Perucho, M., Hanahan, O., Lipsich, L., Wigler, M.: Isolation of chicken thymidine kinase gene by plasmid rescue. - Nature **285**: 207-210, 1980.

Sambrook, J., Fritsch, E., Maniatis, I.: Molecular Cloning: A Laboratory Manual. - Cold Spring Harbor Laboratory, Cold Spring Harbor 1990.

Sanford, J., Skubik, K., Reisch, B.: Attempted pollen-mediated plant transformation employing genomic donor DNA. - Theor. appl. Genet. **69**: 571-574, 1985.

Sawahel, W.: Transgenic plants: performance release and containment. - World J. Microbiol. Biotechnol. **10**: 139-144, 1994.

Sawahel, W., Cove, D.: Gene transfer strategies in plants. - Biotechnol. Adv. **10**: 393-412, 1992.

Sawahel, W., Fukui K.: Gene cloning in plants: innovative approaches. - Bio/Techniques **19**: 106-113, 1995.

Sawahel, W., Sastry, G., Knight, C., Cove, D.: Development of an electro-transformation system for *E. coli* DHIOB. - Biotechnol. Techniques **7**: 26 1 -266, 1993.

Schwartz, D., Cantor, D.: Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. - Cell **37**: 67-75, 1984.

Shillito, R., Carswell, G., Johnson, C., DiMaio, J., Harms, C.: Regeneration of fertile plants from protoplasts of elite inbred maize. - Bio/Technology **7**: 581-588, 1989.

Soyfer, V.: Hereditary variability of plants under the action of exogenous DNA. - Theor. appl. Genet **55**: 225-235, 1980.

Soyfer, V., Titow, Y.: The absense of a definite gene-specific effect in wheat after seed treatment with exogenous DNA. - Mol. gen. Genet. **182**: 361-363, 1981.

Wigler, M., Pellicer, A., Silerstein, S., Axel, R.: Biochemical transfer of single copy eukaryotic genes using total cellular DNA as donor. - Cell **14**:725-731, 1978.