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METHOD

Construction and Validation of a Dual-Transgene Vector System for Stable Transformation in Plants

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ABSTRACT

In this study, we constructed dual-transgene vectors (pDT1, pDT7, and pDT7G) that simultaneously co-expressed two genes in plants. *ACTIN2* and *UBQ10* promoters were used to control the expression of these two genes. The *4×Myc*, *3×HA*, and *3×Flag* reporter genes allowed for the convenient identification of a tunable co-expression system in plants, whereas the dexamethasone (Dex) inducible reporter gene *C-terminus of the glucocorticoid receptor* (*cGR*) provided Dex-dependent translocation of the fusion gene between the nucleus and cytoplasm. The function of pDT vectors was validated using four pairwise genes in *Nicotiana benthamiana* or *Arabidopsis thaliana*. The co-expression efficiency of two genes from the pDT1 and pDT7G vectors was 35% and 42%, respectively, which ensured the generation of sufficient transgenic materials. These pDT vectors are simple, reliable, efficient, and time-saving tools for the co-expression of two genes through a single transformation event and can be used in the study of protein–protein interactions or multi-component complexes.

KEYWORDS: Co-expression; Dual-transgene vector; pDT1; pDT7; pDT7G

INTRODUCTION

The relationship between multiple genes or proteins is a major research focus. However, obtaining tools or methods that can provide the efficiency required to transform multiple genes into plant genomes remains a challenge. Several types of traditional methods have been developed, including crossing, co-transformation, and retransformation. Crossing is a traditional approach used to obtain transgenic lines that carry marker-free genes of interest (Dafny-Yelin and Tzfira, 2007). As an example of co-transformation, three wheat genes encoding fructan biosynthesis enzymes were co-transformed into tobacco to increase the plant's tolerance to abiotic stresses (Bie et al., 2012). Similarly, five carotenogenic genes were simultaneously expressed to increase the levels of three vitamins (Zhu et al., 2008), and golden rice transgenic lines were produced by co-transforming two foreign genes, *psy* and *crtI* (Naqvi et al., 2009). As an example of retransformation, an RNA interference (RNAi) construct was retransformed into Gerbera transgenic plants to suppress the activity of flavonoid 3′,5′-hydroxylase (F3′5′H), thereby resulting in the

Abbreviations: cGR, C-terminus of the glucocorticoid receptor; CIB1, cryptochrome 2-interacting basic-helix-loop-helix 1; CRY1, cryptochrome 1; CRY2, cryptochrome 2; Dex, dexamethasone; pDT, dual-transgene vector; PP2-B7, phloem protein 2-B7; SPA1, suppressor of phytochrome A1; TCP2, teosinte-like1, cycloidea, and proliferating cell factor 2.

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accumulation of pelargonidin (Seitz et al., 2007). In addition, double-gene transgenic lines were created by transforming the glyoxalase pathway genes *glyoxalase I* (*gly I*) and *glyoxalase II* (*gly II*) one by one, and they showed increased salt tolerance (Singla-Pareek et al., 2003).

Over the last decade, plasmid co-expression systems have also been used as tools for delivering multiple genes through a single transformation event (Naqvi et al., 2010; Peremarti et al., 2010). A triple vector was constructed containing three polyhydroxybutyrate (PHB) synthesis genes, and the expression of these genes resulted in the accumulation of PHB (up to 4% fresh weight) in transgenic plants (Bohmert et al., 2000). The expression of the secondary wall synthesis regulator genes *SND1* and *NST1* was inhibited by simultaneously expressing their RNAi fragments *via* a single pSND1/NST1-RNAi construct in *Arabidopsis* (Zhong et al., 2007). Seven key enzyme genes (*idi*, *crtE*, *crtB*, *crtI*, *crtY*, *crtW*, and *crtZ*) were cloned into pZK3BCSPS with the help of homing endonucleases in a tandem manner to generate transgenic plants with increased carotenoid and ketocarotenoid (Fujisawa et al., 2009). A binary vector, pTRANS3458, was also developed, and it has the ability to transform six genes into plant genomes with the help of six auxiliary vectors (Goderis et al., 2002).

