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Bleomycin resistance: a new dominant selectable marker for plant cell transformation

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Summary

Plant cells are sensitive to the antibiotic bleomycin, a DNA damaging glycopeptide. A bleomycin resistance determinant, located on transposon Tn5 and functional in bacteria, has been cloned in a plant expression vector and introduced into *Nicotiana plumbaginifolia* using *Agrobacterium tumefaciens*. The expression of this determinant in plant cells confers resistance to bleomycin and allows selection of transformed plant cells.

Introduction

The development of *Agrobacterium tumefaciens* derived plant gene vectors (1, 2, 15, 28) as well as the direct gene transfer methodology (17, 23, 11, 18, 24, 16) have enabled the transfer of new genetic information into dicotyledonous and monocotyledonous plant cells. In most cases, resistance to the antibiotic kanamycin has been used to distinguish successfully transformed plant cells from non-transformed plant cells. Other dominant selectable markers which have been developed to be used in plant cell transformation include chimaeric genes which confer resistance to kanamycin (3, 12), chloramphenicol (13), methotrexate (12) and hygromycin (27, 26). For more flexibility in plant cell transformation experiments, it would be advantageous to have disposal of additional selectable plant cell markers with different molecular target sites. Here, we report on such a new marker.

The antibiotic bleomycin, used as a cytotoxic drug in human cancer therapy, is a glycopeptide which interacts with DNA, resulting in single and double stranded breaks (22). It has been demonstrated that a genetic locus, endocing a polypeptide of 126 amino acids, is present on transposon Tn5, which confers resistance to bleomycin in *Escherichia coli* (9, 21, 7). In this work it is demonstrated that regenerating *N. plumbaginifolia* protoplasts are sensitive to the presence of bleomycin in the culture medium and become resistant following introduction of a chimaeric bleomycin resistance gene.

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Materials and methods

DNA manipulations

Plasmid DNA was isolated according to the alkaline lysis procedure as described by Birnboim and Doly (5). All restriction endonucleases, T4-ligase, DNA polymerase I and the Klenow large fragment of DNA polymerase I were used according to the instructions of the supplier. DNA restriction fragments were separated by electrophoresis on a low melting agarose gel, excised from the gel, phenol-extracted at 65 °C and concentrated by alcohol precipitation. Ligations were performed at 14 °C using concentrations of 4–8 nM DNA fragment termini in order to promote the formation of dimeric molecules. *E. coli* strain HB101 was transformed by the $CaCl_2$ method of Cohen *et al.* (6), and suitable dilutions were plated at 37 °C on solidified Lb medium containing the appropriate antibiotics (amplicillin, 50 μg/ml; spectinomycin, 50 μg/ml).

Northern blot analysis

Total RNA from *N. plumbaginifolia* calli was isolated as described by Govers *et al.* (10) yielding approximately 1 mg per 5 gr of tissue. The RNA was electrophoresed in 1% agarose gels in 0.01 M NaH_2PO_4 pH 7.0 after denaturation in DMSO/glyoxal as described by Maniatis *et al.* (10), and blotted onto Gene Screen filters in 0.025 M NaH_2PO_2 pH 7.0. Filters were incubated at 42 °C in 10 ml hybridization solution [50% (vol/vol) deionized formamide, 1 M NaCl, 0.05 M Tris-HCl pH 7.5, 5 × Denhardt's solution, 0.1% $NaDodSO_4$ and 100 μg/ml denatured salmon sperm DNA] and hybridized with a nick-translated probe (4.10^6 cpm/10 ml hybridization mixture). Hybridizations were carried out for 24 hours and subsequently the filters were washed twice for 15 minutes in 2×SSC, 0.1% $NaDodSO_4$ at 42 °C and twice for 30 minutes in 0.5×SSC, 0.1% $NaDodSO_4$ at 42 °C. The blots were autoradiographed to Kodak XAR-5 films for 2 days at −70 °C using an intensifying screen.

