A method combining TA cloning and fluorescence screening for rapid acquisition of transgenic seeds

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ABSTRACT

The establishment of transgenic plants has greatly promoted the progress of plant research. However, traditional selection methods using antibiotics or herbicides may miss any positive transformants with growth defects. Additionally, screening with antibiotics/herbicides requires a huge amount of seeds, sterile work conditions and a large amount of space to germinate plants, making the selection process time- and labor-consuming. In this study, we constructed a novel stable transformation vector, plasmid of OLE1-GFP T-DNA vector (pOGT), which can shorten the steps of cloning foreign genes into expression vectors by using TA cloning. Additionally, selection of transformed seeds with fluorescence overcomes the difficulties of conventional selection with antibiotics/herbicides and simplifies the screening process for transgenic plants.

METHOD SUMMARY

We constructed a stable transformation vector, plasmid of OLE1-GFP T-DNA vector (pOGT) with an OLE1-GFP fusion gene driven by the OLE1 promoter (*ProOLE1*) as a selection marker. The OLE1 promoter controls the expression of an OLE1-GFP fusion protein and makes the transformed seeds fluorescent. Digestion of pOGT with *XcmI* generates a single thymidine (T) overhang at the 3' end which enables the introduction of foreign genes into pOGT through one-step TA cloning. We generated transgenic *Arabidopsis* lines transformed with the pOGT-exogenous gene and screened transformed seeds using fluorescence.

KEYWORDS

Arabidopsis thaliana • OLE1 gene • green fluorescent protein (GFP) • selection marker • stable expression vector • TA cloning

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Generation of transgenic plants, especially for the model plant *Arabidopsis thaliana*, is essential for characterization of gene functions and investigation of molecular mechanisms [1,2]. Identification of the transgenic plants is conventionally achieved with a selection marker, which allows transformed plants to grow on agar plates with an antibiotic or herbicide. However, such traditional screening methods have several major drawbacks. First, the expression of some genes may cause developmental growth defects in the transformants, and conventional selection with antibiotic or herbicide may miss such transformed plants [3]. Second, screening for transformants able to grow in the presence of antibiotics/herbicides requires sterile work conditions, a large amount of seeds and space to grow the plants. The development of a system that overcomes these difficulties could greatly facilitate the screening of transgenic plants.

OLE1 (GenBank accession name: At4G25140) is a type of oleosin, which are themselves structural proteins embedded in the membranes surrounding the oil bodies [4]. Studies have shown that OLE1 is specifically localized on the oil body membrane in the seed cells and can be used as a screening marker for *Arabidopsis* [5]. Therefore, a fusion gene with an OLE1 promoter driving a fluorescent reporter (*ProOLE1::OLE1-GFP*) may be used as an endogenous selection marker to identify transgenic *Arabidopsis* at the seed stage. The pCXS vector is a commercial plant-stable expression vector, which contains a *ccdB* lethal gene. Digestion of the pCXS vector with *XcmI* removes *ccdB* and generates a 3'-T overhang, allowing the introduction of foreign genes using TA cloning [6].

In this study, we report a novel technique which combines pCXS plant expression vector as vector backbone with *Pro_{OLE1}::OLE1-GFP* as screening marker. The recombinant vector is named pOGT (plasmid of OLE1-GFP T-DNA vector). pOGT could be used as T-DNA vectors which have 3'-T overhangs after restriction endonuclease digestion. Therefore, TA cloning could be performed for cloning of PCR-amplified fragments into the pOGT vector. The resulting construct could be introduced into *Arabidopsis*, and the transformed seeds could be screened by fluorescence.

MATERIALS & METHODS

Plant materials & growth

Arabidopsis thaliana seeds (Col-0) were sterilized with 70% ethanol and incubated at 4°C for 2–3 days to break seed dormancy, then sown on nutrient soil for 1 week. The seedlings were transferred to 10-cm² pots at a density of four plants/pot for growth. *Arabidopsis* plants were grown at 22°C under long-daylight conditions (light 9–12 h/day) with sufficient fertilizers to obtain a large number of flowers. The seeds were provided by Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresources.

