Plant Mol Biol Rep (2007) 25:115–120
DOI 10.1007/s11105-007-0013-4

An Improved pPZP Vector for *Agrobacterium*-mediated Plant Transformation

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Published online: 5 September 2007

Abstract We report a new and improved pPZP vector (pPZP3425) for efficient plant transformation. This vector is derived from the widely used pPZP100 series of binary *Agrobacterium* vectors. One disadvantage of these vectors is the use of chloramphenicol resistance for selection in *Escherichia coli* and Agrobacteria. We have therefore included a kanamycin resistance gene for selection in *Agrobacterium*. Furthermore, the strong 35S CaMV promoter driving the plant resistance gene has been replaced by the weaker *nos* promoter because it has been shown that the 35S promoter driving the plant resistance marker can lead to ectopic expression of the transgene. During replacement of the 35S promoter, the NcoI site within the plant resistance gene has been removed, and NcoI can now be used for cloning purposes within the expression cassette which consists of an intron-containing *gus* gene driven by a strong constitutive promoter (35S promoter with doubled enhancer plus omega-element as translational enhancer). Thus, a single vector can conveniently be used for two purposes: (1) for overexpression of proteins by replacing the *gus* gene by the coding sequence of choice and (2) for creation of promoter:*gus* fusions by substituting the constitutive promoter by any other promoter. We demonstrate the usefulness of this vector for cloning a promoter:*gus* fusion and in planta transformation of Arabidopsis.

Keywords *Agrobacterium tumefaciens* · *Arabidopsis thaliana* · Binary vector · Chloramphenicol · Kanamycin · Plant transformation

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Introduction

Agrobacterium tumefaciens is able to transform a variety of plant and fungal species by transferring its T-DNA into the host cell. The use of this transformation system has benefited very much from the development of binary vectors such as pBIN19 (Bevan 1984). This once very popular vector has now been largely replaced by a variety of different vector systems. One widely used group of vectors is the pPZP vectors (Hajdukiewicz et al. 1994). They are smaller and have a higher copy number as compared to pBIN19. However, the CaMV 35S promoter to drive the plant selectable marker is problematic, as it has been shown that the 35S promoter can affect the expression of the transgene (Yang et al. 2005; Yoo et al. 2005). Furthermore, chloramphenicol is used as a selectable marker for *Escherichia coli* and Agrobacteria in the pPZP100 series, but many *Agrobacterium* strains (including C58 and its derivative GV3101 which is routinely used for Arabidopsis transformation) have an inducible resistance mechanism against this antibiotic (Rogers et al. 2002; Tennigkeit and Matzura 1991). Additionally, the *Nco*I restriction site between 35S promoter and plant selectable marker excluded the use of this enzyme in the expression cassette. *Nco*I is one of a few enzymes that contain the ATG start codon in the recognition sequence, and can therefore conveniently be used to connect promoters and coding sequences. For all these reasons, we decided to construct an improved pPZP vector without these limitations. Furthermore, we inserted a *gus*-intron reporter driven by a strong constitutive promoter to create a vector which could be used for two applications: for promoter:*gus* fusions and for the overexpression of genes.

Materials and Methods

Construction of pPZP3425

The 35S promoter of the vector pPZP111 (Hajdukiewicz et al. 1994) was replaced by a *nos* promoter which also removed the *Nco*I site. We digested the vector pPZP111 with the two restriction enzymes *Nco*I and *Bst*XI and the large vector fragment corresponding to the vector without the 35S promoter was isolated from an agarose gel. The *nos* promoter was amplified by polymerase chain reaction (PCR) from the vector pPE13 (Epple et al. 1997) with the primer pNOSfor [5′-tgttcaatcatgagaaacgatccagatc-3′] to introduce a new *Bsp*HI restriction site and pNOSrev [5′-gaaacgaccaccatgttgggagcggagaat-3′] to replace the *Bsp*HI restriction site with a *Bst*XI site. The PCR fragment was cut with *Bsp*HI and *Bst*XI and ligated into the vector backbone from pPZP111 to yield the intermediate vector pPZP141. Sequencing confirmed the replacement of the *Nco*I site by the *Bsp*HI site and the presence of the *nos* promoter.

