Development of the binary vector pTACAtg1 for stable gene expression in plant: Reduction of gene silencing in transgenic plants carrying the target gene with long flanking sequences

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Abstract  Genetic modification in plants helps us to understand molecular mechanisms underlying on plant fitness and to improve profitable crops. However, in transgenic plants, the value of gene expression often varies among plant populations of distinct lines and among generations of identical individuals. This variation is caused by several reasons, such as differences in the chromosome position, repeated sequences, and copy number of the inserted transgene. Developing a state-of-art technology to avoid the variation of gene expression levels including gene silencing has been awaited. Here, we developed a novel binary plasmid (pTACAtg1) that is based on a transformation-competent artificial chromosome (TAC) vector, harboring long genomic DNA fragments on both sides of the cloning sites. As a case study, we cloned the cauliflower mosaic virus 35S promoter:β-glucuronidase (35S:GUS) gene cassettes into the pTACAtg1, and introduced it with long flanking sequences on the pTACAtg1 into the plants. In isolated transgenic plants, the copy number was reduced and the GUS expressions were detected more stably than those in the control plants carrying the insert without flanking regions. In our result, the reduced copy number of a transgene suppressed variation and silencing of its gene expression. The pTACAtg1 vector will be suitable for the production of stable transformants and for expression analyses of a transgene.

Key words: binary vector, gene expression, gene silencing, TAC vector, transgenic plants.

Introduction  Genetic transformation in plants sheds lights on new functions of genes. The transgenic technology provides “instant” breeding without reciprocal crossing and classical selection, and increases speeds to improve crops. Genetically modified plants open up new opportunities for the production of food nutrients, beneficial metabolites, and biomaterials. However, as transferring DNA into plants is normally carried out via random insertion by a Rhizobium radiobacter [former name, Agrobacterium tumefaciens (Agrobacterium)]-mediated genetic transformation or DNA bombardments (Chen et al. 1998; Clough and Bent 1998; Klein et al. 1992), the value of transgene expression sometimes varies widely among independent lines and generations (Kohli et al. 1998).
2 Stable transformation with a novel binary vector

Adjusted amounts of proteins or enzymatic products produced by the transgene often vary from those expected (Mlynarova et al. 1996; Odell et al. 1985). The reduced products cause some difficulties in applying the benefits to basic and applied sciences.

Chromatin structure at the site of integration often influences transgene expressions; transcription is active around euchromatin but inactive near heterochromatin (Ojolo et al. 2018). When a transgene is integrated into heterochromatin, transcription factors could not reach the promoter region. The transcriptional silencing affected by the integration locus of a transgene is termed as a position-effect variation. The position effects are observed among many species, such as yeast, insects, mammals, and plants (Iglesias et al. 1997; Matzke and Matzke 1998; Schubert et al. 2004; Sherman and Pillus 1997; Wallrath 1998).

Other reasons for the variation in transgene expression levels are ascribable to the copy number of the transgenes in plants. Either Agrobacterium-mediated transformation or direct gene transfer via electroporation into plant cells often introduces multiple copies of the transgene (Schubert et al. 2004). The copy number affects the expression levels; increased copy number sometimes simply provides more gene products, however tandem repeated transgenes are often silenced in plants (termed as repeat-induced gene silencing (RIGS)) (Ye and Signer 1996). Increasing copy number also results in homology-dependent gene silencing (HDGS) (Davies et al. 1997). An artificially introduced gene sometimes interacts with endogenous or exogenous nucleic acid molecules, both DNA and RNA, and is silenced by endogenous duplicated sequences: DNA-DNA, DNA-RNA, and RNA-RNA interactions result in the gene downregulation such as transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). RNA-induced silencing complex (RISC) containing 21-24 nucleotide (nt) long sRNAs mediates the TGS and PTGS, which normally occurs when a transgene is under the control of a strong promoter (Baulcombe 2004; Day et al. 2000; Guo et al. 2016). The complete inhibition of transgene expressions is often observed (Elmayan et al. 1998). These silencing effects cause variation of transgene expressions among independent individuals of transgenic plants. To avoid the gene silencing, the copy number of the transgene should be minimized.

To control the transgene expressions, a couple of methods such as an in planta gene targeting and introducing DNA with an insulator sequence have been attempted so far (Fauser et al. 2012). An in planta gene targeting is a method based on site-directed homologous recombination (Paszkowski et al. 1988; Puchta and Fauser 2013). However, despite recent advances in gene-editing technologies (Nishizawa-Yokoi et al. 2012; Osakabe et al. 2010), the recombination rate is relatively low to produce transgenic plants. Another approach to reduce the variation of transgene expressions is developed by utilizing an insulator, which is chromatin boundary elements, such as matrix-attachment regions (MARS), to prevent mislocalization of transgenes in heterochromatin (Breyne et al. 1992; Nagaya et al. 2001). Though it was reported that the vector with an insulator at the borders of T-DNA indeed reduced the variability in β-glucuronidase (GUS) activities (Nagaya et al. 2001), the copy number of a transgene is still uncontrollable. More effective and longer flanking regions are awaited.

