

Full Length Research Paper

A novel expression cassette for the efficient visual selection of transformed tissues in florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.)

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Constructs carrying visual reporter genes coupled with efficient promoters could facilitate the process of identification and selection of stable transformants in recalcitrant crops. Here, a novel construct utilizing a ribulose-1,5-bisphosphate carboxylase (RbcS) promoter combined with the green fluorescent protein (GFP) reporter gene to initiate very high expression of GFP in florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) was described. Based on this expression cassette, a new regeneration protocol using leaf discs as explants was developed for the *Agrobacterium*-mediated transformation of *Chrysanthemum* genotype '1581', and a transformation efficiency of 7% was obtained. The expression of two different GFP constructs targeted to either cytosol or plastids was compared in transgenic lines. Both GFP constructs were expressed at such a high level that the green fluorescence dominated red fluorescence in the leaf tissues, allowing easy observation and microdissection of transformed tissues even without a GFP filter. Under normal light, plants with GFP targeted to plastids had a light green phenotype deriving from the high GFP expression. Quantitative reverse transcriptional PCR analysis showed that the plastid targeted construct with intron had significantly higher steady state transcript levels of GFP mRNA. This novel expression cassette may allow direct visual selection of transformed tissues independent of antibiotic selection in a wide range of plant species.

Key words: Florist's chrysanthemum (*Chrysanthemum morifolium*), genetic transformation, GFP gene, RbcS promoter, RbcS targeting signal.

INTRODUCTION

Florist's chrysanthemum has thousands of commercial cultivars and is one of the most important flower crops in the global floricultural industry today. The needs for improvement of ornamental traits and resistance to biotic and/or abiotic stress in relation to the commercial value of the crop have created increasing interests in molecular genetic approaches for the improvement of florist's

chrysanthemum. Transgenic improvement of *Chrysanthemum* cultivars is usually initiated by exploring an efficient genetic transformation system through the investigation of the regeneration potential of different genotypes, explants, and phytohormones (Petty et al., 2003; Teixeira da Silva, 2006). Transformation efficiencies for florist's chrysanthemum depend on the selected genotype, and unfortunately most genotypes resist transformation. Transformation may be influenced by the process of transformant selection such as pre-culture period, addition of antibiotics in the medium, *Agrobacterium* strain and the type of construct used for transformation (Jeong et al., 2002; Ahsan et al., 2007).

Constructs carrying visual reporter genes could facilitate the process of identification and selection of stable transformants and even allow selection of transformants

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Abbreviations: BA, 6-Benzyladenine; GFP, green fluorescent protein; MS, Murashige and Skoog's medium; NAA, naphthalene acetic acid; NPTII, neomycin phosphotransferase; RbcS, ribulose-1,5-bisphosphate carboxylase.

without the use of antibiotics (Jordan, 2000; Jaiwal et al., 2002). GFP is widely used as a reporter gene for the evaluation of genetic transformation parameters such as transformation efficiency, optimization of pre- or post-transformation steps (Cardoza and Stewart, 2003; Zhou et al., 2004) and also as a useful marker for promoter analysis combined with constitutive, inducible or tissue-specific promoters (Nehlin et al., 2000; Potenza et al., 2004; Hraska et al., 2006). The small subunit of ribulose-1, 5-bisphosphate carboxylase (RbcS) is plastid-localized, though, encoded by nuclear DNA. It represents the most abundant protein found in plant leaves (Dean and Leech, 1982). In contrast to the commonly used 35S promoter, the RbcS promoter from *Chrysanthemum morifolium* cloned together with its own terminator was shown to be much more active in *Chrysanthemum* in a fusion construct with the GUS gene (Outchkourov et al., 2003). Therefore, RbcS promoter-based expression cassettes represent attractive systems for expression of heterologous genes at high levels in green tissues of most plant species. Furthermore, the RbcS targeting signal can be used to direct different proteins to the plastid (Dean et al., 1989).

In this study, our objective was to investigate the potential of a construct with the RbcS promoter fused to the GFP reporter gene as a visual reporter of transformation in leaf tissues of florist's (*Chrysanthemum morifolium* Ramat.). The effect of plastid targeting on the level of GFP expression compared to default expression in the cytoplasm was examined, and with this construct, a novel protocol was developed using leaf disc-based transformation of genotype '1581'.