Plasmid transfer

Plasmids pAGS112 (25) and pCTW 400 (this work) were transferred from *E. coli* to *A. tumefaciens* by using a triparental mating procedure. Briefly, 1 ml of a logarithmically growing culture of HB101, containing either pAGS112 or pCTW400, was mixed with 1 ml of logarithmically growing MM294 (pRK2013), (8), and 1 ml of logarithmically growing *A. tumefaciens* strain LBA4404 (pAL4404), (14). Hundred microliter of these mixtures were spotted on membrane filters placed on fresh LB agar plates and incubated for 24 hours at 29 °C in order to allow plasmid transfer. Filters containing the bacteria mixtures were resuspended in 2 ml of 0.9% NaCl and dilutions were plated on LB medium containing both 20 μg/ml rifampicin (to select *A. tumefaciens*) and 2.5 μg/ml tetracyclin (to select for the presence of plasmids pAGS112 and pCTW400 respectively). After 3 days of incubation at 29 °C, single transconjugants were repurified on the same medium. Following two additional purifications, *A. tumefaciens* strains were considered to being pure.

Plant cell culture

Leaf mesophyll protoplasts of *N. plumbaginifolia* were isolated from sterile grown plants and purified according to Maliga (19). Protoplasts were cultured at a cell density of 10^5/ml in K3-medium (19), containing 0.4 M glucose, 1 mg/l 1-naphthalene acetic acid (NAA) and 1 mg/l benzyladenine (BA) pH 5.6. On day 3 following protoplast isolation, 15 μl of an overnight culture of *A. tumefaciens* were added to 3 ml protoplast suspension and cocultivation was carried our for three days. On day 6, protoplasts were washed and resuspended in twice the volume of original culture medium containing 100 μg/ml cefotaxim. On day 8, the suspension was diluted two-fold again and selection was started, either for kanamycin (50 μg/ml) or for bleomycin (10 μg/ml) resistance. After 4−8 weeks, mini-calli were transferred to solid RM-medium (19) containing 3% sucrose, 1 mg/l NAA, 1 mg/l BA, pH 5.6, 100 mg/l cefotaxim and either 100 mg/l kanamycin or 10 mg/l bleomycin.

Results

Plant cells are sensitive to bleomycin

N. plumbaginifolia protoplasts were isolated and cultured for eight days to allow the formation of microcalli consisting of approximately 8 cells. Bleomycin was then added at concentrations ranging from 0−25 μg/ml and incubation of the regenerating protoplasts was continued. Bleomycin concentrations higher than 0.5 μg/ml severely inhibited growth of the microcalli; whereas at 5 μg/ml growth was almost completely arrested as observed 3 and 7 weeks after the addition of bleomycin (see Fig. 1). Sensitive plant cells were not killed immediately by contact with bleomycin, but rather continued to grow without dividing. This resulted in giant cells which eventually stopped growing and died.

Lycopersicon esculentum was also found to be sensitive to bleomycin. Regeneration of tomato leaf discs was almost completely inhibited at a bleomycin concentration of 5 μg/ml. Germination of tomato seeds started normally at bleomycin concentrations up to 10 μg/ml, but sensitive seedlings did not develop an extensive root system and, especially at higher concentrations (5 and 10 μg/ml),