Plasmid DNA construction of the pOGT vector

The forward and reverse primers pOLE1-F and pOLE1-R (Table 1) were designed based on the *Arabidopsis Pro_{OLE1}::OLE1* sequence from The Arabidopsis Information Resource website [7]. Extraction of *Arabidopsis* genomic DNA was performed according to the cetrimonium bromide (CTAB) method [8]. The *Pro_{OLE1}::OLE1* gene was amplified by PCR using the *Arabidopsis* genomic DNA and specific primers pOLE1-F and pOLE1-R. The

Table 1. Primer sequences used in this study.

Name	Forward primer sequence	Reverse primer sequence	Function	Product size (bp)
pOLE1-F/R	ACGAACGATACTCGAGCACCCCTACTTAGATCAACACATA	ACGAACGATACTCGAGCACCCCTACTTAGATCAACACATA	Cloning <i>Pro_{OLE1}::OLE1</i>	2136
pOGT-F/R	GCAGGCATGCAAGCTTCACCCCTACTTAGATCAACACAT	GGCCAGTGCCCAAGCTTCTTGACAGCTCGTCCATG	Cloning <i>Pro_{OLE1}::OLE1-GFP</i>	2868
PM-XcmI-F/R	TCGGTCTTGGTCACACAAGGAAGAACTCTCTGGTAA	TTACCAGAGAGTTCCCTTGTTGTGACCAAGACCCGA	Site-specific mutation of <i>XcmI</i> in <i>OLE1</i> promoter	13759
PA30-F/R	ATGTCGGCTGAAACACTTTCTT	TTAAAATTGGCCTCCTTCAAAGGTC	Cloning <i>MeEXPA30</i> gene	755
Q-GFP-F/R	CGACCACTACCAGCAGAACA	TCTCGTTGGGGTCTTTTGCTC	Amplification of <i>GFP</i> fragment	108
Q-ATC2-F/R	GGTAACATTGTGCTCAGTGGTGG	GGTGCAACGACACCTTAATCTTCAT	Reference gene primer of <i>Arabidopsis thaliana</i>	188
GFP-F/R	ACAGTTTGCCAGTGATACACA	AAAGTTCGACAGCGTCTCC	Amplification of <i>GFP</i> fragment	272
HygR-F/R	TACCCCGACCACATGAAGC	TTCACTTGATGCCCGTTCTT	Amplification of <i>HygR</i> fragment	444

pA7-GFP vector (BioVector) was digested with *XhoI* and *SaII* (New England Biolabs), and the resulting 4591-bp fragment was purified by gel recovery using the Omega gel extraction kit following the manufacturer's instructions. Next, the *Pro_{OLE1}::OLE1* fragment and pA7-GFP fragment were linked by homologous recombination using Vazyme ClonExpress II one-step cloning kit.

The primers pOGT-F and pOGT-R were designed according to the sequence of *Pro_{OLE1}::OLE1-GFP* (Table 1). The *Pro_{OLE1}::OLE1-GFP* gene was amplified by PCR using the pA7-OLE1-GFP plasmid as a template and the corresponding primers. The pCXSN vector [6] was digested with *HindIII* (New England Biolabs), the 10,802-bp fragment was purified by gel recovery (Omega). After that, the *Pro_{OLE1}::OLE1-GFP* fragment and digested pCXSN fragment were linked by homologous recombination (Vazyme), generating pCXSN-OLE1-GFP.

The *XcmI* recognition site of the *OLE1* promoter on the pCXSN-OLE1-GFP vector was eliminated by site-specific mutation, using the primers (PM-XcmI-F, PM-XcmI-R) listed in Table 1, according to the method of the Quick-Mutation™ Site-Directed Mutagenesis Kit (Beyotime). The complete sequence of pOGT was deposited in NCBI with the accession number BankIt2292911 pOGT MN832866.

Plasmid DNA construction of pOGT-MeEXPA30 vector

MeEXPA30 (Manes.S055800) was amplified by PCR using DNA polymerase (Takara primeSTAR GXL DNA polymerase) and primers (PA30-F, P30-R) listed in Table 1. The A-addition procedure for the amplified product was performed using the Takara DNA A-Tailing Kit. The plant expression vector pOGT was digested with *XcmI* restriction enzyme (New England Biolabs) to obtain a T-DNA vector. The digested pOGT plasmids and the *MeEXPA30* were purified from agarose gels after electrophoresis using the Omega gel extraction kit and ligated with T4 DNA ligases (Thermo). The ligation reaction was carried out in a total volume of 20 μl containing 60 ng of T-DNA vector and the corresponding volume of PCR product with an insert-to-vector molar ratio of around 3:1. The ligation reaction mixture was transformed into *Escherichia coli* strain *Trans1-T1* (TransGen Biotech, Beijing, China) by heat-shock transformation (42°C, 30 s).

