SHUTTLE VECTORS FOR DIRECT *IN FRAME* CLONING AND CONSTRUCTION OF *GFP* AND *GUS/GFP* REPORTER GENE FUSIONS

K. Stefanova, A. Atanassov, I. Atanassov
AgroBioInstitute, Dragan Tzankov 8, 1164 Sofia, Bulgaria

ABSTRACT

*Two shuttle plasmid vectors have been constructed that facilitate the direct **in frame** cloning upstream of **gfp** and **gus/gfp** reporter genes. Coding sequences of **gfp** and **gus/gfp** reporter genes were inserted into multicloning site of pUC19 cloning vector in a way to create frame shift and prevent **gfp** and **gus/gfp** expression from the **lac** promoter. Blunt end PCR amplified fragments, corresponding to signal peptide or other translated gene regions, were cloned into **Sma I** site upstream of **gfp** and **gus/gfp** genes. The recombinant colonies, containing correct **in frame** fusion, were directly selected after appearance of green (GFP) fluorescence under UV light. The use of the developed cloning vectors for construction of gene fusions and plant transformation vectors are discussed.*

Introduction

Fusions between green fluorescent protein (*gfp*), b-glucuronidase (*gus*) and *gus/gfp* reporter genes and translated regions of studied genes have found growing application into various fields of plant transgenic research including: (a) visual selection of transgenes and monitoring of the levels and mode of transgene expression [1, 2, 3, 4]; (b) functional characterization of gene domains [5]; (c) extracellular protein trafficking [6]; (d) characterization of signal peptide properties and sub-cellular protein targeting and accumulation [7, 8, 9, 10]. Although the number of plant transformation vectors developed so far, the construction of fusions with reporter genes is still laborious and time-consuming procedure. The direct cloning into plant transformation vectors restricts the testing of the functionality of the constructed fusion protein through expression in *E. coli*, prior starting of the lengthy plant transformation. This makes difficult the evaluation of the results, especially when no reporter gene expression is detected in the obtained transgenic plants. The lack of efficient in frame cloning procedure also substantially restricts the characterization of signal peptides from plant genes, whereas *in frame* cloning of relatively small fragments has to be performed.

Here, we have created two GFP- and GUS/GFP- fusion cloning vectors facilitating the *in frame* cloning of PCR-amplified gene fragments. The restoration of recombinant *gfp* expression, due to shift of reading frame, allows direct identification of the positive colonies.

Materials and Methods

Construction of pFG and pFGG cloning vectors

Standard molecular cloning procedures were employed in all performed experi-

ments, [11]. pGFPuv (Clontech, USA, [12]) and pCAMBIA 1305.1 (http://www.cambia.org) plasmids were used as sources for PCR amplification of *gfp* and *gus* gene coding sequences. pUC 19 was used as a backbone plasmid for construction of pFG and pFGG cloning vectors.

The pFG vector was made through insertion of *gfp* coding sequence into multicloning site /MCS/ of pUC19 vector. An 743 bp fragment of *gfp* gene was amplified by PCR with GFP-F (CCCCGGGTAGCAAAGGAGAAGAAC) and GFP-R (CAAGCTTGAGCTCTGAGTCGACCTTGTACAGCTCGTCCA) primers. *Sma I* and *Sac I /Sal I* sites were introduced in the forward and reverse primers respectively. Following digestion with *Sma I* and *Sac I* enzymes, the PCR fragment was inserted into *Sma I / Sac I* sites of MCS-pUC19.

The pFGG vector was constructed through subsequent insertion of *gus* coding sequence upstream of *gfp* gene in pFG plasmid. The *gus* gene was PCR amplified by GUS-F (CCCGGATCCCCGGGCCGACGAACTAGTCT) and GUS-R (CTAGCGTTCTTGTAGCCGAA) primers. *BamH I / Sma I* sites were inserted into the forward primer. *BamH I* digested PCR fragment was inserted upstream of the *gfp* gene into *BamH I / Sma I* sites of the pFG plasmid.

