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Short Communication

pCYOs: Binary vectors for simple visible selection of transformants using an albino-cotyledon mutant in *Arabidopsis thaliana*

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Abstract Several selection markers for the screening of transformants have been developed; however, simple and reliable methods are generally preferred. We have developed a novel visible selection system for the identification of transformants in *Arabidopsis thaliana* that does not require any special reagent and/or equipment except using the albino-cotyledon mutant *cyo1*. In this system, the pCYO vector carrying the *CYO1* genomic fragment as a selection marker is introduced into the *cyo1* mutant. Transformation is performed by the *Agrobacterium*-mediated floral dip method and resultant T_1 seeds are sown in soil. Seedlings with green cotyledons, not albino, are expected to be 'complemented' transformants with the transgene of interest. This system provides a very simple selection method that can be performed without any special equipment, reagent, sterile conditions, or UV illumination. We have constructed three vectors, (1) pCYO1, an empty vector; (2) pCYO2, an overexpression vector carrying CaMV35S promoter; and (3) pCYO3, a vector for genome editing, carrying the CRISPR/Cas9 cassette. Example transformation experiments using these vectors, including genome editing, are shown.

Key words: *Arabidopsis thaliana*, cotyledon, CYO1, selection marker, transformation.

Transformation is an essential technology in molecular biology used to study various organisms including the model plant *Arabidopsis thaliana*. In *Arabidopsis*, transformation is mainly performed by the *Agrobacterium*-mediated floral dip method that does not require sterile plant cultures (Clough and Bent 1998). Antibiotic selection markers are commonly used for selection of transformed plants; however, they do require a sterile culture process. Selection methods for non-sterile plant cultures include the use of herbicides such as BASTA on non-sterile soil (Block et al. 1987) and pFAST, a visible GFP/RFP marker that is driven by a seed-specific promoter (Shimada et al. 2010).

We developed the idea that complementation of visible mutants with wild-type genes could create a simple selection system for transformants. To demonstrate this idea, a mutant whose phenotype could be clearly distinguished from the wild type under normal culture conditions at the early stages of development was required. The ovule is the target of the floral dip transformation, but transgenes carried by the maternal-tissue cells such as seed coat are not transmitted to the progeny (Ye et al. 1999). Therefore, mutants whose phenotype is observed in cotyledons, which are derived from the egg, are the best candidates for this method. Based on this strategy, we have developed a novel visible selection vector system for screening transformants in *Arabidopsis* that uses the albino-cotyledon mutant *cyo1*.

The *cyo1*/*sco2* mutant in *Arabidopsis* has albino cotyledons and normal green true leaves (Albrecht et al. 2008; Shimada et al. 2007). CYO1/SCO2 is a thylakoid membrane-localized protein disulfide isomerase, which interacts with various photosynthesis proteins in the thylakoid membrane and maintains their function (Muranaka et al. 2012; Shimada et al. 2007; Tanz et al. 2012). Its deficiency results in impairment of chloroplast biogenesis. The albino phenotype of *cyo1* is restricted to cotyledons, although some effect on chloroplast function can be observed in true leaves under certain conditions (Zagari et al. 2017). The *cyo1* mutant can be easily distinguished from the wild-type within a week of sowing and transformants are expected to be reliably isolated by 'green/white selection' from a large number of seedlings grown on non-sterile soil.

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR associated protein 9; CTR1, CONSTITUTIVE TRIPLE RESPONSE1; SCO2, SNOWY COTYLEDON 2; GFP, Green Fluorescent Protein; gRNA, guide RNA.

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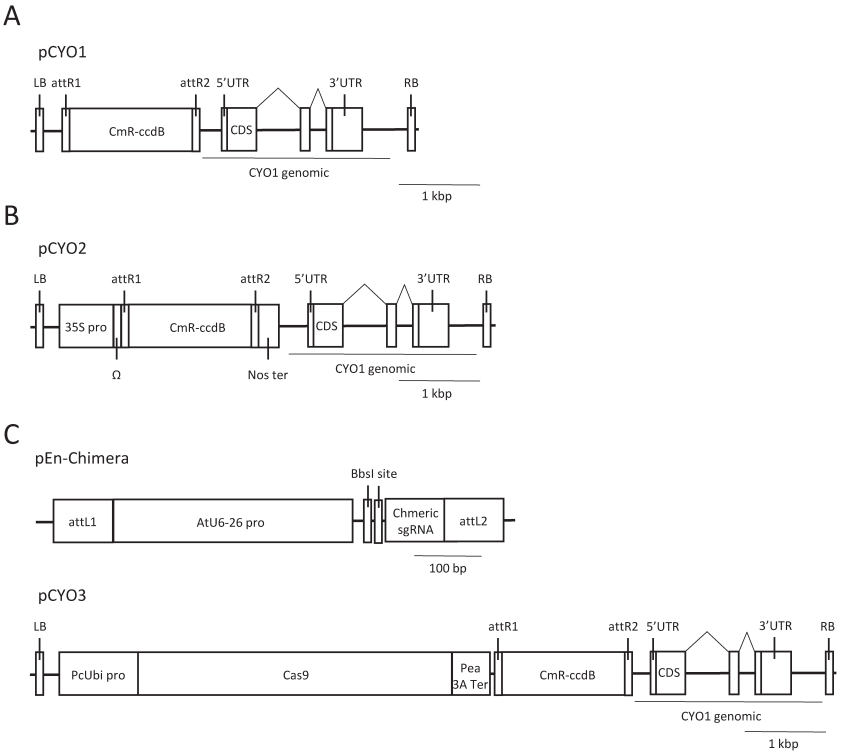