A transformation-competent artificial chromosome (TAC) vector shuttles a more-than-100 kb DNA fragment between Escherichia coli and Agrobacterium, and integrates the large DNA fragment into a plant genome (Liu et al. 1999). This vector has the advantage of stable transformation with the long DNA fragment in Agrobacterium. Previously, we reported an Arabidopsis thaliana (Arabidopsis) genomic library cloned into the TAC vector (Hirosue et al. 2015; Liu et al. 1999). Here, to prevent the variation of transgene expressions and copy number, and to achieve stable expressions of a foreign gene, we developed the TAC-clone-based binary vector, pTACAtg1, with native genome fragments next to both sides of the cloning sites; this vector includes +41 kb and +32 kb flanking sequences (Figure 1). Utilizing the pTACAtg1 vector can prevent both increase of copy number and the variation of transgene expressions.

Materials and methods

Materials and plant growth condition

A TAC clone K8K14 was isolated from an Arabidopsis TAC genomic library [accession number AB007645 (Nakamura et al. 1997)]. Host E. coli cell DH10B and restriction enzymes were purchased from Invitrogen (Carlsbad, USA) and Toyobo (Osaka, Japan). Arabidopsis Wasselewskija (Ws) plants were grown at 22°C under long-day conditions (16 h Light/8 h Dark cycles) for 1 month for the Agrobacterium-mediated transformation; the Agrobacterium used in this study is a Rhizobium radiobacter strain C58C1. The constructs on binary vectors were introduced into Arabidopsis plants by

![Figure 1. Schematic representation of the pTACAtg1 vector](image)

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the Agrobacterium-mediated dipping method (Clough and Bent 1998). T1 plants were selected on 20 mg l⁻¹ hygromycin plates. Amounts of sown seeds were calculated as 10,000 seeds per 200 mg. To measure the GUS activity, plants were grown for 10 days on 1/2 Murashige-Skoog (MS) salt-1% sucrose plates including vitamins and 15 mg l⁻¹ hygromycin, and then transferred onto the 1/2 MS-1% sucrose plates without antibiotics.

**Vector construction for stable gene expression**
The K8K14 genomic fragment was originally cloned at the HindIII site on the pYLTAC7 vector. The TAC clone K8K14 was digested with AscI and SrfI, filled with a T4 DNA polymerase to create blunt ends, and self-ligated with a Takara ligation kit (Takara, Otsu, Japan). Similarly, we removed FseI and NotI sites by digestion and self-ligation. To introduce a DNA cassette coding rare-cut restriction enzyme sites, SfiI-AscI-SfiI-SrfI-FseI-NotI, the K8K14 clone was digested with SfiI; two SfiI recognition sites were located on the K8K14 DNA sequence as described. The DNA cassette, GCC CAC TCG CCG CCG CCG CCG CCG GCC TAA TTG GCC GCC GCC CCT GCA GCC TAT GCC C, was introduced into SfiI sites on the K8K14. As a control vector, the AscI, SfiI, SrfI, FseI, and NotI sites on the pYLTAC7 were removed by digestion and filled with a T4 DNA polymerase (TaKaRa), and the DNA cassette coding rare-cut restriction enzyme sites described above was inserted into the HindIII site on the TAC vector. As a reporter, a GUS cassette from pSGL2 (Kato et al. 1991) was cloned between AscI and NotI in these vectors. The resultant plasmids were introduced into E.coli DB10B cells via electroporation with a GenePulser (Bio-Rad, Hercules, USA); the electric field condition is 2.5 kV/0.2 cm, 25 µF, and 100 Ω. The plasmids for stable expression were introduced into Agrobacterium cells by electroporation with the GenePluser (Bio-Rad, Hercules, USA); the plasmids for stable expression were introduced into Agrobacterium cells by electroporation with the GenePluser; 2.5 kV/0.2 cm, 25 µF, and 100 Ω.

