A Novel Binary T-Vector with the GFP Reporter Gene for Promoter Characterization

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Abstract

Several strategies have been developed to clone PCR fragments into desired vectors. However, most of commercially available T-vectors are not binary vectors and cannot be directly used for Agrobacterium-mediated plant genetic transformation. In this study, a novel binary T-vector was constructed by integrating two AhdI restriction sites into the backbone vector pCAMBIA 1300. The T-vector also contains a GFP reporter gene and thus, can be used to analyze promoter activity by monitoring the reporter gene. On the other hand, identification and characterization of various promoters not only benefit the functional annotation of their genes but also provide alternative candidates to be used to drive interesting genes for plant genetic improvement by transgenesis. More than 1,000 putative pollen-specific rice genes have been identified in a genome-wide level. Among them, 67 highly expressed genes were further characterized. One of the pollen-specific genes LOC_Os10g35930 was further surveyed in its expression patterns with more details by quantitative real-time reverse-transcription PCR (qRT-PCR) analysis. Finally, its promoter activity was further investigated by analyzing transgenic rice plants carrying the promoter::GFP cassette, which was constructed from the newly developed T-vector. The reporter GFP gene expression in these transgenic plants showed that the promoter was active only in mature but not in germinated pollens.

Introduction

Several strategies have been explored to clone DNA fragments from polymerase chain reaction (PCR) into desired vectors. One of the strategies is to incorporate restriction enzyme sites into oligonucleotide primers to create sticky ends by digesting the PCR products. However, the digestion efficiency is low for many restriction endonucleases when their recognition sequences are located within a few base pairs of the end of the PCR products [1]. The second strategy is to clone PCR products as blunt-ended fragments. The strategy requires an enzymatic processing to remove a 3’ overhang linear vector. Currently, considerable reports have been published to introduce the application of this enzyme in the generation of 3’ overhang linear vector. In addition, most of commercially available T-vectors are not binary vectors and cannot be directly used for Agrobacterium-mediated genetic transformation. In this study, we have constructed a novel binary T-vector by inserting a 1,317-bp DNA sequence with two AhdI restriction sites.

A T-vector can be constructed by two ways. One way is to artificially add T-overhang to blunt-ended plasmid DNA by terminal deoxynucleotidyl-transferase (TdT) or Taq polymerase in the presence of dideoxythymidine triphosphate (ddTTP) and dTTP, respectively [3–5]. By this way, the prerequisite is that one should first construct a plasmid DNA with blunt-ended restriction enzyme sites. Another way is to directly generate a 3’ overhang linear vector by certain restriction enzymes. Several restriction enzymes could be used for that purpose and the examples include BclI, BfiI, HphI, MluI, TaaI, XcmI and AhdI (Eam1105I) [6–9]. The widely employed enzyme is XcmI. The enzyme has a flexible recognition sites “CCANNNN/NNNNTGG” (N = A/C/T/G). Thus, a plasmid DNA with the sequence “CCANNNTNNNNTGG” can be recognized by XcmI and be digested to generate a 3’ overhang linear vector. Currently, considerable reports have been published to introduce the application of this enzyme in the generation of 3’ overhang linear vectors for cloning PCR products [7,10–12]. Besides XcmI, little is known about the application of the other enzymes on generating the 3’ overhang vector. In addition, most of commercially available T-vectors are not binary vectors and cannot be directly used for Agrobacterium-mediated genetic transformation. In this study, we have constructed a novel binary T-vector by inserting a 1,317-bp DNA sequence with two AhdI restriction sites. The T-vector contains a GFP reporter gene and thus, can be used to analyze promoter functions by the GFP gene.

Identification and characterization of various promoters not only benefit the functional annotation of their genes but also provide alternative candidates to be used to drive interesting genes for genetic improvement of targeted organisms by transgenesis. Among different types of promoters, we are interested in pollen-specific promoters. These promoters can be used to drive expression of some genes to disturb pollen development by genetic transformation and as a result, to develop male sterile lines. Genetically stable male sterile lines are the prerequisite to...
commercially utilize crop heterosis to improve crop production. Thus, pollen-specific promoters are potentially useful in crop genetic improvement by developing hybrid crop cultivars [13–15].

Some of pollen-specific genes and their promoters were isolated and characterized more than twenty years ago from plants [16,17]. Since then, more numbers of pollen-specific promoters have been characterized and these include petunia PA2 [18], tomato LAT52 and LAT59 [19,20], rapeseed Bp10 [21], maize Zm13 [22,23], tobacco NTP307 [24], Lycopersicon esculentum promoters TUA1 [25], AtPTENI [27], AtSTP6 [28], AtSTP9 [29] and AWE1 [30]. Among them, the tomato LAT52 promoter has been widely used to drive pollen-specific expression. In rice, promoters from numbers of genes showed activities in mature pollen and/or pollen tubes and examples include OsSCP1, OsSCP2, OsSCP3, OSPA, OSPK, and OSIPF3 [31–35]. Recently, Oo et al. (2014) reported 6 genes whose promoter activities were detected in the late stage of pollen formation in the transgenic Arabidopsis carrying the promoter::GUS/GFP constructs [15].