MATERIALS AND METHODS

Plant materials

Sterile cuttings of the florist's chrysanthemum cultivar '1581' were provided by Plant Research International (PRI) and grown on Murashige and Skoog (MS) medium containing 3% w/v sucrose and 6-benzyladenine (BA) 0.1 mg l⁻¹ (pH 5.8) to proliferate enough materials for regeneration and transformation. For regeneration and transformation experiments, 0.5×0.5 cm leaf discs were cut from 6-8 weeks-old *in vitro*-grown plants and inoculated on media with different combinations of BA and NAA.

Regeneration medium and hormone combinations

Adventitious shoots were induced directly from leaf discs on a concentration matrix of 16 combinations of cytokinin (6-BA, at 1.0, 2.0, 3.0, 5.0 mg l⁻¹) and auxin (NAA, at 0.1, 0.2, 0.5, 1.0 mg l⁻¹) added to basal MS medium containing 0.7% w/v agar and 3% w/v sucrose. The pH value of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were maintained in a tissue culture room under a photoperiod regime of 14-h light (3000 lux) and 10-h darkness at a constant temperature of 25°C.

After 40 days of inoculation, explants were evaluated in terms of the explant percentage regenerating shoots and the number of shoots formed on the cut edge of each explant. The number of

shoots per explant was based on only those shoots with a stem axis of more than 1 cm divided by the total number of explants for each replicate. Each treatment consisted of three to five replications with ten inoculated leaf discs per Petri dish. The significance of the data was analyzed by the SAS software package.

Plant transformation

The leaf explants of *Chrysanthemum* '1581' were pre-cultured for 2 days on medium supplemented with BA 1.0 mg l⁻¹ and NAA 0.5 mg l⁻¹, then soaked in LB medium that contains suspended *Agrobacterium tumefaciens* AGL0 (OD₆₀₀=0.5) for 5 min and blotted on sterilized filter paper. They were then placed on direct regeneration medium and cultured at 25°C under dark conditions for about 48 h until small white colonies of *Agrobacterium* were observed on the medium. After this co-cultivation period, the explants were subcultured for regeneration on medium supplemented with 30 mg l⁻¹ kanamycin and 200 mg l⁻¹ cefotaxime under the same light and temperature conditions and transferred to fresh media every 3 weeks until 5 cm-long shoots were formed.

Plasmids

For transformation, DNA constructs ImpactVector1.1-GFP and ImpactVector1.4-GFP were used (www.impactvector.com). The RbcS promoter and terminator were cloned from *Chrysanthemum* '1581' (Outchkourov et al., 2003). ImpactVector1.1 targets GFP to the cytoplasm, and the ImpactVector1.4 contains the natural chloroplast targeting signal with its native intron from the RbcS gene. This plastid targeting is expected to direct GFP protein accumulation in the chloroplast stroma of transgenic lines. The GFP reporter gene was inserted into NcoI-SacI restrictions sites of both vectors, and then the 2.8 kb *Ascl-Pacl* expression cassette "RbcS promoter-(plastid targeting) GFP gene-RbcS terminator" was subcloned into an *Ascl-Pacl*-digested pBinplus vector (Van Engelen et al., 1995) which harbors the NPTII antibiotic selection marker (Figure 1). The constructs were electroporated into *Agrobacterium tumefaciens* (AGL-0) using a Gene Pulser electroporator (Bio-Rad).

Gene expression assay

GFP expression in shoots grown under selective conditions was observed with a Nikon epi-fluorescence microscope Eclipse TE2000 equipped with a UV light unit producing a 400 nm activation wavelength and a 420 nm GG420 barrier filter to observe 500 nm GFP fluorescence in combination with natural red fluorescence from chlorophyll.