All recombinant plasmids identified from positive colonies were verified by sequencing.

Transformation of *Arabidopsis* plants

The pCXS-OLE1-GFP, pOGT and pOGT-MeEXPA30 plasmids were introduced into *Agrobacterium tumefaciens* GV3101 (Weidi Biotechnology, Shanghai, China) by a freeze-thaw method. *Arabidopsis* Col-0 plants were transformed via the floral-dip method as described previously [9].

Screening of transgenic *Arabidopsis*

The dried seeds of wild-type (Col-0) and transformed *Arabidopsis* were collected and screened under fluorescence microscope (Leica M205FA/DFC550). Transgenic *Arabidopsis* seeds (T_1 seeds) with green fluorescent protein (GFP+) were grown on soil media (vermiculite:nutrient soil = 1:1), generating T_1 transgenic plants. Seeds collected from T_1 plants were T_2 seeds. For genotyping T_1 plants, the genomic DNA of T_1 plants was extracted by the CTAB method [8]. Amplification of $Pro_{OLE1}::OLE1$ -GFP, MeEXP30, GFP, HygR and *Actin2* in T_1 plants was performed using the corresponding primers listed in Table 1.

Quantitative real-time PCR

For examining the transcript levels of GFP in plants transformed with pOGT and pCXS-OLE1-GFP, the total RNA was extracted from leaves of 2- to 3-week-old plants with the RNA-plant Plus Kit (Tiangen Biotech, Beijing, China), and reverse transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China) following the product instructions. Levels of GFP mRNA in these two transgenic plants were detected using the SYBR real time PCR Kit (TaKaRa, Dalian, China). Three independent experiments were performed. Expression levels were calculated using the double-standard curves method [10]. *ACT2* (GenBank accession name: AT3G18780) was used as the reference gene for normalization [11] and the primers for amplification are listed in Table 1. Statistical analyses were performed using two-sample unpaired *t*-tests.

RESULTS & DISCUSSION

Construction of pOGT vector

A schematic illustration of the pOGT construction is shown in Figure 1. The fragment of *OLE1* gene with its native ▶

Figure 1. Schematic illustration of pOGT construction. The $Pro_{OLE1}::OLE1$ fragment was amplified from the *Arabidopsis* genomic DNA and cloned into the pA7 vector. The fragment of $Pro_{OLE1}::OLE1$ -GFP was amplified from the recombinant pA7 vector and cloned into the pCXS vector. The *XcmI* site in the promoter of *OLE1* was mutated, resulting in the final construct of the pOGT vector. In the primers, the 16-bp upstream sequence from restriction sites on the vector is represented by lowercase letters, and the restriction sites are represented by red lowercase letters. pOGT: Plasmid of OLE1-GFP T-DNA vector; $Pro_{OLE1}::OLE1$: *OLE1* promoter driving a fluorescent reporter.