Figure 1. Schematic organization of three purpose-designed variations of the pCYO vector. (A) pCYO1: [(no promoter, no terminator)]. (B) pCYO2: [(CaMV35S promoter, Nos terminator)]]. (C) pEn-Chimera[(entry vector for CRISPR/Cas9)], pCYO3:[(CRISPR/Cas9)]]. ccdB, the negative selection marker for bacteria; CmR, chloramphenicol acetyl transferase; LB, left border; RB, right border; PcUbi pro, Ubiquitin4–2 promoter from *Petroselinum crispum*; Cas9, SpCas9 open reading frame codon-optimized for *A. thaliana*; Pea3A Ter, pea3A terminator from *P. sativum*. *CYO1* genomic fragment is used as a selection marker.

To build this system, a null allele of *cyo1* is required. The original *cyo1* is a null allele but it was isolated by an activation tagging system (Shimada et al. 2007), indicating that it carries enhancer elements and a selection maker gene, which could cause a problem or inconvenience in the experiments and/or analyses. We tried to generate a typical null allele of *cyo1* in Col-0 by CRISPR/Cas9-mediated genome editing. We designed the guide RNA (gRNA) in the second exon of *CYO1*, which corresponds to the conserved C4-type zinc finger motif among CYO1 and related proteins (Shimada et al. 2007; Supplementary Figure 1A). We successfully isolated several lines showing the *cyo1* phenotype in the T_3 generation. One of the lines was found to have a 31-bp deletion in the second exon, which causes a frame shift (Supplementary Figure 1B). We named this probable complete loss-of-function allele *cyo1-11*. To segregate out the CRISPR cassette and off-target mutations, the T_4 generation of this line was backcrossed four times to Col-0. The *cyo1* phenotype was stably observed in the selfed progeny of the backcrossed line, suggesting that this *cyo1* mutant is appropriate for the selection system (Supplementary Figure 1C).

In this study, three binary vectors, pCYO1, pCYO2, and pCYO3, were constructed (Figure 1). Details of vector construction and transformation procedure are described in the Supplementary data. All the vectors adopt the GATEWAY system, and arbitrary sequences can be introduced between attR1 and attR2 by using an entry vector such as pENTR. pCYO1 is the basic binary vector that does not contain a promoter or terminator (Figure 1A). pCYO2 is the binary vector for overexpression in which the gene of interest can be recombined between the CaMV35S promoter and Nos terminator (Figure 1B). pCYO3 is the vector designed for genome editing with CRISPR/Cas9 using a pEn-Chimera carrying a gRNA cassette (Figure 1C; Fauser et al. 2014). As the "selection marker gene", the 2.3-kbp genomic fragment containing the entire *CYO1* gene region was used (Shimada et al. 2007). Since the genomic clone contains its own promoter and terminator, *CYO1* from the transgene is expressed in exactly the same manner as the wild type gene does, and thus can complement the *cyo1-11* albino phenotype.

To examine the effectiveness of the pCYO vector, transformation was carried out using pCYO2-GFP in which *GFP* was recombined into the pCYO2 vector. Seeds transformed by floral dip method using *Agrobacterium* EHA 105 carrying pCYO2-GFP were sown in soil after 4 days of cold treatment. One week after sowing, the cotyledons of most individuals showed an albino phenotype of *cyo1*, but some individuals had

green cotyledons, which are thought to be transformants complemented by the *CYO1* genomic fragment (Figure 2A, B). Only the individuals with green cotyledons emitted GFP fluorescence under ultraviolet irradiation (Figure 2C). These results suggest that the CYO1 visible maker worked in normal soil culture.

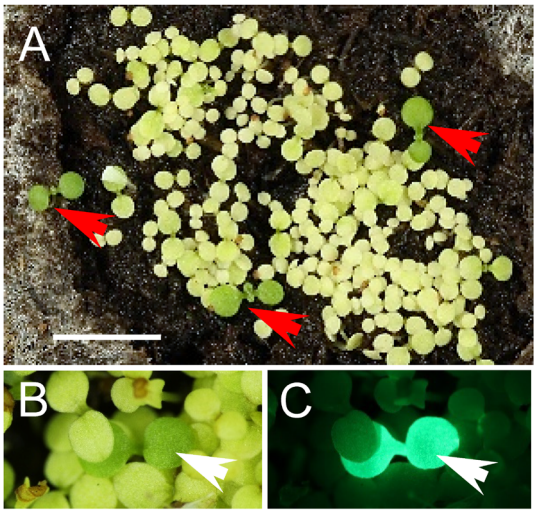

Figure 2. *cyo1-11* T_1 sprouts transformed by pCYO2 harboring GFP. (A) and (B) show the T_1 sprouts, and (C) shows a GFP fluorescence image of (B). Red arrows indicate individuals with green cotyledon, which are possible transformants. The bar indicates 1 mm.