Positive shoots were further analyzed using RealTime-PCR to compare the effects of plastid targeting on the mRNA transcript levels of the GFP gene. Total RNA was extracted by the TriPure™ small sample method. cDNA synthesis was done using the TaqMan™ Reverse Transcription Reagents. Reverse transcription was performed in the GeneAmp PCR system at the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. A volume of 2 µl of cDNA (2 µg) was used for qRT-PCR, adding 10 µl BIO-RAD iQ™ SYBR® Green Supermix, 2 µl 3 µM (diluted) specific forward and reverse primers, and a volume of 20 µl water. The primers were designed to amplify a 200 bp fragment of the GFP sequence (GFP-Forward: GCAGAAGAACGGCATCAAGGT; GFP-Reverse: AGTGGTTGTCGGGCAGCAG). The actin housekeeping gene from *Chrysanthemum* '1581' was first cloned and sequenced to design the primers that amplify a 200 bp fragment (Actin-Forward: CCTCTTAATCCTAAGGCTAATC; Actin-Reverse: CCAGGAATCCAGCACAAATACC). Primer design was done with Beacon Designer 7. Amplification and real-time measurement were

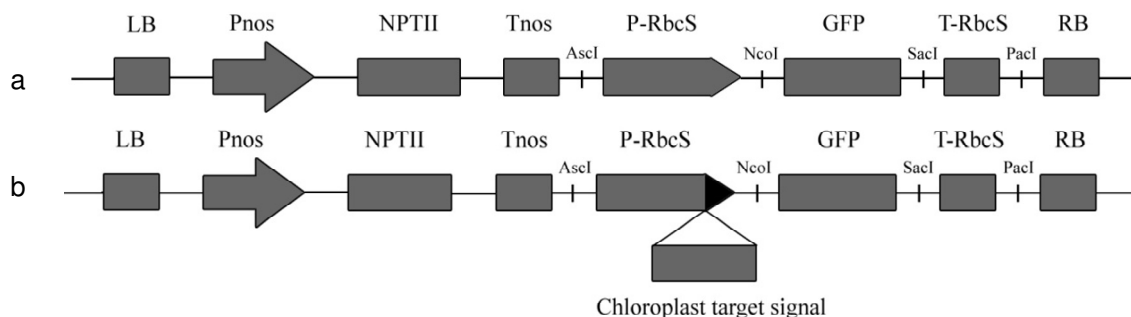


Figure 1. The RbcS promoter-GFP expression cassettes with (b) and without (a) plastid targeting in pBinplus.

performed in the iCycler iQ5 (Bio-Rad, USA) (95°C, 3 min; 40 cycles of 95°C, 10 s and 60°C, 30 s; 95°C, 1 min; 60°C, 1 min) The results were analyzed using the IQ5 Optical System Software and $2^{-\Delta\Delta Ct}$ CT Method where $-\Delta\Delta Ct = C_{T \text{ target gene}} - C_{T \text{ reference gene}}$ (Livak and Schmittgen, 2001). The experiments shown were repeated twice with similar results.

Statistical analysis

Data obtained from all experiments were presented as the means \pm SE of the three to five replications and subjected to analysis of ANOVA using SAS software Version 8.1 (SAS Institute 2004).

RESULTS AND DISCUSSION

Direct shoot regeneration of *Chrysanthemum* '1581'

Leaves were harvested from 6-8 weeks old *in-vitro* plants and excised along the edges for full contact on the media. Cell enlargement along cut edges of most explants was observed within 3 days of culture initiation and some primordial shoots started to appear as early as 7 days after incubation on regeneration media. The effects of BA and NAA on shoot induction were highly significant ($P \leq 0.01$). The basal medium with BA 2.0 mg l⁻¹ significantly promoted shoot formation compared to the concentrations of 1.0 and 3.0 mg l⁻¹ ($P \leq 0.05$), while on medium of BA 5.0 mg l⁻¹ the average number of induced shoots decreased (Figure 2). NAA at a concentration of 0.5 mg l⁻¹ also significantly induced shoot formation compared to 0.1, 0.2, and 1.0 mg l⁻¹ ($P \leq 0.05$). From all regeneration media, more than 1.0 shoot per explant was obtained on average and 85% of explants regenerated into shoots (data not shown here). The best result of regeneration was obtained for the combination of BA 1.0 mg l⁻¹ and NAA 0.5 mg l⁻¹ with an average of 2.0 induced shoots per explant 40 days after inoculation with *Agrobacterium*.

Variation in regeneration frequencies can be caused by a series of reasons such as explant types, phytohormone ratios, and choice of genotypes (Welandar, 1988). Seiichi et al. (1995) was the first to report on direct regeneration and transformation of cultivar '1581' using stem segment

as explants, but failed to mention the exact regeneration frequencies. The stem segments were derived from greenhouse grown flower stems making the protocol less convenient for year round use. In this study, *in vitro* leaves of '1581' with an adapted hormone ratio and concentration yielded a high regeneration frequency of 85% and 2.0 shoots per explant.