Figure 2. Fluorescence and molecular detection of transgenic *Arabidopsis* seeds transformed with the pOGT and pCXSN-OLE1-GFP vectors. (A–E) Seeds viewed under bright-field microscopy. (F–J) The same fields under fluorescence microscope. (A & F) Col-0 (WT) *Arabidopsis* seeds. (B & G) T₁ generation seeds of pOGT transgenic *Arabidopsis*. (C & H) T₂ generation seeds of pOGT transgenic *Arabidopsis*. (D & I) T₁ generation seeds of pCXSN-OLE1-GFP transgenic *Arabidopsis*. (E & J) T₂ generation seeds of pCXSN-OLE1-GFP transgenic *Arabidopsis*. (K) Quantification of transformation efficiency represented by the ratio of fluorescent seed versus the total seed number in each random detection view under the microscope. Graphed are means $\pm$ SE, representative of three independent replicates ($n = 3$). Lowercase letters indicate significant groupings ($p < 0.01$) using the *t*-test. T₂ generation seeds were the progeny of the T₁ seeds with the strongest fluorescence intensity. (L) mRNA levels of GFP measured by quantitative real-time PCR from leaves of 2–3-week-old Col-0 (WT) and transgenic *Arabidopsis* transformed with pOGT and pCXSN-OLE1-GFP. Transcript levels were normalized to the amount of ACT2 transcripts detected in each sample. Data were pooled from 3 replicates ($n = 3$). Lowercase letters indicate significant groupings ($p < 0.01$) using the *t*-test. (M) PCR identification of T₁ transgenic plants expressing pOGT. Ten independent primary plants (Lanes 1–10) were selected for the analysis. Lane 11 shows no amplification of *Pro_{OLE1}::OLE1-GFP* gene from *Arabidopsis* Col-0 (wild-type). Lane 12 shows no amplification of *Pro_{OLE1}::OLE1-GFP* gene without template (negative control). pOGT: Plasmid of OLE1-GFP T-DNA vector; *Pro_{OLE1}::OLE1-GFP*: OLE1 promoter driving a fluorescent reporter; WT: Wild type.

promoter was amplified from the *Arabidopsis* genome by PCR (Figure 1 & Supplementary Figure 1A). The forward primer contained a 16-bp upstream sequence and the sequence of *XhoI* restriction sites on the pA7 vector, and

the reverse primer contained a 16-bp downstream sequence and the sequence of *SalI* (Figure 1). The amplified PCR product was inserted into the pA7 vector in the position between *XhoI* and *SalI* by homol-

ogous recombination (Figure 1 & Supplementary Figure 1B & C). The fragment of *OLE1* promoter::*OLE1-GFP* (*Pro_{OLE1}::OLE1-GFP*) was amplified from the recombinant pA7 plasmid with primers containing 16-bp upstream/

Figure 3. Heterologous expression of cassava *MeEXPA30* in *Arabidopsis* using the pOGT vector. (A) Diagrammatic representation of the pOGT-MeEXPA30 construction. The fragment of *MeEXPA30* was amplified from cassava genome and cloned into the pOGT vector through TA cloning. (B & C) Images from bright-field microscopy. (D & E) The same fields under fluorescence microscope. (B & D) T₁ generation seeds of pOGT-MeEXPA30 transgenic *Arabidopsis*. (C & E) T₂ generation seeds of pOGT-MeEXPA30 transgenic *Arabidopsis*. (F) Quantification of transformation efficiency represented by the ratio of fluorescent seeds versus the total seed number in each random detection view. Graphed are means $\pm$ SE, representative of three independent replicates ($n = 3$). (G) PCR identification of T₁ transgenic plants expressing pOGT-MeEXPA30. Ten independent primary plants (Lanes 1–10) were selected for the analysis. Lane 11 shows no amplification of *MeEXPA30* from *Arabidopsis* Col-0 (wild-type). Lane 12 shows the amplification of *MeEXPA30* from pOGT-MeEXPA30 plasmid. pOGT: Plasmid of OLE1-GFP T-DNA vector.

downstream sequences from the *Hind*III site, and introduced into the stable transformation vector pCXS_N, generating the construct of pCXS_N-OLE1-GFP (Figure 1 & Supplementary Figure 1D–F). Because the promoter of *OLE1* contains an *Xcm*I recognition site, which would affect the vector used as *Xcm*I digestion-generated T-DNA vector, we mutated the *Xcm*I site by site-directed mutagenesis (Figure 1A & H). *Xcm*I digestion of the vector with the mutated promoter (pOGT vector) generated two bands, whereas digestion of the construct with the original promoter (pCXS_N-OLE1-GFP) resulted in three bands, confirming the elimination of the *Xcm*I site in the OLE1 promoter (Supplementary Figure 1G). Thus we generated the final

construct of the pOGT vector.