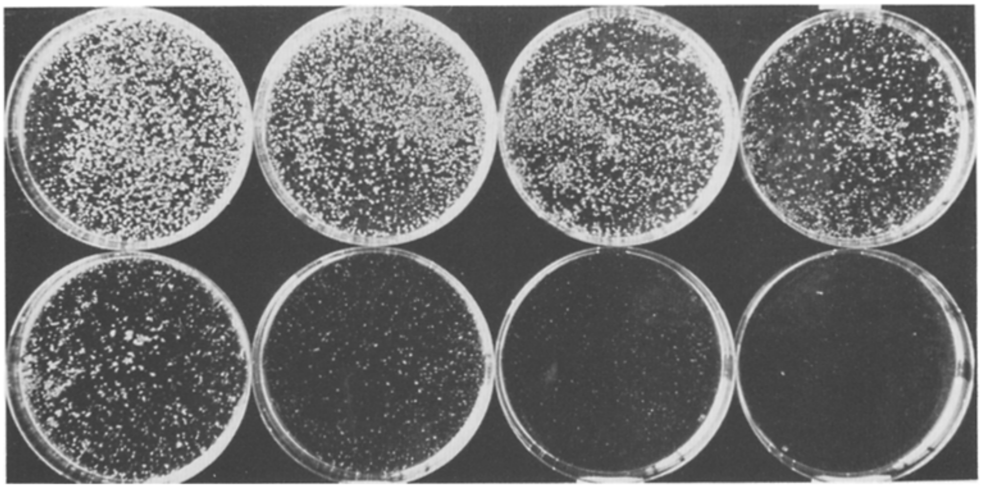

Fig. 1. Sensitivity of *N. plumbaginifolia* protoplasts to bleomycin. Eight days after protoplast isolation bleomycin was added to the culture medium at concentrations of (from top left to bottom right): 0, 0.25, 0.5, 1.0, 2.5, 5, 10 and 25 μg/ml. The effect of bleomycin on growth of the micro-calli was photographed 3 weeks after its addition.

often lost their apical meristem (data not shown).

Construction of a chimaeric bleomycin resistance gene and its introduction into a plant vector

The structural bleomycin resistance gene from transposon Tn5, subcloned between a *Bgl*II and a *Bam*HI site in plasmid pUT13, was provided by Prof. dr. Tiraby (Toulouse, France). The 494 bp *Bgl*II-*Bam*HI fragment, containing the translation initiation triplet of the bleomycin resistance gene near the *Bgl*II site, was cloned in the *Bam*HI site of plasmid pA10C3 [a derivate of pCaMV35S (4), in which a spectinomycin resistance gene has been introduced] between the Cauliflower Mosaic Virus 35S promoter and the polyadenylation signal of the nopaline synthase gene (see Fig. 2). By restriction endonuclease analysis followed by agarose gel electrophoresis, a suitable recombinant plasmid was selected, pCTW390, in which the *Bgl*II site was fused to the *Bam*HI site downstream from the 35S promoter. This plasmid, pCTW390, then contains the chimaeric bleomycin resistance gene conveniently located on a 1.8 kb *Hin*dIII-*Eco*RI fragment. The structure of the chimaeric resistance gene was confirmed by sequencing overlapping fragments containing the *Bam*HI/*Bgl*II and *Bam*HI sites of plasmid pCTW390 (data not shown). The 1.8 kb *Hin*dIII-*Eco*RI fragment of plasmid pCTW390 was subsequently cloned in the plant vector pAGS127 (25), which contains a chimaeric kanamycin resistance gene between the T-region border sequences of the Ti-plasmid of *A. tumefaciens*. This resulted in plasmid pCTW400 which then harbours both the antibiotic resistance genes but in opposite orientation between the T-region border sequences (see Fig. 2).

Introduction of bleomycin resistance into N. plumbaginifolia.

As a first step to the incorporation of bleomycin resistance into plant cells, plasmid pCTW400 (see Fig. 2) was conjugatively transferred from *E. coli* to *A. tumefaciens* strain LBA4404 (pAL4404), a non-oncogenic strain devoid of *onc*-genes but containing functional *vir*-genes (14). The purified transconjugant, LBA4404 (pAL4404, pCTW400), was used in a cocultivation experiment with *N. plumbaginifolia* protoplasts. At day 8 after protoplast isolation, selection for resistance was started; to half of the batch 50 μg/ml kanamycin was added and to the other half 10 μg/ml bleomycin. About 30 days after protoplast isolation, kanamycin resistant calli became visible. Bleomycin resistant calli could be observed about 45 days after protoplast isolation indicating a lower growth rate of calli on medium containing bleomycin. At 55 days after protoplast isolation the number of kanamycin and bleomycin resistant calli did not differ, indicating