**Assay of GUS activity**
Leaves from 28-day-old plants were harvested and frozen in liquid nitrogen. Samples were ground to a fine powder under liquid nitrogen and mixed with an extraction buffer [0.1 M phosphate buffer (pH 7.8), 2 mM EDTA, 5% glycerol, 2 mM dithiothreitol (DTT)]. The samples were centrifuged at 18,000 g for 5 min at 4° C, and then the supernatants were collected. For fluorometric assay, a 4-methylumbelliferyl-β-D-glucuronide (4-MUG) (final 1 M) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the supernatants and incubated at 37° C. The enzyme activity was measured by using a multiwavelength fluorescence reader GENios (Tecan, Männedorf, Switzerland) with 360 nm excitation and 465 nm emission filters. The activity was estimated by the amount of products, 4-MU, synthesized from the 4-MUG per min by 1 mg protein.

**Estimation of copy number of a transgene**
Genomic DNA was isolated with a DNeasy Plant Mini Kit (QIAGEN, Maryland, USA) and quantified with a PicoGreen dsDNA Quantitation Kit (Molecular Probe, Eugene, USA). Real-time quantitative PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Forster City, CA, USA) and a TaqMan probe quantitative PCR kit (Applied Biosystems). Gene-specific primers and labeled probes were designed with a Primer Express Version 1.0 (Applied Biosystems) (Supplementary Table S1). To estimate the copy number, DNA fragments amplified from Arabidopsis PHOTOSYNTHETIC ELECTRON TRANSFER C (PETC) gene (At4G03280) were used as a standard molecular. Each value was quantified in comparison with the standard molecular amounts and estimated as a round number (Supplementary Table S2).

**Genomic Southern blot analysis**
Southern blot analysis was performed as described previously (Sambrook and Russell 2001). We selected 7 lines that were expected to exhibit a single copy of the GUS fragments confirmed by qPCR. Genomic DNA from the 7 lines and control plants (Ws and plants with the TAC clones, pYLTAC7-K8K14) were isolated. Five micrograms of each of the DNA were digested to completion with a restriction enzyme PstI, separated in 1% agarose gels, and transferred to a Hybond-N nylon membrane (Amersham, Buckinghamshire, UK). DNA probes shown in Figure 7A were prepared by PCR amplification with oligonucleotide primers (Supplementary Table S1), and labeled with [α-³²P]-dCTP by using a Takara BcaBEST™ Labeling Kit (Takara). Stringent wash was conducted at 65°C in 0.1X Saline Sodium Citrate Buffer (SSC) containing 0.1% SDS.

**Detecting small RNAs in northern hybridization**
Small RNA was isolated with a Concert Plant RNA Reagent according to the manufacturer manual (Invitrogen). The RNA was centrifuged at 18,000 × g, for 10 min at 4° C, in 0.5 M NaCl-5% polyethylene glycol mol wt 8000 solution, to remove larger RNAs, then small RNAs were collected as the supernatant. RNA was separated on 15% polyacrylamide gel and blotted on a Hybond N+ (Amersham, Little Chalfont, Buckinghamshire, UK) by UV-crosslinking. Antisense GUS RNA was detected in hybridization with a ³²P-labeled sense RNA probe prepared in a MAXIscript In vitro transcription Kit (Ambion, Huntington, Cambridge, UK).

**Results**
To achieve a stable transformation, the pTACAtg1 vector was constructed
We hypothesized that a transgene with long-franking regions would be stably transcribed by avoiding the positional effect around the integrated regions and multiple insertions causing transcriptional or post-transcriptional gene silencing. First, we chose a TAC clone K8K14 with a 72,698 bp insert from
the Arabidopsis TAC library (Hirose et al. 2015; Liu et al. 1999). The K8K14 includes two recognition sequences, GGC C NN NN NG GCC, by a rare-cut enzyme SfiI with an approximately 300-base interval in the middle of clone; the GGC C AC TCA GG CC and GGC C AC TAT GG CC sequences are located at the positions from 31,565 to 31,577 and from 31,833 to 31,845 on K8K14 (Accession #AB007645), respectively (Supplementary Figure S1). The position from 31,565 to 31,845 is estimated as a non-coding region between At5g67370 and At5g67380, which encode an unknown protein, annotated as a conserved in the green lineage and diatoms 27 protein (CGLD27), and a casein kinase II subunit (CKA1), respectively. As both these genes located at the site are expressed under various conditions from the data in microarray experiments (Hanano et al. 2002; Yamada et al. 2003), the locus is plausible not to be influenced by the gene silencing. Thus, we considered that the K8K14 is suitable for construction of a novel vector with the long flanking regions to provide stable expression of a transgene.

For the construction, we removed A scI, SfiI, SrfI, FseI, and NotI sites at the cloning sites on both flanking regions of K8K14 (originally located on the pYLTAC7), and inserted a cassette coding cloning sites, SfiI, Ascl, SfiI, SrfI, FseI and NotI between the SfiI sites on the middle of the K8K14 insert (Supplementary Figure S1). The resultant plasmid was termed as pTAC_Arabidopsis_thaliana_genome_1 (pTACAtg1), which is the binary vector with long genomic sequences at both ends of the cloning site (Figure 1). A TAC vector (pYLTAC7-cs1) without K8K14 sequences but with the same cloning sites was also prepared as a control.