Generation of *Arabidopsis* transgenic lines transformed with pOGT vector

To investigate the feasibility of generating transgenic lines transformed with the pOGT vector, we introduced the vector into *Arabidopsis* via *A. tumefaciens*-mediated floral-dip method. The fluorescence of T₁ seeds was inspected under the fluorescence microscope (Figure 2B & G). Only 0.5% of the T₁ seeds exhibited green fluorescence (Figure 2B & G & K). T₁ seeds with strong green fluorescence were chosen for the propagation of next generation (T₂) seeds. Compared with T₁ seeds, the proportion of fluorescent seeds in the T₂ generation

increased to 26% (Figure 2C & H & K). To examine the introduction of Pro_{OLE1}::OLE1-GFP, ten independent T₁ plants were selected for PCR analysis using their genomic DNA. Amplifications of the full length of Pro_{OLE1}::OLE1-GFP were detected in all ten plants, confirming the results of fluorescence detection (Figure 2M).

Because we made the mutation in the *OLE1* promoter within the pOGT vector, we also transformed the construct with the original promoter (pCXS_N-OLE1-GFP) to compare the effect of the mutation on the transformation efficiency and the expression activity. Fluorescence detection showed that seeds transformed with pOGT vector and pCXS_N-OLE1-GFP had similar rates of fluorescent

seeds in both T_1 and T_2 seeds (Figure 2A–K). To examine the expression activity of the mutated *OLE1* promoter, we compared the expression level of *GFP* in T_1 plants expressing pOGT versus pCXS-*OLE1*-*GFP*. The results of qPCR showed that the transcript levels of *GFP* were similar in the transgenic lines transformed with these two constructs (Figure 2L). These results indicate that the mutation does not affect the transformation efficiency or activity of the *OLE1* promoter.

To compare the effectiveness of fluorescent screening versus hygromycin selection, 20 independent T_1 plants were selected for PCR analysis using their genomic DNA. Amplifications of a fragment of *GFP*, *HgyR*, were detected in all 20 plants (Supplementary Figure 2), confirming the equal effectiveness of these two methods.

Heterologous expression of cassava *MeEXPA30* in *Arabidopsis* using pOGT vector

To investigate the utilization of pOGT vector to introduce foreign genes into *Arabidopsis*, we generated transgenic lines expressing cassava *MeEXPA30*. The vector pOGT was first digested with *XcmI* restriction enzyme. The fragment of *MeEXPA30* was amplified from cassava genome followed by the addition of adenosine overhangs at the 3' end of the PCR products. Thus, the PCR fragments could be directly cloned into *XcmI*-digested pOGT vector through TA cloning (Figure 3A). The construct of pOGT-*MeEXPA30* was transformed into *Arabidopsis*, and a similar screening process as for the plants transformed with pOGT was performed. Fluorescence detection showed that nearly 0.5% of T_1 seeds and 28% of T_2 seeds had fluorescence (Figure 3B–F). We also examined the amplification of *MeEXPA30* by PCR analysis using genomic DNA from T_1 plants. Ten primary individual plants had showed the correct amplification, confirming transformation. These results demonstrate that the pOGT vector is an effective system to generate transgenic *Arabidopsis* lines expressing foreign genes.

In recent years, fluorescent proteins have been widely used in screening transgenic seeds and plants [12–14]. Unlike methods using 4-methylumbelliferyl- β -D-glucopyranoside or Pdf2.1::DSRED as a selection marker, the pOGT system does not require pretreatments to induce the

expression of fluorescent proteins. Although *OLE1*-*GFP* has also been previously used as a screening marker in the fluorescence-accumulating seed technology (FAST) method [5], the FAST method uses the gateway cloning system to introduce the foreign genes. This method contains two steps: cloning the DNA fragment into a general donor plasmid and transferring into expression vectors [5]. Such two-step cloning and expensive reagents limit the gateway system to use for large-scale cloning [6]. The pOGT system we developed in this study utilizes a one-step TA cloning strategy for engineering the expression construct, simplifying the cloning method.

FUTURE PERSPECTIVE

The *Agrobacterium*-mediated floral-dip method was originally used for generating transgenic *Arabidopsis thaliana*. In recent years, it has also been used for other plants such as wheat [15]. Therefore, the principle of pOGT may also be applied to other oil-rich plants, giving the pOGT system broader impact.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0141

AUTHOR CONTRIBUTIONS

L-J Luo, Q Liu, L-Y Jiang and M Li designed the research. M Li and Q Liu performed the experiments. M Li, Q Liu, L-Y Jiang, Y-H Wu, L-J Luo, Y-H Chen and L-J Luo analyzed the data. L-Y Jiang and M Li wrote the paper. All authors contributed to writing this manuscript and approved of its final version.

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