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A family of binary gene vectors with low inter-transformant variation

Bernd Reiss[1,*], Csaba Koncz[1,2], Ian Moore[1] and Jeff Schell[1]

[1]Max-Planck-Institut für Züchtungsforschung, Carl von Linne Weg 10, D-50829 Köln, F.R.G. [2]Institute of Plant Physiology, Biological Research Center of Hungarian Academy of Sciences, H 6701 Szeged PO.Box 521, Hungary

ABSTRACT

We have constructed a family of binary vectors for use in Agrobacterium-mediated transformation of plants. These vectors yield transformants with high efficiency and lead to remarkably reproducible expression of co-transferred genes. This low degree of inter-transformant variation is most likely due to the combination of a highly efficient selectable marker gene, methotrexate resistance, with its localisation close to the left T-DNA border. Both, a Cauliflower Mosaic Virus 35S promoter and the light regulated Rubisco small subunit promoter, which exhibit a high degree of variation with other vector systems, gave reproducible levels of expression between transformants with these vectors. Several useful features such as a poly-linker, additional marker or reporter genes (kanamycin, hygromycin, ß-glucuronidase), and an expression cassette were added to the basic vector.

INTRODUCTION

A general approach used to study regulatory or developmental processes in plants relies on the re-introduction of genes modified in vitro into the plant genome. The last step, transformation, is dependent on the availability of suitable transformation vectors and selectable marker genes. Despite the development of techniques allowing direct uptake of naked DNA, the highest frequencies are still obtained by Agrobacterium-mediated transformation of tobacco. Although a variety of Ti-plasmid based vectors and selectable markers already exist, their use is still somewhat restricted because most of the current vectors contain either kanamycin or hygromycin resistance genes as selectable markers (Reviews: An et al., 1988; Walden et al., 1990). This is a problem for example in experiments which involve several transformations of the same plant material in sucession. In order to provide an addition to the repertoire of existing vectors, we constructed a family of binary vectors based on a methotrexate (MTX) resistance gene as the dominant selectable marker.

Dihydrofolate reductase (DHFR) catalyses the formation of tetrahydrofolate, a compound essential in nucleotide metabolism. The addition of methotrexate to sensitive cells leads to cell death by thymidilate starvation as a result of the inhibition of DHFR activity. This inhibition is efficiently overcome by a methotrexate resistant dhfr gene isolated from mouse. Therefore, MTX provides a tight selection in mammalian (Simonsen and Levison, 1983) and plant cells (Eichholtz et al., 1987).

*Author to whom correspondence should be addressed

Expression levels of transgenes in a population of transgenic plants may vary considerably, a phenomenon generally referred to as inter-transformant variation. Systems for selection which provide borderline selectibility are likely to contribute to this variability. Such systems may favor the selection of abnormal integration events. These could affect the expression of the co-transferred gene in a quantitative manner. Therefore, an efficient selection system like MTX selection is probably one of the prerequisites to obtain reproducible expression of transgenes.

Another factor which might be considered is the location of the selectable marker gene in the vector. Many current vectors contain the selectable marker gene at the right border end of the vector (Reviews: An et al., 1988; Walden et al., 1990). However, the transfer of the T-DNA is initiated at the right border, proceeds through the T-DNA and is terminated at the left border (Zambryski et al., 1989). Consequently, any disturbance during the transfer process would lead to integrated T-DNA's that are missing parts of the left end, but still contain sequences located close to the right end of the T-DNA. Therefore, in vectors carrying the selectable marker at the right border, deletions affecting the cloned genes could be relatively frequent.

Taking these considerations into account, a vector for use in Agrobacterium-mediated transformation was constructed in which the MTX resistance marker gene was inserted close to the left border of the transferred T-DNA. Two well characterised promoters: 1.) the CaMV 35S promoter (Guilley et al., 1982) and 2.) the promoter of the small subunit of Rubisco (SSU) gene ss3.6 from pea (Herrera-Estrella et al., 1984) which is regulated in a light and chloroplast-associated manner, were analysed with this vector system. Both promoters exhibited considerable inter-transformant variation using other vector systems for transformation. Hundreds of fold variation in expression levels were described for the SSU (Jones et al., 1985; Dunsmuir et al., 1983) and CaMV 35S (Hobbs et al., 1990) promoters. In contrast, very little variation of expression levels was observed in transformants obtained using our vector system.