Construction of fusions with reporter gene.

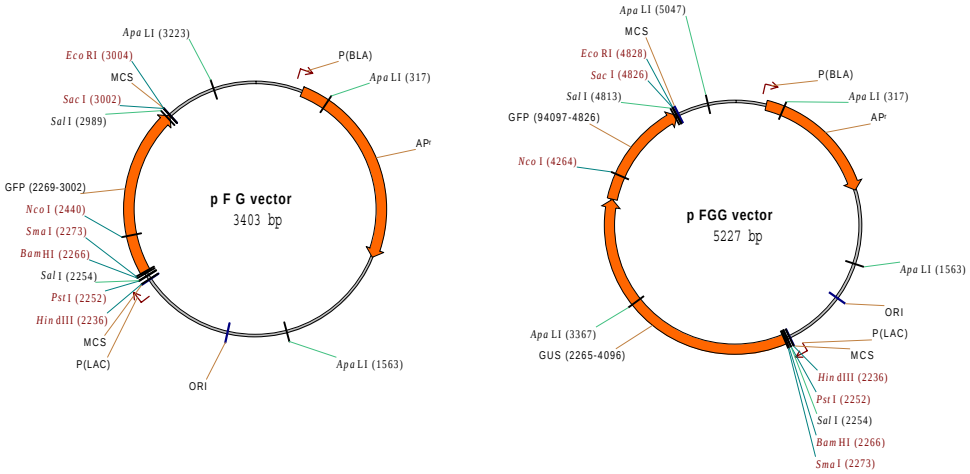
The region corresponding to putative signal peptide of b- D-glucane exohydrolase gene from *Nicotiana plumbaginifolia* was PCR amplified from p2EH plasmid containing the genomic region of the gene (Stefanova et al unpublished), using SP-F (GGA TGGGGAGAATGTCA) and SF-R (GGGTGTTTTGGGTCCTT) primers. Both *Tag I* and *Pfu* DNA polymerases (Fermentas) were used for PCR amplification. The *Tag I* amplified PCR fragment was additionally treated with *Klenow I* enzyme (Fermentas) for generating of blunt ends. The PCR fragment was inserted into *Sma I* site of pFG and pFGG vectors. Following of E. coli (JM109) transformation, the obtained ampiciline resistant colonies were screened under UV (254 nm) light by placing of reverted petri dishes on the top of transiluminator. The fluorescent colonies were tested for presence and correct orientation of the insert through PCR amplification with specific primers and digestion with *BamH I*, *Sma I* and *SacI* enzymes of the PCR fragment amplified from recombinant plasmid DNA by pUC reverse and forward primers.

Results and Discussion

Vector construction

The pFG plasmid was obtained following directional cloning of PCR amplified fragment, containing the translated region of green fluorescent protein /gfp/ reporter gene, into *Sma I / Sac I* sites of pUC19 cloning vector. The PCR primers were selected in a way to introduce a frame shift, preventing correct translation of b-galactositase /*gal*/ and *gfp* genes expressed from the *lac* promoter in pFG, **Fig. 1.** Thus, white non-fluorescent colonies bearing pFG plasmid were obtained after transformation of *E. coli* cells.

The pFGG plasmid was constructed through insertion of PCR amplified coding sequence of b-glucuronisidase reporter /*gus*/ gene into *BamH I /Sma I* sites of the pFG plasmid, upstream of the *gfp* coding sequence. The PCR primers were selected in a way to obtain in frame fusion between *gus/gfp* coding sequences, without restoration of the correct translation

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A.1.        gag gat ccc cGG GTA GCA AAG
            BamHI SmaI
A.2.                                x   R   G   S   K
      gag gat ccc cGG ATG-/PCR/-xxC CCG GGT AGC AAA
      BamHI                       SmaI