Next, we removed the *Bam*HI site in the MCS of the pPZP141 clone by digestion with *Bam*HI and filling up with Klenow enzyme. After purification and religation, the vector was transformed into *E. coli* and selected on chloramphenicol plates (25 μg/ml) to yield pPZP142. We introduced the high-level constitutive promoter of the expression vector pPE14 which has already been used successfully (Epple et al.

1997). For that, we transferred the complete expression cassette from the vector pPE14 (including thionin cDNA and *nos* terminator) into pPZP142 using *Hin*dIII. After transformation into *E. coli* and selection via chloramphenicol, we also checked the orientation of the cassette via digestion at the *Eco*RI site (within the pPZP backbone sequence) and *Bam*HI (within the cassette). The resulting intermediate vector pPZP1424 was digested with *Nco*I and *Bam*HI to substitute the thionin cDNA with the intron containing *uidA* gene derived from the pSH4GUS vector (Holtorf et al. 1995) which was cut with the same restriction enzymes. The intermediate vector pPZP1425 was again confirmed by sequencing.

Finally, we introduced the kanamycin resistance gene from pBIN19. The gene was amplified with the primers KAN_Rfor (5′-cagcatcatgcataattgtggtttca-3′) and KAN_Rrev (5′-gttgcgatgcatctaggtactaaaacaat-3′), which each introduced a Mph1103I restriction site. The PCR fragment was cut with Mph1103I and introduced into the single Mph1103I site of the pPZP backbone. The transformation into *E. coli* was now selected on medium containing kanamycin (50 μg/ml) and yielded positive clones of the final vector pPZP3425 shown in Fig. 1.

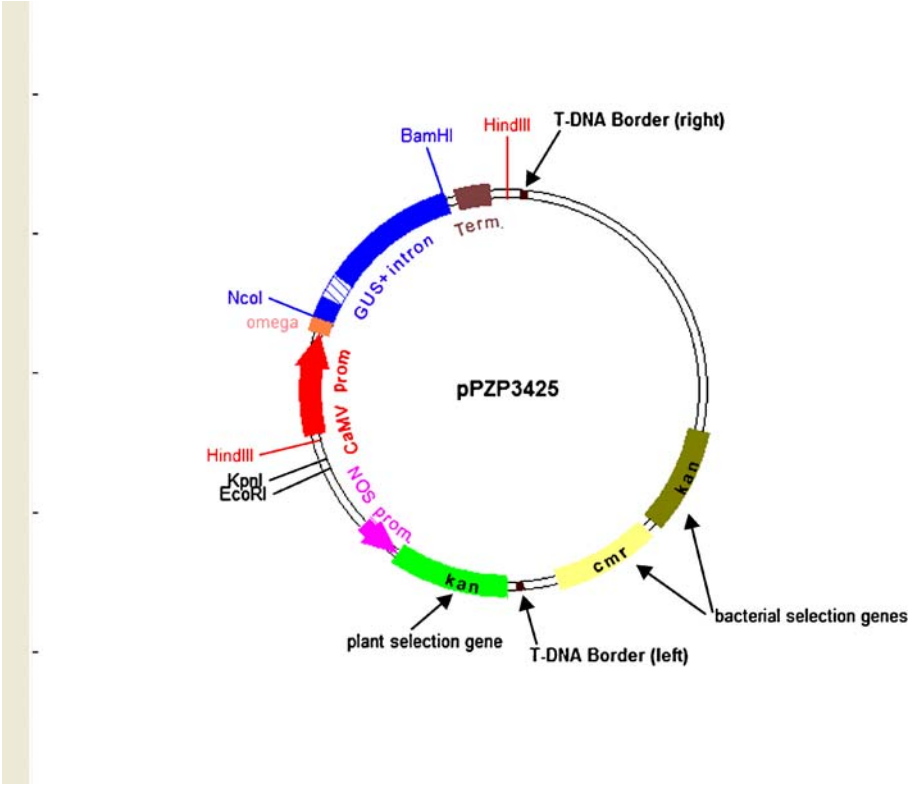

Fig. 1 Scheme of the new binary cloning vector pPZP3425

Fig. 2 **a** Selection plate containing transformed Arabidopsis seeds on MS medium supplemented with kanamycin. **b** GUS staining of the kanamycin-resistant seedlings from **a**