In this study, we combined expression cassettes harboring two constitutive promoters with different reporter genes into a single plasmid to provide tunable co-expression vectors (pDT1, pDT7 and pDT7G) for different research goals. The co-expression system and co-expression efficiency were validated by expressing several pairwise genes using an in-fusion cloning method (Sleight et al., 2010). The results presented here show that the pDT vectors successfully and efficiently co-expressed two genes in plants in a single transformation event, which allows us to conveniently examine the co-expression of two proteins/genes in plants. Furthermore, pDT7G was used to express *cryptochrome 2* (*CRY2*) and *green fluorescent protein* (*GFP*), which were fused to cGR, thereby leading to a length difference in *Arabidopsis* seedling hypocotyls under blue light and GFP protein translocations between the nucleus and cytoplasm. These results demonstrate the utility of the Dex-inducible co-expression system.

RESULTS

Design and construction of pDT1 and pDT7 vectors

The binary vector pCAMBIA3301-*ACTIN2::3×HA-LUC* derived from pCAMBIA3301 is a high-copy-number, stable, fully sequenced plasmid vector. These characteristics are related to the presence of a replication origin sequence from the plasmid pBR322, which is stably maintained in *Agrobacterium tumefaciens* because of the *rep* and *sta* functions of plasmid pVS1 and carries the *kana* gene that confers resistance to kanamycin in both *E. coli* and *A. tumefaciens*. We used this vector to incorporate two genes into a single vector to create the backbone of pDT vectors. Detailed construction information is provided in the Materials and Methods section. Three independent transcriptional units are included in this vector system, and they are controlled by different promoters and terminators (Figs. 1, S1, and S2). pDT1 and pDT7 vectors, have the same first and second transcriptional units. The first transcriptional unit contains the *bar* gene under the control of the *CaMV*35S promoter, and it confers resistance to the herbicide Basta in plants (White et al., 1990). The second transcriptional unit contains the *ACTIN2* promoter, which is another constitutive promoter from *Arabidopsis*, and it controls the *4×Myc* fusion gene. The third transcriptional unit contains the *UBQ10* promoter, which controls the *3×HA* fusion gene in pDT1 and the *6×His-cGR* fusion gene in pDT7. The truncated rat GR protein cGR (amino acid residues 508 to 795) is sufficient for Dex-dependent nuclear translocation (Yu et al., 2007; Sarmah, 2012).

Co-expression of pairwise genes with pDT1 and pDT7 vectors

To evaluate pDT vector system for dual gene delivery, the cDNAs of *teosinte-like1*, *cycloidea*, and *proliferating cell factor 2* (*TCP2*) and *cryptochrome 1* (*CRY1*) were cloned and inserted into *Spe* I and *Mef* I, respectively, within the pDT1 vector (Fig. 1), to produce the *TCP2-CRY1*-pDT1 construct. A five amino acid linker (GPPPG) (Yu et al., 2007) was designed into the cloning primers and used as a flexible spacer between the target genes and epitope tags. The plasmid was then transformed into *Arabidopsis* and tobacco using *Agrobacterium* transfection. The pDT1 constructs with single-gene inserts (*TCP2-Myc*-pDT1 and *HA-CRY1*-pDT1) were also constructed as controls for transient expression in tobacco. The transgenic tobacco leaves and 20 Basta-resistant T_1 transgenic lines of *Arabidopsis* were used for an expression analysis of *TCP2* and *CRY1*. *TCP2* and *CRY1* were simultaneously co-expressed in both tobacco (Fig. 2A) and *Arabidopsis* (Fig. 2B).

In a manner analogous to constructing *TCP2-CRY1*-pDT1, the *CRY1-TCP2* pair and the *cryptochrome 2-interacting basic-helix-loop helix 1* (*CIB1*) and *phloem protein 2-B7* (*PP2-B7*, hereafter referred to as *B7*) pair, were incorporated into pDT7 to generate the *CRY1-TCP2*-pDT7 and *CIB1-B7*-pDT7 constructs, respectively. Transgenic lines of *Arabidopsis* were first selected using Basta. Immunoblotting of Basta-resistant transgenic lines showed that *CRY1-Myc* and *CIB1-Myc* were over-expressed in *Arabidopsis* (Fig. 2C and E). The mRNA expression levels of *TCP2* and *B7* were higher than those of the wild type (Fig. 2D and F), suggesting that *TCP2* and *B7* were over-expressed in *Arabidopsis*.

Construction of the pDT7G vector

When using the pDT7 vector to stack two genes for transformation into plants, we observed two problems that had to