Auxins and cytokinins represent the most important external addition for regulating growth and morphogenesis in plant tissue and organ cultures (Gaspar et al., 1996, Kaul et al., 1990) and basal medium with BA 1.0 mg l⁻¹ and IAA 0.1 mg l⁻¹ was used for direct shoot induction from stems of *Chrysanthemum* '1581' (Seiichi et al., 1995; Visser et al., 2007). In this study, BA was optimal at the same concentration, and IAA was replaced by NAA and optimal at a five times higher concentration of 0.5 mg l⁻¹.

Transformation and selection of GFP transformants

The optimized regeneration protocol was applied to introduce the GFP gene into *Chrysanthemum* '1581'. Transgenic regenerating callus and shoots were observed under a fluorescence microscope with a UV of 420 nm barrier filter. Four shoots transformed with Impactvector1.1-GFP and six shoots transformed with Impactvector1.4-GFP were regenerated from 60 and 80 explants, respectively representing a transformation efficiency of ca. 7%. The putative transformation status was directly visible based on the very strong green fluorescence signal which was even stronger than the endogenous red signal. Hence, the GFP expression cassette allows early selection of transformed shoots and tissues even without GFP filter, and it also prevents the selection of escapes.

All positive shoots were taken to the greenhouse after they were rooted. Plantlets transformed with Impactvector1.4-GFP taken from the greenhouse showed stronger green fluorescence under the fluorescence microscope than the Impactvector1.1-GFP leaves (Figure 3). In the greenhouse, the potted plants of Impactvector1.1 lines were normal in stature, and showed a mildly lighter

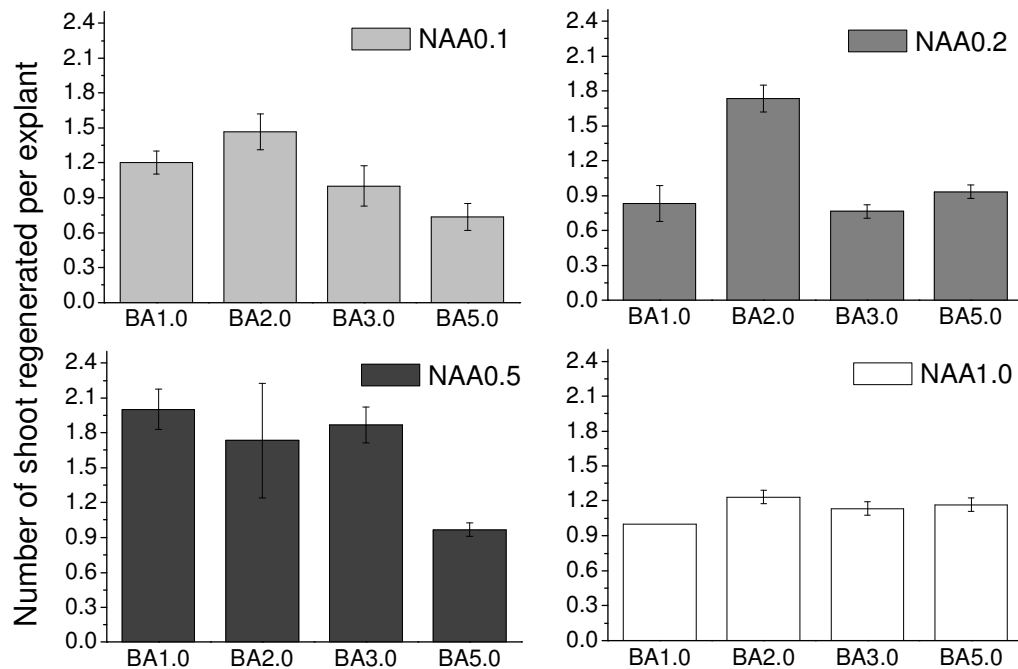


Figure 2. Shoot formation on leaf explants of florist's *Chrysanthemum* '1581' through direct regeneration after 40 days on 16 different combinations of BA (mg l^{-1}) and NAA (mg l^{-1}) on MS basal media. Mean \pm SE.