Cloning of At5g05340promoter: Gus Fusion

The vector pPZP3425 was digested with the restriction enzymes *Kpn*I and *Nco*I to cut out the constitutive CaMV promoter. The vector backbone was isolated from a 1% agarose gel. The promoter sequence (800 pb) of At5g05340 was amplified by using the following primer pair: At5g05340for (5′-caaaccttgggtaccaacaa-3′) containing a *Kpn*I restriction site and At5g05340rev (5′-gtatcaatgataaagccatggcgata-3′) containing an *Nco*I site. The PCR product was digested with *Kpn*I and *Nco*I and inserted upstream of the *uidA* gene into the purified backbone of pPZP3425. One isolate containing an insert for the predicted size was confirmed by sequencing and was transformed into *A. tumefaciens* strain GV3101.

Arabidopsis Transformation and Histochemical Assay

Arabidopsis plants (Columbia) where grown in short day conditions at 23°C and induced to flowering by shifting to long day conditions (16:8 h). Transformation was performed as described by Vignutelli et al. (1998), and transformants were selected on Murashige and Skoog (MS) agar including kanamycin (30 μg/ml) and timentin (250 μg/ml) to prevent bacterial overgrowth. After approximately 2 weeks, GUS staining of the putative transgenic seedlings was conducted essentially as described earlier (Jefferson et al. 1987).

Results and Discussion

The vector pPZP3425 (Fig. 1) provides an expression cassette consisting of an intron containing *gus* gene driven by a strong constitutive promoter. This vector can be used for two applications: for the overexpression of proteins, the *gus* gene is replaced by the coding sequence of choice, and for promoter:*gus* fusions, the constitutive promoter is substituted by the promoter of choice. pPZP3425 is a derivative of the pPZP100 series of binary vectors (Hajdukiewicz et al. 1994), but has several improvements. The strong 35S CaMV promoter driving the plant resistance gene has been replaced by the weaker *nos* promoter because it has been shown that the 35S promoter driving the plant resistance marker can lead to ectopic expression of the transgene (Yang et al. 2005; Yoo et al. 2005). During replacement of the 35S promoter, the *Nco*I site within the plant resistance gene has been removed, and *Nco*I can now be used for cloning purposes within the expression cassette. As discussed in the "Introduction", chloramphenicol selection is not possible with many *Agrobacterium* strains (Rogers et al. 2002; Tennigkeit and Matzura 1991). We have therefore included a kanamycin resistance gene for selection in *Agrobacterium*. Finally, we have introduced an expression cassette consisting of an intron-containing *gus* gene driven by a strong constitutive promoter (35S promoter with doubled enhancer plus omega-element as translational enhancer). The designation of the new pPZP vector follows that for the original pPZP vectors with pPZP3xxx now indicating that the bacterial resistance is based on kanamycin and pPZPx4xx indicating plant selectable kanamycin resistance driven by the *nos* promoter.

The vector pPZP3425 is routinely used in our lab for both applications, and we have already produced dozens of transgenic Arabidopsis lines via the in planta transformation method with some alterations (Bechtold et al. 1993; Vignutelli et al. 1998) using the *Agrobacterium* strain GV3101. We usually obtain at least approximately ten transformants per plant. An example containing a promoter:*gus* fusion for the gene At5g05340 is shown in Fig. 2. From approximately 100 seeds that were plated on kanamycin containing MS medium, two seedlings survived (Fig. 2a) and both stained positive for GUS (Fig. 2b).

It should be mentioned that although the vector does not contain extensive multi-cloning sites, we have always been able to clone the desired constructs using the available restriction sites. If the promoter or the coding sequence that is to be cloned contains an restriction site that is used in the vector (*Kpn*I or *Eco*rRI, *Nco*I, *Bam*HI), it is usually possible to select an enzyme that provides cohesive ends which are compatible with the enzyme used in the vector. *Nco*I for instance is producing cohesive ends that are compatible with those produced by *Bsp*HI, *Fat*I, and *Pci*I.

Acknowledgments The authors thank the Austrian Science Fund (FWF) for the financial support for this research project under grant number P16296-B06. We also thank Sabine Daxböck-Horvath and Sabrina Köllner for their assistance in the lab.

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