MATERIALS AND METHODS

Recombinant DNA techniques: Standard techniques were used according to procedures described by Sambrook et al. (1989).

Construction of the methotrexate resistance gene: pFR400 (Simonsen and Levinson, 1983) contains a MTX resistant dhfr gene from mouse. A plant gene-expression cassette consisting of the CaMV 35S promoter and polyadenylation site separated by a poly-linker is available in plasmid pDH51 (Pietrzak et al., 1986). pFR400 was digested with SacII, the ends rendered blunt by filling in with E. coli polymerase Klenow fragment and the resulting product digested by XbaI. The fragment containing the dhfr gene was isolated and inserted in plasmid pDH51 linearised with SphI and from which 3' overhanging ends were removed by Klenow fragment and which was redigested by XbaI. This step allowed the insertion of the dhfr gene between the 35S RNA promoter and polyadenylation site in the orientation required for expression of the dhfr gene. In order to reduce the number of restriction sites in the marker gene, pDHFR-1 was constructed. The recognition sequences for XbaI, BamHI, and SmaI were removed by linearisation with XbaI, filling in, redigestion with SmaI, and religation. pDHFR-1 contains the MTX resistance marker cassette on an EcoRI fragment cloned in pUC18. To obtain pDHFR-11 and pDHFR-12, pDHFR-1 was digested with EcoRI, the ends were filled in, BglII linkers were added, and the 35S RNA promoter/dhfr gene expression unit cloned in the BglII site of a derivative of pUC18 obtained by the insertion of a BglII linker next to the BamHI site. In pDHFR-11 and pDHFR-12 (Fig. 1), the MTX marker is inserted in opposite orientations with regard to the polylinker.

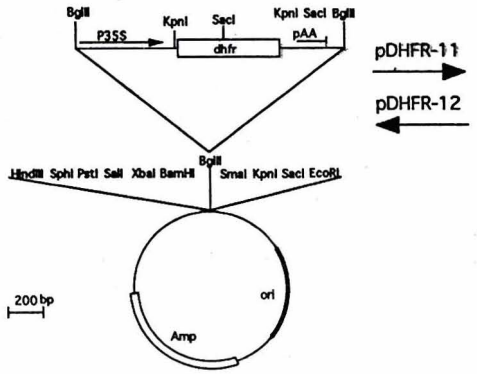

Fig. 1: The methotrexate resistance marker.
The restriction map indicates all sites useful for cloning. The CaMV 35S promoter (P35S), the dhfr gene (dhfr), and polyadenylation signal (pAA) are indicated. The ampicillin resistance gene (Amp) and the origin of replication in pUC18 are marked. Arrows below plasmid names indicate the orientation of the methotrexate resistance gene relative to the polylinker.

Construction of binary vectors: Plasmid pM001was obtained in two steps. First, the BclI site close to the right border in pPCV603 (Koncz et al., 1989) was filled in by Klenow polymerase fragment, a HindIII linker was added, then sequences between the EcoRI site (coordinate 0 in the pBR322 sequence (Sutcliffe, 1978) and the modified BclI site were replaced by an EcoRI/HindIII fragment containing the polylinker from M13mp18 (Yanisch-Peron et al., 1985). In the second step, the methotrexate resistance gene was introduced into this plasmid. For this purpose, pDHFR-1 DNA was digested with EcoRI, the ends were filled in with Klenow polymerase fragment and NdeI linkers added. The EcoRI digested and NdeI linker containing pDHFR-1 and the BglII digested pDHFR-11 plasmids were cut with BstXI and a NdeI/BstXI fragment corresponding to the amino-terminal half and a BglII/BstXI fragment corresponding to the carboxy-terminal half were inserted into the pPCV603 derivative cut by NdeI and BglII. This step resulted in the replacement of pBR322 sequences between nucleotides 1666 and 2297 with the 1.2 KB methotrexate marker gene in an orientation in which the dhfr gene is transcribed towards the left T-DNA border.