To investigate whether the pTACAtg1 provides a stable expression of a transgene, we cloned a cauliflower mosaic virus 35S promoter:GUS (35S:GUS) fusion gene from pSLG2 (Kato et al. 1991) into the both binary vectors, pTACAtg1 and pYLTAC7-cs1 as a pilot experiment. The GUS cassette also included a 5′ untranslated region (UTR) of a soybean lipoxygenase gene to enhance the translation and a NOS terminator. The 35S:GUS cassettes with or without long genomic fragments (on pTACAtg1 or pYLTAC7-cs1, respectively) were introduced into Arabidopsis Ws ecotype plants by an Agrobacterium-mediated dipping method. By selection on hygromycin-containing plates and by confirmation of the GUS fragment in PCR amplification described below, we obtained 57 and 80 of pTACAtg1 and control lines carrying the GUS cassettes (pTACAtg1-GUS and pYLTAC7-GUS), respectively.

The pTACAtg1 vector reduced the copy number

To investigate whether the pTACAtg1 vector reduced the copy number of transgenes, we measured the copy number and the GUS activity. The copy number was estimated in quantitative PCR (qPCR) (Figure 2). Most of the pTACAtg1-GUS plants only exhibited a couple of copies; in our study, more than half lines had less than three copies. In the pTACAtg1-GUS lines, maximum 8 copies were detected, while the copy number varies widely between 1 to 23 copies in the control pYLTAC7-GUS lines. A single copy was confirmed in 35% of pTACAtg1-GUS plants (vs. only 17% in the control); the plants carrying a single copy of transgene were 19 out of 57 pTACAtg1-GUS and 14 out of 80 control lines, respectively. In transformation by using a common binary vector, multiple copies of a transgene are ordinarily observed, and the copy number of foreign DNA often affects its gene expression, such as gene silencing. In fact, more than 80% of the control plants (pYLTAC7-GUS) carried multiple copies of the transgene. In contrast, the copy number was reduced and less variable in the pTACAtg1-GUS plants.

The pTACAtg1 vector suppressed variation of expression levels and gene silencing

To make it clear about the correlation between copy numbers and expressions, we assayed the GUS activity from these plants (Figures 3, 4). In pTACAtg1-GUS plants, the GUS activity was detected at the range between 0 to 600,000 pmol of 4-methylumbelliferone (4-MU) min⁻¹ mg⁻¹ protein. All pTACAtg1-GUS plants carrying less than 3 copies exhibited the GUS activity (Figures 3A, 4A–C). The single-copy lines of the pTACAtg1-GUS plants displayed stable expressions, and the GUS activity of the 2 copy lines seemed to be double of that in the single lines (Figure 3A). Gene silencing was observed only in approximately 15% of plants, which harbored...
more than 4 copies (Figures 3A, 4D, E). More than $10^4$ pmol of 4-MU GUS activity was detected in three quarters of these pTACAtg1-GUS plants. On the other hand, in control plants, the level of activity was observed in a wide range; 0 to 300,000 pmol of 4-MU GUS activity was detected (Figure 3B). Only 40% of the pYLTAC7-GUS transformants exhibited more than $10^4$ pmol of 4-MU GUS activity (Figures 3B, 4F–J). Silenced GUS activity was observed even in the control plants carrying only 2 copies (Figures 3B, 4G). More than 30% of lines harboring multiple copies did not display the GUS activity because gene silencing occurred frequently (Figures 3B, 4G–J). All of the plants carrying a single copy of the transgene exhibited the GUS activity observed in the control plants carrying only 2 copies (Figures 3B, 4G). Gene silencing did not occur in the lines that harbored a single copy of the transgene. Consistent with the previous studies reported that the positional effects on gene expressions were not observed in the plants with a single copy of transgene (Iglesias et al. 1997; Nagaya et al. 2005; Schubert et al. 2004), silencing of gene expressions did not occur in the lines that harbored a single copy of the transgene. Therefore, the copy number of transgenes influenced the variability of GUS activities; silencing and variation in gene expressions ordinarily occurred in the plants harboring multiple copies. The pTACAtg1 vector reduced copy number of a transgene, resulting in avoidance of gene silencing and in steady gene expressions.

Figure 3. GUS activity from individual lines was measured. (A) pTACAtg1-GUS and (B) pYLTAC7-GUS (control). Copy numbers of GUS genes were shown on the X-axis. Data are represented as mean ± standard deviation (S.D.).