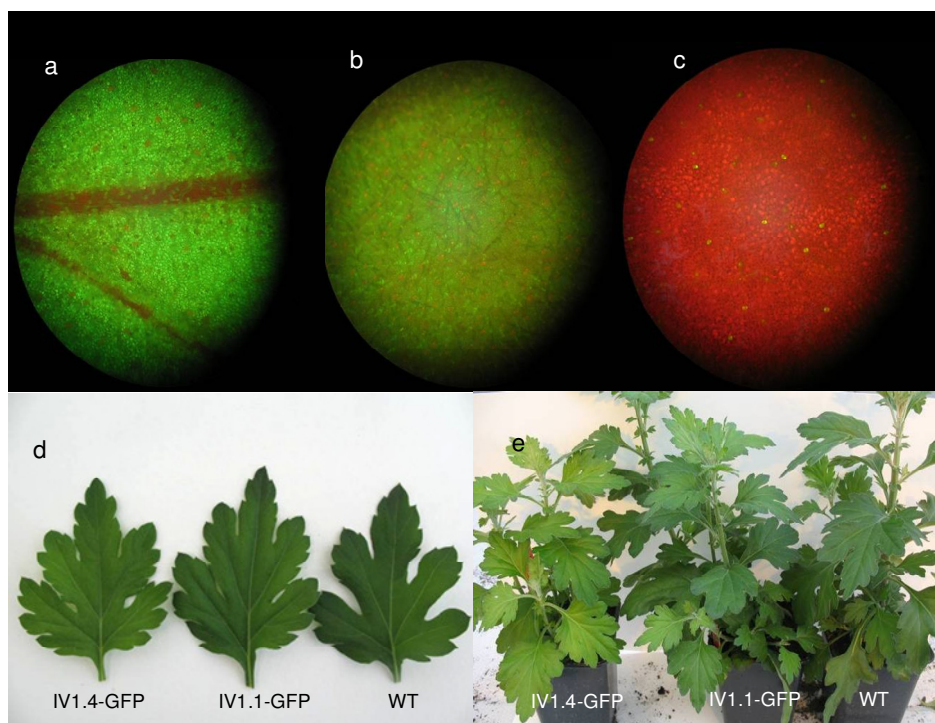


Figure 3. Fluorescence images and phenotypes of transgenic and wildtype *Chrysanthemum morifolium*. GFP fluorescence of *Chrysanthemum* '1581' leaves expressing (a) ImpactVector IV1.4-GFP (transgenic line G3), (b) IV1.1-GFP (transgenic line G8) using a 420 nm barrier filter for viewing both green and red fluorescence. Panel (c) shows the natural red chlorophyll fluorescence of wild type '1581' leaves with the same microscope settings as in (a) and (b). (d) Differences in shades of green of transformed *Chrysanthemum* '1581' leaves versus wildtype under normal light. (e) Phenotypes of transgenic and non-transgenic '1581'.

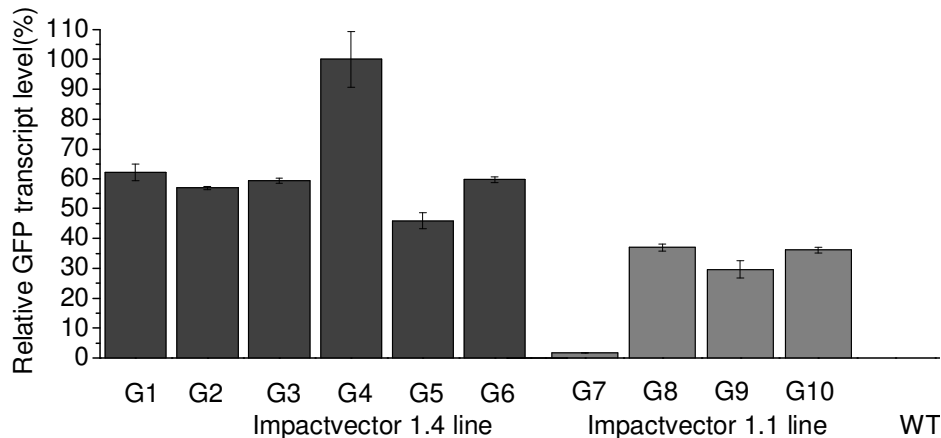


Figure 4. Relative transcript level of GFP mRNA in leaves of transgenic lines of *Chrysanthemum* '1581' measured by quantitative RealTime-PCR. G1-G6 are Impactvector 1.4 transgenic lines and G7-G10 are Impactvector 1.1 lines expressing GFP. WT is a non-transgenic *Chrysanthemum* '1581'. The Y-axis shows the relative transcript level of the GFP gene by the percentage value of each transgenic lines relative to the line (G4) with highest expression level. The GFP expression level of G4 line is valued as 100%.

green phenotype compared to wildtype. Impactvector1.4-GFP shoots displayed a stronger color change to light green, and also displayed a slightly dwarfed phenotype (Figure 3).