To obtain pMN001, the NPT II gene from pKm4 (Reiss et al., 1984a) was isolated as a BamHI/SmaI fragment, fused to the ocs polyadenylation sequence isolated as a PvuII/HindIII fragment from plasmid pAGV40 (Herrera-Estrella et al., 1983) and inserted between the BamHI and HindIII sites of pM001. Plasmid pMG001 was obtained by the insertion of the BamHI/EcoRI fragment from pBI101 (Jefferson et al., 1987) containing the GUS gene and polyadenylation signal between the BamHI and HindIII sites of pM001 after filling in the EcoRI site and adding HindIII linkers.

Plasmid pMG002 was obtained by insertion of the 35S promoter from pDH51 as an EcoRI/BamHI fragment into the corresponding sites of pMG001.

Plasmid pMH001 was constructed by the exchange of a HindIII/XhoI fragment from pM001 containing the right border and adjacent Ti-plasmid sequences for a HindIII/XhoI fragment from pPCV5013Hyg (Koncz et al., 1989) that contains the identical Ti-plasmid sequences connected to a hygromycin gene under the control of the promoter of the nopaline synthase gene.

To construct pMEX001, pM001 was linearised with HindIII, the ends were filled in with Klenow enzyme, and the DNA was digested with EcoRI. Plasmid pDH51 was linearised with SacI, the ends were rendered blunt with Klenow enzyme, and the DNA digested with EcoRI. The EcoRI/SacI fragment containing the CaMV 35S promoter and polyadenylation signal from pDH51 was isolated and inserted into the corresponding sites of pM001.

Plasmid pMN024 that contains the NPT II gene under the control of the SSU gene 3.6 promoter was constructed by inserting into the EcoRI and BamHI sites of pMN001 the 970 bp EcoRI/HindIII SSU promoter fragment (Herrera-Estrella et al., 1984) after filling in the HindIII ends and adding BamHI linkers.

Tissue culture and plant transformation: Binary vectors were mobilised by conjugation from E. coli to Agrobacterium tumefaciens strain GV3101 pMP90RK (Koncz and Schell, 1986). Tobacco leaf disk infection with Agrobacterium, plant tissue culture and regeneration were according to published methods (Marton et al., 1982; De Block et al., 1984; Marton, 1984; Horsch et al., 1985). Transformants were selected on media containing 0,5 mg/l methotrexate or 15 mg/l hygromycin.

Determination of NPT II activity: Protein extracts were prepared as described by Schreier et al. (1985) and separated by non-denaturing polyacylamide gel electrophoresis. NPT II activity was determined in situ as described by Reiss et al. (1984b).

Determination of GUS activity: Protein extracts were prepared and GUS activity determined using the fluorogenic assay protocol as described by Jefferson et al. (1987).

Results and Discussion

Construction of the chimeric dhfr gene.

A methotrexate resistance marker modified for the use in plant cells was obtained by the insertion of the mouse methotrexate resistant dhfr gene from pFR400 (Simonsen and Levinson, 1983) into a plant expression cassette (Pietrzak et al., 1986) containing the CaMV 35S promoter and polyadenylation signal. This plasmid was modified by eliminating a number of restiction sites located within the marker gene and by adding poly linkers at the ends of the cassette (Fig. 1).

Binary vectors with the methotrexate marker.

Plasmid pPCV603 (Koncz et al., 1989) that contains the T-DNA borders, replication functions and an ampicillin (Amp) resistance marker for selection in E. coli and Agrobacterium was modified by the introduction of the methotrexate resistance gene between the left border and the pBR322 replicon. Sequences at the right border were replaced by a poly-linker resulting in plasmid pM001 (Fig. 2). Several genes were added to this basic construct. These include promoterless reporter genes like neomycin phosphotransferase II (NPT II) (Beck et al., 1982) and ß-glucuronidase (GUS) (Jefferson et al., 1987), an independent selectable marker gene: hygromycin resistance (Van den Elzen et al., 1985), or a plant expression cassette. A compilation of these plasmids is given in figure 2.