Figure 4. Histogram of GUS activity measured from individual lines. (A–E) pTACAtg1-GUS and (F–J) pYLTAC7-GUS (control). (A, F) 1 copy, (B, G) 2 copies, (C, H) 3 copies, (D, I) 4 copies, and (E, J) more than 5 copies.

A single copy of the transgene introduced by the pTACAtg1 provided steady expression levels beyond the generation

Next, we also analyzed segregation and expressions of the GUS gene in the T2 population of the pTACAtg1-GUS lines (Figure 5), because the variation of expressions is also observed among generations of genetically identical individuals in transformants produced by using a common vector. The GUS genes in the T2 population from the T1 hemizygous lines were segregated in 6 and 8 of hemizygous: 4 and 2 of homozygous in pTACAtg1 and control plants, respectively, when the transformants were selected on antibiotic plates. It is consistent with Mendelian inheritance: 1 homozygote and 2 heterozygote plants. In the assay of GUS activity, these values were corresponded to the copy number of GUS gene (Figure 5A, B). We concluded that the transgene introduced
with the genome fragments by the pTAC Atg1 vector was stably expressed in the T2 population generated from the identical T1 plant that harbors a single copy.

**PTGS occurred in the plants with multiple copies of transgene**

We also analyzed the segregation of gene silencing in the plants carrying multiple copies of the transgene. In some of these lines with more than 4 copies, gene silencing was observed even in the pTAC Atg1 lines, as seen in the control plants (Figures 3, 5C, D). However, some of these T2 lines segregated recovered from the silencing even in the silenced T1 lines. We wondered whether the silencing was mediated by double-strand small RNAs (Guo et al. 2016; Schubert et al. 2004). Therefore, to investigate the double-strand small RNAs for the GUS gene, we isolated small RNAs from the silenced and non-silenced lines. In northern blot analysis, the small anti-sense GUS RNA hybridized with sense-GUS RNA probe was detected only in the silenced plants displaying no GUS activity (Figure 6). The detected anti-sense GUS RNA was estimated as 20–25 nucleotide length. In contrast, the anti-sense GUS small RNAs were not detected in the plants exhibiting GUS activity. It did not depend on either pTAC Atg1 or control vector, which was utilized for the transformation of 35S:GUS cassettes. Our results suggested that the silencing in the pTAC Atg1-GUS lines was mediated via small antisense-RNA.

**The pTACAtg1 has enough efficiency for transformation**

We wondered about the transformation efficiency of the pTAC Atg1 plasmid, because such a long plasmid ordinarily has low efficiency (Liu et al. 1999). The pTAC Atg1 vector includes more than 70 kb extra-sequences, consisting of 31.6 kb and 40.9 kb flanking sequences at the outside of the cloning sites as long as the K8K14. The transformation efficiency was calculated by counting hygromycin-resistant T1 transformants per sown seeds; the ratio of T1 plants generated by pTAC Atg1-GUS and pYLTAC7-GUS plasmid were 215/50,000 and 543/61,150, respectively. Although the transformation efficiency of pTAC Atg1-GUS was about half of that in control, it was enough ratio to produce transgenic plants. We generated 200 T1 plants, but no obvious alternation on phenotype was observed. This suggested that the flanking sequences from At5g67290 to At5g67450 on the K8K14 have no invasive effects on the plant phenotypes. Thus, the pTAC Atg1 vector can be useful as a binary vector for transformation with high efficiency as same as the control TAC vector.

**More than three quarters of the pTACAtg1-GUS transformants contained GUS cassettes**

In handling a long DNA insert, we were apprehensive about the integration of intermediated DNA fragments. It is well-known that transformants sometimes contained a partial DNA fragment, because transferring a long insert is partially interrupted in incomplete Agrobacterium-mediated transformation (Kim et al. 1998). To investigate how many lines indeed contain a full-length GUS cassette, we amplified the GUS DNA fragments from 75 individual T1 plants introduced by the pTAC Atg1. The GUS DNA fragments from 57 of pTAC Atg1 individual lines were detected. Approximately 24% of T1 plants with pTAC Atg1-GUS lacked the GUS regions. In
our experiments described above, we excluded these GUS-lacking lines and investigated a total of 57 T1 of pTAC Atg1-GUS plants for further analysis. In contrast, all of T1 control plants included the full length of GUS.

We expected that 57 of the pTAC Atg1-GUS plants with GUS fragments contained the complete DNA sequences between the right border (RB) and the GUS region (Figure 1), because the T-DNA transformation mediated by Agrobacterium occurs from right to the left border (LB). As we were interested in how many transgenic lines exhibited a full T-DNA insertion until LB, we analyzed the Bacillus subtilis levansucrase (SacB) DNA fragments next to the LB in PCR amplification. The SacB DNA fragments were amplified in 45 out of 75 T1 pTAC Atg1-GUS lines, whereas those were detected in all of 80 transgenic plants introduced by pYLTAC7-GUS. We concluded more than half of the pTAC Atg1-GUS lines included a full-length insert.