Genome editing was tested using the pCYO3 vector. *CTR1*, a negative regulator of ethylene signaling in *Arabidopsis*, was used as a target gene (Kieber et al. 1993). *ctr1* seedlings show 'triple response' under the dark conditions and retard the growth of small leaf blades under the light conditions. gRNA designed for the first exon of *CTR1* was cloned into the *Bsb*I sites of pEn-Chimera, which is an entry vector for CRISPR/Cas9 system (Figure 3A, (Fauser et al. 2014)). Then, the *CTR1* gRNA cassette was recombined into the destination vector pCYO3 harboring *Cas9*. *cyo1-11* was subjected to floral dip transformation and T_1 seeds were obtained. In the 'naked-eye' screening, 10 independent T_1 individuals with green cotyledons were selected and T_2 seeds from each T_1 individual were harvested. Screening of dark-grown seedlings of the T_2 generation identified *ctr1*-like individuals in 3 of the 10 lines. Sequence analysis of the *ctr1*-like individuals revealed that all the 3 lines had a 1-bp deletion 3 bp upstream of the PAM sequence, which causes frame shifts (Figure 3B). This allele was named *ctr1-201*. Three-day-old dark-grown seedlings and 3-week-old plants of the T_3 generation of *ctr1-201* showed typical *ctr1* phenotypes, suggesting that the traits inherit stably (Figure 3C). These observations suggest that the pCYO3 vector is useful for genome editing of *Arabidopsis*.

In summary, we developed the binary vectors pCYO1,

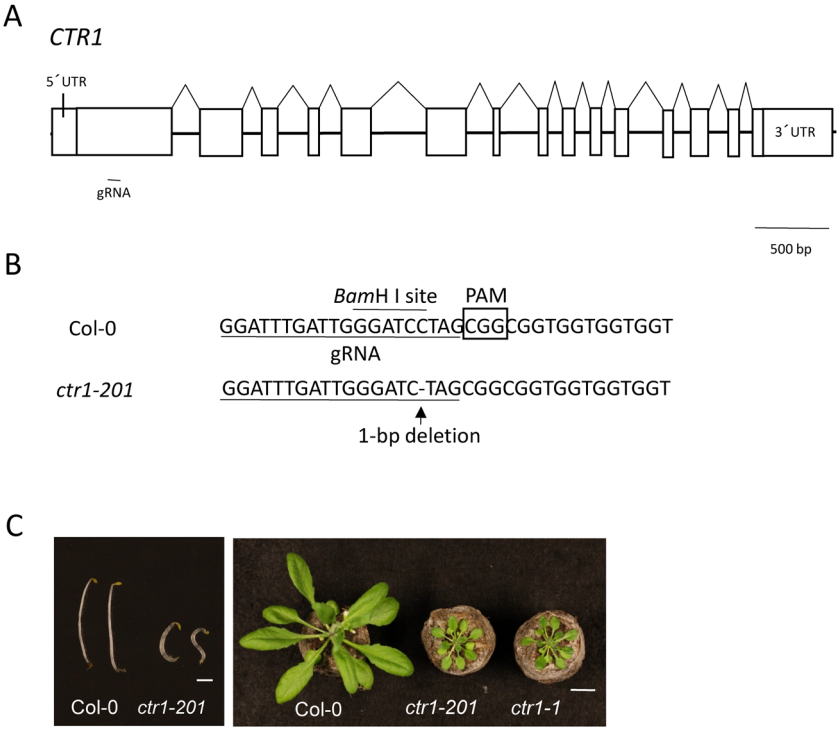

Figure 3. Genome editing using pCYO3. (A) Structure of the *CTR1* gene and guide RNA (gRNA) for CRISPR/Cas9. White boxes show exons. (B) Mutation in *ctr1-201*. The arrow indicates the 1-bp deletion in the first exon of *CTR1*. The box and vertical line show PAM and gRNA, respectively. (C) Phenotype of *ctr1-201*. The left panel shows 3-day-old dark grown seedlings. The bar shows 2 mm. The right panel shows 3-week-old plants grown under the long day condition. The bar shows 1 cm. (C) The phenotype of *cyo1* mutant, *cyo1-11* sprouts have albino cotyledons. Plants were grown for 4 days on 1×MS medium under the long day light conditions. The bar shows 1 cm.

pCYO2, and pCYO3, which have a novel visible selection marker for *Arabidopsis* transformation working in the *cyo1* albino mutant. This marker system is based on the complementation of the *cyo1* mutation by the wild-type *CYO1* genomic fragment (Supplementary Figure 2). The transformants can be easily distinguished from non-transformants by cotyledon color. Thus, pCYO vectors may serve as a user-friendly selection system that does not require any additional equipment and/or reagents except for the *cyo1* mutant. The *cyo1* mutation can be removed through backcross to wild-type and segregation in its progeny, which is particularly useful in genome editing.

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