B.1.        gag gat ccc cgg gCC GAC GAA
            BamHI SmaI
B.2.                                x   P   G   R   R
      gag gat ccc cGG ATG-/PCR/-xxC CCG GGC CGA CGA
       BamHI                        SmaI
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Fig. 1. Map and MCS region of vectors pFG and pFGG.
(A) Maps of pFG and pFGG vectors. (B) 5'-end of *gfp* coding region prior /B.1./ and after /B.2./ cloning of PCR fragment into pFG. (C) 5'-end of *gus/gfp* coding prior /C.1./ and after /C.2./ cloning of PCR fragment into pFGG.
The MCS-pUC19 sequence is presented with small letter. The coding sequences of *gfp* and *gus/gfp* reporter genes are shown in bold capital letter. The position of the PCR fragment insertion is marked with -/PCR/- and the sequences of the both ends are shown in italic capital letter. The amino acid sequences of the carboxyl terminal regions of *gfp* and *gus/gfp* reporter genes, after frame shift followed PCR cloning, are present in /A.2./ and /B.2./.

of *gal/gus/gfp* fusion gene expressed from *lac* promoter. Again only white non-fluorescent colonies, bearing pFGG plasmid, were obtained after transformation of *E. coli* cells.

In frame cloning into pFG and pFGG vectors

The use of GFP expression for the direct selection of recombinant plasmids, containing *in frame* cloned PCR fragment into pFG anf pFGG vectors, was tested. Blunt end PCR fragment, containing the putative signal peptide /SP/ region of b-D-glucan exohydrolase gene from *Nicoti-*

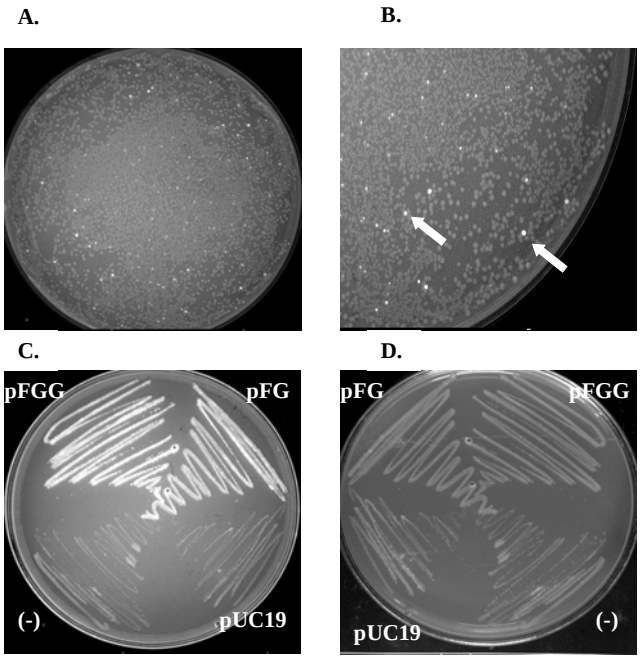

Fig. 2. Direct selection of GFP fluorescent colonies following *in frame* cloning of PCR fragments upstream of *gfp* and *gus/gfp* reporter genes into pFG and pFGG vectors.
(A-B) GFP fluorescent colonies following transformation of *E. coli* cells with plasmid DNAs obtained after insertion of PCR fragments.
(C-D) UV-light /C/ and visible light /D/ illuminations of plated *E. coli* cells contained PCR-pFG , PCR-pFGG; pUC19 plasmids. The plasmid-less *E.coli* cells are pointed with (-).