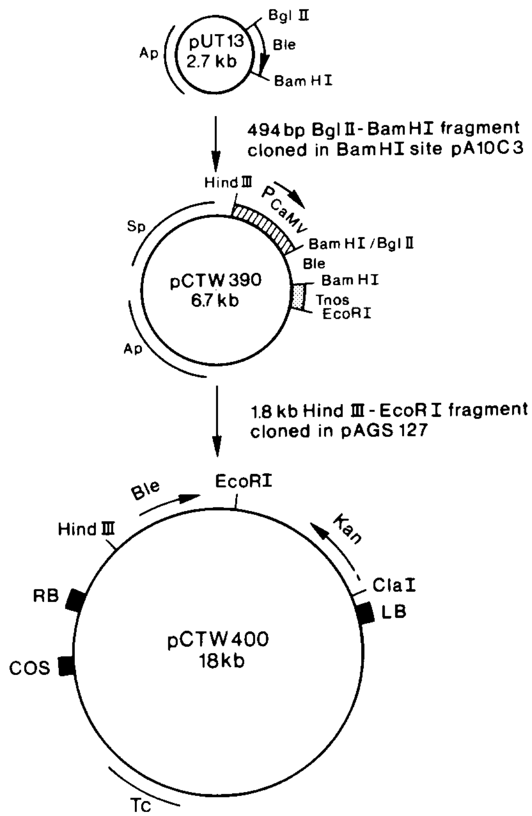

Fig. 2. Incorporation of the bleomycin resistance gene into a plant vector. Details of the construction are described in the text. Abbreviations used are: Ap, ampicillin resistance; Sp, spectinomycin resistance; Tc, tetracyclin resistance; Ble, bleomycin resistance; Kan, kanamycin resistance; P_{CaMV}, 35S promoter of Cauliflower Mosaic Virus; T_{nos}, polyadenylation signal of the nopaline synthase gene; cos, cohesive ends of bacteriophage lambda; RB, right border of the T-region; LB, left border of the T-region.

that the plating efficiency of *N. plumbaginifolia* micro-calli was comparable for both markers. In control experiments using untransformed protoplasts under similar conditions, neither kanamycin nor bleomycin resistant calli were obtained.

In another series of experiments, calli which had been selected for resistance to one antibiotic were subsequently tested for resistance to the other antibiotic. Thus, 5 independently-isolated, kanamycin resistant calli were found to be resistant to bleomycin at concentrations (10 μg/ml) which were lethal to untransformed calli. Northern blot analysis, using the structural bleomycin and kanamycin genes as probe, revealed these calli to contain transcripts from both the kanamycin resistance gene with the expected lenght of 1400 n. and 1650 n., and the bleomycin resistance gene with the expected length of 900 n. (see Fig. 3). Similar results were obtained with calli which had been selected originally for bleomycin resistance. Nine of these calli, randomly chosen, were found to be also kanamycin resistant and to contain transcripts from both genes.