To investigate whether the region between the GUS and LB was completely introduced, we also carried out genomic Southern blot analysis in several lines lacking the sacB DNA (Figure 7). We selected 7 lines that were expected to exhibit a single copy of the GUS fragments confirmed by qPCR; the line 1 and 2 exhibited the SacB fragment, while the others lacked it (Figure 7A). We detected the bands with probe A to C on all lines, which carried DNA regions between RB to probe C (Figure 7B). On the other hand, the bands with probe F were only detected on lines 1 and 2; others only contained partial regions even though the GUS cassette was included (Figure 7C). The differences between the Ws and transformants detected with probe A, D, and F represented the polymorphisms between Ws and the background ecotype Columbia (Col) of the TAC clone K8K14. Thus, incomplete transformation was sometimes observed in the experiments carried with pTAC Atg1.

**Discussion**

Here, we developed a novel binary plasmid vector (pTAC Atg1) that is based on a transformation-competent artificial chromosome (TAC) clone, harboring long genomic DNA fragments on both sides of the cloning sites. As the starting material, we chose a TAC clone K8K14 with a 72,698 bp insert from the Arabidopsis TAC library (Hirose et al. 2015; Liu et al. 1999), which includes two constitutively-expressed genes At5g67370 and At4g67380. To facilitate easier cloning of a foreign DNA in such a long plasmid, we created unique cloning sites for rare-restriction enzymes, SbfI, AscI, SfiI, SrfI, FseI, and NotI, at the non-coding region between these genes. To evaluate the effects of the flanking sequences on copy number and gene expression of transgenes in the transgenic plant population, we introduced, as a case study, the 35S promoter:β-glucuronidase (35S:GUS) gene cassette in the cloning sites of pTAC Atg1 and also in that of pYLTAC cs1 as a control. These plasmids were used to transform Arabidopsis plants with the Agrobacterium-mediated transformation protocol. In isolated transgenic plants,
the copy number was reduced and the GUS expressions were detected more stably than those in the control plants carrying the insert without flanking regions. Thus, the pTACAtg1 vector will be suitable for the production of stable transformants and for expression analyses of a transgene.

Our results seem to be inconsistent with a previous study of Dean and coauthors (1988), in which they reported that long-flanking sequences with ~10-kb and ~13-kb length of Petunia rbcS gene at both 5’ and 3’ ends of the cloning site did not provide steady expressions in tobacco transgenic plants. As the pTACAtg1 vector contains ~70kb flanking sequences longer than that of the rbcS gene, this may explain why the pTACAtg1 suppresses variability of transgene expressions in contrast with the previous report.

Large size of flanking regions reduces multiple insertions in the use of pTACAtg1 vector (Figure 2). In fact, more than half of the pTACAtg1-GUS plants harbored less than three copies. Even if multiple insertions happen, the distance among the transgenes will be at least ~80kb length based on genomic sequences on the pTACAtg1 vector. In previous studies, multiple insertions of foreign genes in a single locus were reported to cause the TGS in transgenic plants (Furner et al. 1998). However, as the TGS is normally known in tandem repeated insertions, the TGS is unlikely to happen by transformation with a long flanking region in pTACAtg1. In a few lines of pTACAtg1-GUS carrying multiple copies, silencing of gene expression was still observed even though the frequency was low. We found small anti-sense RNAs in the silenced lines, suggesting that the silencing observed in this study was mediated by the TGS or PTGS via small RNAs. Though the copy number reduced by the longer flanking regions causes less variability of the expressions among independent lines and generations, further technology avoiding silencing is necessary.

The length between LB to RB on a binary vector influences its complete transformation as shown in Figure 7. Expectedly, in the use of the pTACAtg1 vector with the long flanking sequence, a partial DNA insert in transformation was sometimes observed. This incomplete transformation may be a disadvantage in our pTACAtg1 vector. However, we will not need to produce so many T1 lines because the pTACAtg1 transformants display steady expressions as described above. It is noteworthy that more than 75% of transgenic plants (57 out of 75 pTACAtg1-GUS plants) carried the GUS fragments and most of them exhibited the GUS activity. Even though uncompleted insertions sometimes occur, it will be more worth isolating transformants expressing the inserted transgene steady.