Efficient plant transformation by PM vectors.

In order to compare MTX selection with more commonly used selection regimes, tobacco leaf disks were transformed with Agrobacteria harbouring pMH001. After infection, leaf disks were divided and selection was started on either methotrexate or hygromycin containing media. In these experiments, the first shoots appeared after 4 to 6 weeks of selection on hygromycin. At this point, the leaf disks on methotrexate had a brownish appearance, but shortly thereafter, calli appeared on these tissues and started to green. In total, several hundred calli were generated on methotrexate and hygromycin. The number of calli or mini shoots obtained in each selection was evaluated after 8 weeks of selection. On average, after hygromycin selection 3.5 calli and after methotrexate selection 3 calli per leaf disk were scored. Therefore, selection for methotrexate resistance was as efficient as with established markers like hygromycin. However, selection for methotrexate resistance required more time.

To analyse the frequency of co-transfer of an unselected gene, plants obtained by either hygromycin or methotrexate selection were regenerated and tested for resistance to both selective agents. 93% of the plants selected on hygromycin were able to form roots on methotrexate and 89% of the plants resulting from methotrexate selection formed roots on hygromycin. There was therefore no significant difference in co-transfer frequencies depending on the selection regime.

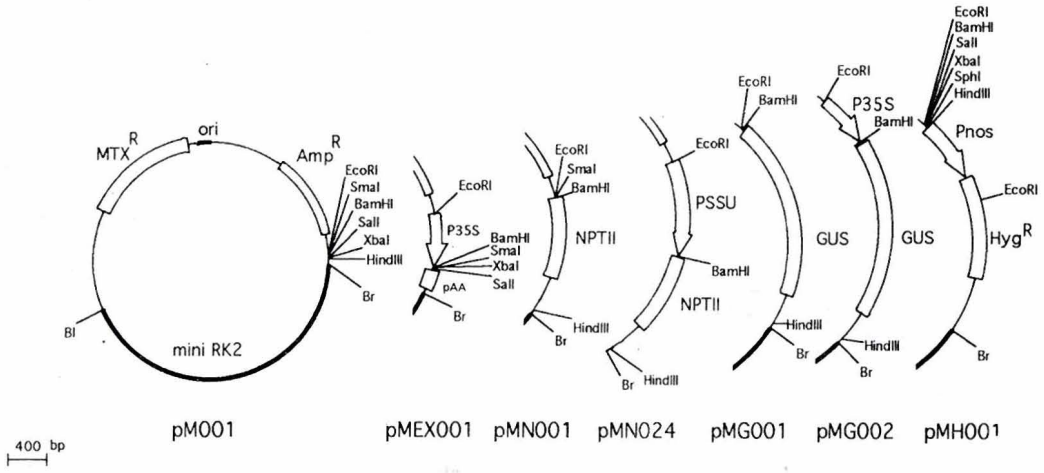

Fig. 2: The family of binary vectors.
All restriction sites included in the map are unique and can be used for cloning. The pBR322 derived origin of replication (ori) and the RK2 derived replication and transfer functions necessary for propagation in Agrobacterium (mini RK2) are marked with bold lines. Ampicillin resistance (Amp^R), methotrexate resistance (MTX^R), neomycin phosphotransferase II (NPTII), ß-glucuronidase (GUS), and hygromycin resistance (Hyg^R) genes are boxed. The CaMV 35S (P35S), Rubisco SSU (PSSU), and nopaline synthase (Pnos) promoters and the CaMV polyadenylation signal (pAA) are indicated. Positions of the right (Br) and left (Bl) borders of the T-DNA are given.

In conclusion, this series of transformation experiments demonstrated that the plasmids are highly efficient plant transformation vectors. The experiments described here were performed with tobacco SR1 and W38, however also N. plumbaginifolia and A. thaliana have been transformed with these vectors (unpublished results).