The intensity of green fluorescence differed significantly between the two expression cassettes although, the promoter and terminator were identical between the two constructs. The only difference was that Impactvector 1.4-GFP carried a plastid targeting signal with intron. Unpublished results (Jongsma, WageningenUR) with these two vectors in tobacco showed that the expression levels of GFP could be high as 24% of total proteins with 1.4 construct and 12% for the 1.1 construct. This means that a significant portion of protein synthesis is invested in the production of GFP. This may cause the dwarfed phenotype of the 1.4 line.

GFP expression level

The difference in expression level of GFP between constructs 1.1 and 1.4 is caused at either the RNA or protein level. Targeting the plastid deposits GFP in a completely different environment which potentially improves the protein half-life. Alternatively, the use of the native targeting signal yields a more efficient translation start compared to construct 1.1. Finally, it is also possible that the extended RbcS sequence and presence of the native intron in the RbcS targeting signal improve the steady state level of GFP transcripts. To examine this last aspect of potentially higher levels of GFP gene transcripts, the authors quantified the transcript level by real-time PCR. The analysis of all ten transgenic lines revealed very high GFP transcript levels in the leaves of transgenic shoots of both impactvector1.1 and

Impactvector 1.4 GFP lines, whereas no signal was detectable in leaves of wildtype plants. The $-\delta Ct$ calibrated relative to the reference gene showed that impactvector1.4-GFP lines required 1 to 2 cycles less for amplification of the GFP transcript compared to impactvector1.1-GFP lines (Figure 4). This may have resulted in 2-4 higher protein levels, which indeed were apparent from the GFP fluorescence levels and the different phenotypes of 1.1 and 1.4 lines. The difference in relative transcript levels was significant between these two lines ($P < 0.006$). Differences in copy number can sometimes also explain differences in expression level between two independent transformation events. However, in this case the differences are consistent for all lines tested between two constructs. Consistent differences in copy number in independently generated lines of these two highly similar constructs are unlikely.

Green fluorescent protein (GFP) is a very useful and reliable marker for screening putative transformants of various crops or ornamental plants including florist's chrysanthemum (Zvereva and Romanov, 2000; Rakosy-Tican et al., 2007; Yong et al., 2006). Here, the ability of the RbcS promoter to support a much stronger expression of the GFP gene compared to the commonly used CaMV35S promoter was shown. With this novel GFP expression cassette, transgenic plants can be selected at an early stage with less or no dependence on antibiotic selection. This is especially relevant for the transformation of recalcitrant crops.

Conclusion

An efficient protocol for the regeneration of *Chrysanthemum morifolium* '1581' using the leaf discs as

explants was developed. Based on this efficient regeneration, the genetic transformation of two GFP expression cassettes based on RbcS promoter and differential targeting to cytoplasm or plastids was tested. Transgenic primordial and shoots could be identified under a simple fluorescence microscope without GFP filter at a very early stage. In situations of chimeric tissues, dissection of transformed tissues will be easier when red (untransformed) and green (transformed) fluorescence can be viewed simultaneously. Potted plantlets could also be easily recognized by naked eye based on the color change to a lighter green caused by the high expression of the GFP gene. Impactvector 1.4 conducted GFP gene expression at significantly higher levels than Impactvector 1.1 at least partly due to higher steady state levels of GFP mRNA. In general, the RbcS promoter-GFP cassettes, described here can be used more effectively than CaMV35S-based constructs to optimize transformation protocols, because CaMV35S promoter-GFP cassettes express 8 times less in tobacco and very poorly in florist's chrysanthemum and other Composite plants (Outchkourov et al. 2003). Furthermore, it could be used as an alternative selectable marker by creating constructs with this cassette next to a gene of interest. For that purpose, the Impact Vector GFP vectors described here allow insertion of additional expression cassettes in the *Ascl* and *Pacl* restriction sites.

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