The transformation efficiency with the pTACAtg1 vector is enough to produce transgenic plants, even though the transformation efficiency was estimated as approximately half of the control vector. Although a modified form of the original TAC vector, TACAtg1, is a long plasmid with 95,346 bp in length, cloning foreign DNA fragments can be done easily in the rare-cut restriction site, and the resulted constructs are introduced into E. coli host at high efficiency, as shown in our previous paper of the TAC clones (Liu et al. 1999). Rather, by using the pTACAtg1 vector, we will be able to isolate the plants exhibiting steady expressions of a transgene from the small number of T1 transformants easily. It is advantageous to isolate transgenic lines, especially from the plants with low efficiency in transformation. The pTACAtg1 vector will also be a beneficial tool in the selection of stable lines, especially when we handle numerous lines, such as genome projects, to reduce cultivate spaces and to save time and workers. By handling the longer plasmid, we will be able to introduce multiple distinct genes, e.g. constructed by precise sequential DNA ligation on a solid substrate (PRESSO) (Fujisawa et al. 2009; Takita et al. 2013). The flanking regions as seen in those in the pTACAtg1 with the PRESSO may provide the steady expression of multiple genes without gene silencing. Further, combining the steady expression on the pTACAtg1 with the PRESSO and genome editing technology (Endo et al. 2019) will bring a more efficient artificial synthesis of metabolites and the creation of artificial plant organs (Fujisawa et al. 2009; Hanano et al. 2020).

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Accession numbers
The nucleotide sequence of pTACAtg1 vector reported in this paper has been submitted to DDBJ database with the accession number AB904499.

Supplemental Data
Supplementary data are available at Plant Biotechnology online.