Quantitative examination of expression levels of co-transferred reporter genes.

In order to test whether the newly constructed vectors indeed lead to reduced inter-transformant variation, two well characterised promoters which exhibited a high level of variability using other vectors were analysed in the PM system.

Plasmid pMG002 contains the CaMV 35S promoter connected to the GUS reporter gene. Tobacco leaf disks were transformed by Agrobacteria harbouring this construct and plants were selected on methotrexate containing media. GUS activity was determined in leaves of individual plants which formed roots on methotrexate. As shown in figure 3, enzymatic activity in all of the 6 transformants

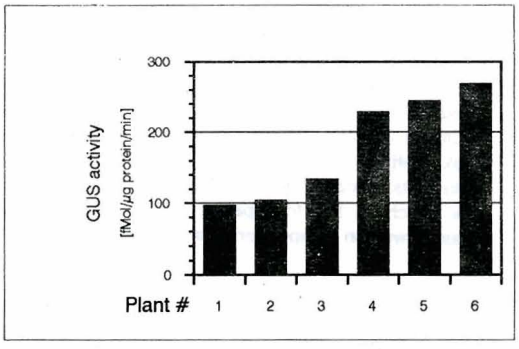

Fig. 3: GUS activity in SR1 leaves after transformation with pMG002.
GUS activity in protein extracts from leaves of six of the transformants obtained by methotrexate selection was determined using the fluorogenic enzymatic assay.

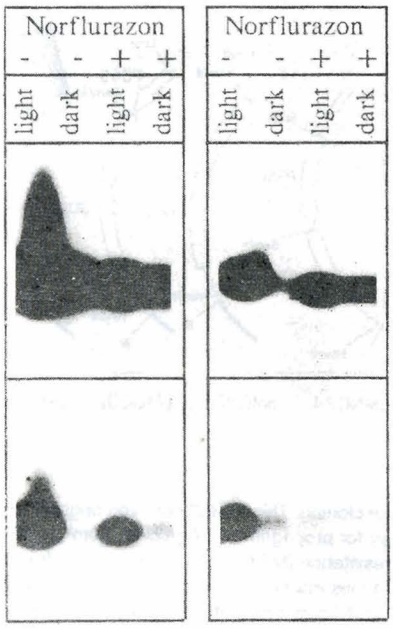

Fig. 4: Regulation of the SSU promoter by light and the presence of functional chloroplasts.
Extracts were prepared from leaves of plants grown in light or kept in the dark for 7 days and proteins separated by non-denaturing gel electrophoresis. NPTII activity was determined in situ. To inhibit the development of functional chloroplasts, plants were treated for several weeks with Norflurazon. Extracts were prepared from white parts of leaves grown in light or after 7 days in the dark. Each of the four panels represent the results obtained with an independent transformant.

expressing the GUS gene was very reproducible. Variation in expression levels was at most about three-fold between different transformants. In contrast, Hobbs et al. (1990) found a 200-fold variation in GUS expression with the same promoter when analysed in another vector system.

This result was confirmed with the Rubisco SSU promoter connected to another reporter gene, NPTII. The SSU promoter is regulated by light and the presence of functional chloroplasts (Herrera-Estrella et al., 1984). Chloroplast-dependent expression can be tested by herbicide treatment (Simpson et al., 1986). Plants selected on methotrexate and transformed with a construct carrying the ss3.6 promoter upstream of the NPT II gene were tested for NPT II activity in the leaves after growth in light or after 7 days in continuous dark with and without Norfluorazon treatment (Fig. 4). Although the expression of NPT II was not quite as homogenous as that obtained with the 35S promoter, inter-transformant variation in this case was also considerably lower than described earlier (Jones et al., 1985; Dunsmuir et al., 1983).

In conclusion, an efficient plant transformation vector was constructed which leads to reproducible expression of transgenes. Low inter-transformant variation observed with this vector system may be attributed to the combination of the highly efficient MTX resistance marker gene with its localisation at the left end of the transferred T-DNA.

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