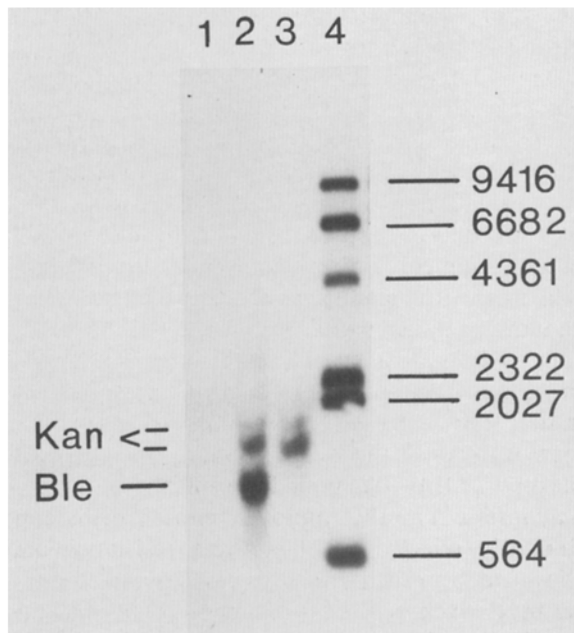

Fig. 3. Transcript analysis of plant calli. Each lane on the Northern blot contains 20 μg of total RNA which was isolated from: lane 1, *N. plumbaginifolia* untransformed calli; lane 2, *N. plumbaginifolia* Calli transformed with the T-region of plasmid pCTW400, and resistant to kanamycin and bleomycin; lane 3, *N. plumbaginifolia* calli transformed with the T-region of plasmid pAGS112 and only resistant to kanamycin. In lane 4, ^{32}P-labeled lambda-DNA digested with *Hin*dIII was run on the same gel as molecular weight marker. The blot was hybridized with a nick-translated probe, which consisted of a 1.3 kb *Xho*I-*Bgl*II fragment from transposon Tn5 containing the structural genes for kanamycin and bleomycin resistance.

Kanamycin resistant calli contain two transcripts corresponding to the resistance gene, one major transcript of 1400 nucleotides and one minor of 1650 nucleotides (lanes 2 and 3). This chimaeric resistance gene contains two nopaline synthase promoters, separated by approximately 250 bp. In case of bleomycin resistance, a 900 nucleotides long transcript is observed, present in the calli transformed with the T-region of plasmid pCTW400 (lane 2), but absent in the other calli (lanes 1 and 3). The size of the transcript, 900 nucleotides, corresponds to the expected size of 800 nucleotides plus a poly A tail of 100 nucleotides. The bleomycin transcript is more abundant than the kanamycin transcripts, indicating transcription from the CaMV 35S promoter to be more efficient as compared to the nopaline synthase promoter.

Discussion

In this work, it is shown that *N. plumbaginifolia* and tomato cells are sensitive to the action of the glycopeptide bleomycin, an antibiotic known to cause lesions in DNA. Especially actively growing tissues seem to be sensitive to this glycopeptide as judged from the frequent loss of apical meristems in young tomato seedlings germinating in bleomycin-containing medium.

Three lines of evidence indicate the chimaeric bleomycin resistance gene introduced into *N. plumbaginifolia* to be functional. First, regenerating *N. plumbaginifolia* protoplasts, cocultivated with *A. tumefaciens* strain LBA4404 (pAL4404, pCTW400), were able to form healthy looking calli in a medium containing bleomycin at 10 μg/ml (lethal to control plants), at the same frequency as kanamycin resistant calli were obtained. Secondly, calli continuously selected for bleomycin appeared to be kanamycin resistant and vice versa. This was indeed to be expected as both chimaeric genes are located between the T-region border sequences of plasmid pCTW400, used to transform *N. plumbaginifolia.* Thirdly, randomly chosen calli, continuously selected for bleomycin resistance and shown to be kanamycin resistant, contained transcripts derived from both chimaeric genes, indicating the genetic transformation of these plant cells and the differential expression of the newly introduced genes.

The mechanism by which resistance to bleomycin is conferred is unknown. Thus far, no enzymatic activity has been ascribed to the 126 amino acid polypeptide encoded by the Tn5-bleomycin resistance gene. As bleomycin causes lesions in DNA, it could be argued that this antibiotic not only induces mutations in sensitive plant cells, but also at a reduced rate in resistant plant cells. Though we can not rule out completely this possibility, all nine randomly chosen calli, which were originally selected for bleomycin resistance, could be regenerated into phenotypically normal plants.

In developing dominant selectable markers for plant cell transformation most attention has been paid thus far to antibiotics which interfere with protein synthesis like kanamycin, hygromycin and chloramphenicol. Here, we show the feasability of using a new selection marker with a different target site, *viz* DNA. In combination with the resistance markers currently being used, bleomycin resistance thus provides an additional mode of selection and, therefore, more flexibility in genetic manipulation of plant cells.

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