Disclosures
The authors have no conflicts of interest to declare.
References

Baulcombe D (2004) RNA silencing in plants. Nature 431: 356–363
Breyne P, vanMontagu M, Depicker N, Ghysen G (1992) Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco. Plant Cell 4: 463–471
Chen L, Marmey P, Taylor NJ, Brizard JP, Espinoza C, DCruz P, Huet H, Zhang S, de Kochko A, Beachy RN, et al. (1998) Expression and inheritance of multiple transgenes in rice plants. Nat Biotechnol 16: 1060–1064
Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743
Davies GJ, Sheikh MA, Ratcliffe OJ, Coupland G, Furner IJ (1997) Gene silencing and methyltransferase activity in transgenic plants. Plant Mol Biol Report 15: 159–168
Day CD, Lee E, Kobayashi J, Holappa LD, Albert H, Ow DW (2000) The effects of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucleic Acids Res 28: 111–122
Davies GJ, Sheikh MA, Ratcliffe OJ, Coupland G, Furner IJ (1997) Gene silencing and methyltransferase activity in transgenic plants. Plant Mol Biol Report 15: 159–168
Davies GJ, Sheikh MA, Ratcliffe OJ, Coupland G, Furner IJ (1997) Gene silencing and methyltransferase activity in transgenic plants. Plant Mol Biol Report 15: 159–168
Furner IJ, Sheikh MA, Collett CE (1998) Gene silencing and methyltransferase activity in transgenic plants. Plant Mol Biol Report 15: 159–168
Fujisawa M, Takita E, Ogata Y, Ozawa K, Suda K, et al. (2012) In planta gene targeting. Front Plant Sci 3: 1–13
Hanamoto N, Nakamura Y, Shibata D, Misawa N (2009) Pathway engineering of Brassica napus seeds using multiple key enzyme genes involved in ketocarotenoid formation. J Exp Bot 60: 1319–1332
Kato T, Shirano Y, Wakahara Y, Tada Y, Itoh E, Shibata D (1991) A modified β-glucuronidase gene: Sensitive detection of plant promoter activities in suspension-cultured cells of tobacco and rice. Plant Mol Biol Report 9: 333–339
Kim YS, Lee MH, Min SR, Yoo OJ, Liu JR (1998) Frequent occurrence of transgene deletion in transgenic plants. Mol Cells 8: 705–708
Klein RM, Wolf ED, Wu R, Sanford JC (1992) High-velocity microprojectiles for delivering nucleic acids into living cells. 1987. Biotechnology 24: 384–386
Kohli A, Gonzalez-Melendi P, Abranches R, Capell T, Sloger E, Christou P (2006) The quest to understand the basic and mechanisms that control expression of introduced transgenes in crop plants. Plant Signal Behav 1: 185–195
Liu YG, Shirano Y, Fukaki H, Yama T, Takase M, Tabata S, Shibata D (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc Natl Acad Sci USA 96: 6535–6540
Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1: 142–148
Mlynarova L, Keizer L, Stekken WM, Nijhof JP (1996) Approaching the lower limits of transgene variability. Plant Cell 8: 1589–1599
Nakamura Y, Sato S, Kaneko T, Kotani H, Asamizu E, Miyajima N, Tabata S, Tabata S, Shibata D (1999) Suppression of Ku70/80 or Lig4 leads to decreased stable transformation and enhanced homologous recombination in rice. New Phytol 149: 651–662
Nishizawa-Yokoi A, Nonaka S, Saika H, Kwon Y, Osakabe K, Toki S (2005) Expression of randomly integrated single complete copy transgenes does not vary in Arabidopsis thaliana. Plant Cell Physiol 46: 438–444
Ojolo SP, Cao S, Priyadarshani SVGN, Li W, Yan M, Aslam M, Zhao H, Qin Y (2018) Regulation of plant growth and development using custom-designed zinc finger nucleases. Proc Natl Acad Sci USA 115: 10394–10399
Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7: 4021–4026
Phillen T, Spiker S, Matzke M, Matzke AJ (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9: 1251–1264
Phelan T, Spiker S, Matzke M, Matzke AJ (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9: 1251–1264
Kim YS, Lee MH, Min SR, Yoo OJ, Liu JR (1998) Frequent occurrence of transgene deletion in transgenic plants. Mol Cells 8: 705–708
Klein RM, Wolf ED, Wu R, Sanford JC (1992) High-velocity microprojectiles for delivering nucleic acids into living cells. 1987. Biotechnology 24: 384–386
Kohli A, Gonzalez-Melendi P, Abranches R, Capell T, Sloger E, Christou P (2006) The quest to understand the basic and mechanisms that control expression of introduced transgenes in crop plants. Plant Signal Behav 1: 185–195
Liu YG, Shirano Y, Fukaki H, Yama T, Takase M, Tabata S, Shibata D (1999) Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc Natl Acad Sci USA 96: 6535–6540
Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1: 142–148
Mlynarova L, Keizer L, Stekken WM, Nijhof JP (1996) Approaching the lower limits of transgene variability. Plant Cell 8: 1589–1599
Nakamura Y, Sato S, Kaneko T, Kotani H, Asamizu E, Miyajima N, Tabata S, Tabata S, Shibata D (1999) Suppression of Ku70/80 or Lig4 leads to decreased stable transformation and enhanced homologous recombination in rice. New Phytol 149: 651–662
Nishizawa-Yokoi A, Nonaka S, Saika H, Kwon Y, Osakabe K, Toki S (2005) Expression of randomly integrated single complete copy transgenes does not vary in Arabidopsis thaliana. Plant Cell Physiol 46: 438–444
Nakamura Y, Sato S, Kaneko T, Kotani H, Asamizu E, Miyajima N, Tabata S (1997) Structural analysis of Arabidopsis thaliana chromosome 3. III. Sequence features of the regions of L1, L191,198 bp covered by seventeen physically assigned P1 clones. DNA Res 4: 401–414
Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812
Ojolo SP, Cao S, Priyadarshani SVGN, Li W, Yan M, Aslam M, Zhao H, Qin Y (2018) Regulation of plant growth and development: A review from a chromatin remodeling perspective. Front Plant Sci 9: 1232
Osakabe K, Osakabe Y, Toki S (2010) Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc Natl Acad Sci USA 107: 12034–12039
Paszkowski J, Baur M, Bogucki A, Potrykus I (1988) Gene targeting in plants. EMBO J 7: 4021–4026
Puchta H, Fauser F (2013) Gene targeting in plants: 25 years later. Int J Dev Biol 57: 629–637
Rhee SY, Beavis W, Berardiniz TZ, Chen G, Dixon D, Doyle A, Garcia-Hernandez M, Huala E, Lander G, Montoya M, et al. (2003) The Arabidopsis Information Resource (TAIR): A model organism database providing a centralized, curated gateway to Arabidopsis biology, research materials and community. Nucleic
Stable transformation with a novel binary vector

Acids Res 31: 224–228
Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York
Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in Arabidopsis T-DNA transformants: The predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16: 2561–2572
Sherman JM, Pillus L (1997) An uncertain silence. Trends Genet 13: 308–313
Takita E, Kohda K, Tomatsu H, Hanano S, Moriya K, Hosouchi T, Sakurai N, Suzuki H, Shinmyo A, Shibata D (2013) Precise sequential DNA ligation on a solid substrate: Solid-based rapid sequential ligation of multiple DNA molecules. DNA Res 20: 583–592
Wallrath LL (1998) Unfolding the mysteries of heterochromatin. Curr Opin Genet Dev 8: 147–153
Yamada K, Lim J, Dale JM, Chen H, Shinn P, Palm CJ, Southwick AM, Wu HC, Kim C, Nguyen M, et al. (2003) Empirical analysis of transcriptional activity in the Arabidopsis genome. Science 302: 842–846
Ye F, Signer ER (1996) RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. Proc Natl Acad Sci USA 93: 10881–10886