ana plumbaginifolia (Stefanova et al., unpublished), was cloned into *Sma I* site of pFG and pFGG vectors, Fig. 1. The positions of the PCR primers were selected so that, the insertion of the PCR fragment in correct orientation to restore the correct triplet order and reading frame of *gal/SP/gfp* fusion gene in pFG plasmid and *gal/SP/gus/gfp* fusion gene in pFGG plasmid. The transformation of *E. coli* cells with plasmid DNAs obtained after blunt end ligation of PCR fragments into pFG and pFGG vectors and observation of obtained colonies under UV light, resulted in selection of a number of green fluorescent colonies, **Fig.2ab**. The positive colonies were readily detected under UV light from standard laboratory UV-transiluminator. In some experiments, especially when the obtained colonies were too small, the colony fluorescence was confirmed through their transfer on fresh media and incubation overnight, **Fig.2cd.** The presence and correct orientation of the cloned blunt end PCR fragment was confirmed through PCR amplification with specific primers, as well as restriction enzyme digestion of PCR fragments amplified with universal pUC reverse and forward primers. Nearly all tested fluorescent colonies contained the cloned PCR fragment in the right orientation. Similar

highly efficient directional *in frame* cloning, using pFG and pFGG vectors, was obtained also with a number of PCR fragments corresponding to wide range of signal peptides and gene coding sequences. In all performed cloning experiments several positive recombinant clones were selected within first the ten tested fluorescent colonies.

Two procedures for obtaining blunt end PCR fragments were applied in the performed cloning experiments: (a) PCR amplification using *Pfu* DNA polymerase and (b) PCR amplification using *Taq* DNA polymerase followed by treatment with *Klenow I* enzyme for producing blunt end fragments. GFP fluorescent colonies were obtained after both treatments, but in some experiments the use of *Pfu* DNA polymerase resulted in obtaining small portion of insert-less fluorescent colonies.

Once prepared the functional fusion with reporter gene could be easily re-cloned downstream of plant promoter region into a corresponding transformation vector using standard cloning techniques. Within our experience, the *SP/gfp* and *SP/gus/gfp* fusions were easily transferred into pBI121 derived binary vectors through replacement of the *gus* reporter gene by excision /ligation in *BamHI / SacI* sites.

In conclusion, plasmid vectors and efficient protocol for direct selection of *in frame* fusion of signal peptides / genes of interest with *gfp* and *gus/gfp* reporter genes were established. The constructed plasmids could be readily used as efficient shuttle cloning vectors for construction of fusions with *gfp* and *gus/gfp* reporter genes, testing of the functionality of fusion gene and subsequent re-cloning into plant transformation vectors. The construction of plant transformation vectors facilitating the second cloning step is in progress.

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REFERENCES

1.**Chalfie M., Tu Y., Euskirchen G., Ward W. and Pasher W.** (1994) Science, **263,** 802-805

2. **Sarwar M., and Maliga P.** (1999) Nature Biotechnol., **17,** 910-915

3. **Arnim A., Deng X., Stacey M.** (1998) Gene, **221,** 35-34

4. **Vickers C., Xue G., Gresshoff G.** (2003) Plant Cell Rep., **22**, 135-140

5. Elmayan T., and Tepfer M. (1994) The Plant J., **6** (3), 433-440

6. **Epel B.L., Padgett H.S., Heinlein M.,** Beachy, R. (1996) Gene, **173**, 75-79

6. **Sansebastiano G., Paris N., Neuhaus S., Neuhaus J.** (1998) Plant J., **15** (4), 449-457

6. **Rojo E., Sharma V., Koleva V., et. al** (2000) The Plant Cell, **14**, 969-997

8. **Borisjuk N., Borisjuk L., Logendra S., Petersen F., Gleba Y., Raskin I.** (1999) Nature Biotechnol., **17**, 466-469

9. **Condrat U., Fiedler U.** (1998) Plant Mol. Biol., **38**, 101-109

10. **Chytilova E., Macas J., Sliwinska E., Rafelski S., Lambert G., Galbraith D.** (2000) Mol. Biol. Cell, **11**, 2733-2741

11. **Sambrook J., Fritsch E. F., Maniatis T.** (1989) Molecular cloning: A laboratory Manual, 2 nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

12. **Crameri A., Whitehorn E., Tate E., Stemmer W.,** (1996) Nature Biotechnol., **14**, 315-319