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 T1 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 (Je 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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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 T3 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 T4 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.

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). ctrl 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 BsaI 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 T1 seeds were obtained. In the 'naked-eye' screening, 10 independent T1 individuals with green cotyledons were selected and T2 seeds from each T1 individual were harvested. Screening of dark-grown seedlings of the T2 generation identified ctrl-like individuals in 3 of the 10 lines. Sequence analysis of the ctrl-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 ctrl-201. Three-day-old dark-grown seedlings and 3-week-old plants of the T3 generation of ctrl-201 showed typical ctrl 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,
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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