Pollen development undergoes a complicated biological process from microspores to mature pollen grains. It has been regarded as an ideal model to study various biological processes such as sexual reproduction, cell fate determination, signal transduction, membrane transport, and polar growth. Pollen-specific promoters may be utilized as tools in annotating biological functions of these genes involved in pollen development and regulation. Thus, it is necessary to identify and characterize various pollen-specific promoters whose activities could be detected at only certain stage of pollen development. There are lots of publicly available rice expression databases such as Gene Expression Omnibus (GEO) datasets [36], ArrayExpress [37] and PLEXdb [38]. However, limited data are available for focusing on pollen-related expression. Besides the Massively Parallel Signature Sequencing (MPSS) rice database [39], two of microarray datasets are available where polons and other tissues were taken for expression analysis. One of the datasets contained the expression data from 8 different samples including leaves, callus cells, roots, uninucleate microspores, bicellular pollens, tricellular pollens, mature pollen grains and germinated pollen grains [40]. Another dataset contained only 3 samples including seedlings, pollen at anthesis and sperm at anthesis [41]. All these data provide the basis for genome-wide identification of pollen-specific genes. In this study, a novel binary T-vector has been developed to facilitate the rapid clone of targeted promoters into the upstream of reporter GFP gene. Considerable numbers of pollen-specific rice genes were also verified in their expression. Additionally, one pollen-specific gene and its promoter were molecularly characterized by using our newly developed T-vector. The data showed that a promoter::GFP construct could be prepared rapidly by using the novel T-vector and the developed construct could be directly used for Agrobacterium-mediated genetic transformation to generate transgenic plants for detecting the promoter activities through the GFP reporter gene. Our data also showed that the 1,492 bp promoter from the rice gene LOC_Os10g35930 was active only at the mature stage of pollen and no GFP activity was observed after pollen germination.

Results

Construction of the novel binary T-vector

The restriction enzyme AhdI has the recognition site GACNNN/NNNGTC (N = A/C/T/G). Thus, a DNA fragment with the sequence GACAAAGTGC can be digested by the enzyme AhdI to generate a 3’ T-overhang end. To introduce this sequence into the binary vector pCAMBIA 1300 (http://www.cambia.org), two bridge fragments were amplified by PCR with the rice genomic DNA as a template using two primer sets as listed in Table S1 (See Methods in details). The Fragment 1 contains two recognition sites including HindIII and AhdI and the Fragment 2 has two recognition sites including AhdI and NcoI. These two fragments were then ligated together by the enzyme SpeI (Figure 1A). The ligated fragment was further digested by both HindIII and NcoI to form a HindIII-NcoI fragment. The fragment was then ligated with a GFP-containing pCAMBIA 1300 binary vector to develop the vector named as pDsTGFP (Figure 1B). The vector has two AhdI sites at the 39th and 329th position, respectively (Figure 1B). Thus, the 3’ T-overhang ends can be generated by digesting the pDsTGFP vector with the enzyme AhdI (Figure 1C).

A PCR-based method for developing a promoter::GFP construct

This novel binary T-vector is specially designed to clone a promoter fragment from PCR amplification using Taq DNA polymerase. For rice and many other plants, their genomes have been sequenced and annotated in a genome-wide level. A promoter sequence can be predicted by further analyzing 2 Kb upstream of a transcription start codon site of a gene. Based on the analysis, the predicted promoter sequence with 2 Kb or less than 2 Kb in length can be selected for designing promoter-specific primer sets, which will be used for PCR-amplification of the candidate promoter. An example was shown in Figure 2 (indicated by the green arrow), where an around 1.5 Kb promoter fragment was amplified. In the meantime, the plasmid DNA pDsTGFP can be digested by AhdI using the method as shown in Figure 2. After digestion, the products were separated by electrophoresis. The fragment with around 10 Kb (indicated by the red arrow in Figure 2) was purified from the gel, which was served as the 3’-overhang T-vector fragment. Both the PCR product and the 3’-overhang fragment were then ligated in the presence of T4 DNA ligase. The ligation products were transformed into E. coli competent cells. Typically, around 10–20 colonies would be obtained, which were generally enough for sub-sequential orientation and verification. The orientation and verification can be carried out by enzyme digestion, PCR and/or sequencing. For PCR and sequencing, both the GFP_Rev primer (Figure 1; Table S1) and promoter-specific primer could be employed.

Genome-wide identification and expression patterns of pollen-specific genes

Based on the Massively Parallel Signature Sequencing (MPSS) rice database [39], a total of 1,013 pollen-specific genes were identified. These genes were then submitted to expression verification by microarray and RNA_Seq datasets. Among the 1,013 pollen-specific genes, no probe is available in the Affymetrix array chips for 212 genes and we analyzed the expression patterns for the remaining 801 genes. By comparing two datasets, most of the genes showed pollen-specific expression. We were interested in highly expressed pollen-specific genes. A total of 70 genes with expression abundance more than 1,000 transcripts per million (TPM) in the MPSS database were selected for further analysis. Among these genes, both LOC_Os01g50810 and LOC_Os08g02880 were not pollen-specific and they showed similar or higher expression in seedling/roots than in pollens by microarray data analyses (Figure 3). For the gene LOC_Os08g39460, it was expressed in pollens with high expression.
level; however, it also showed high expression in callus and roots (Figure 3).

The RNA_Seq data (http://mpss.udel.edu/rice_RNAseq/) was then employed for further verification. In this database, expression data were available in 10 different tissues including shoots, 20-day old leaves, pre-emergence inflorescence, post-emergence inflorescence, anther, pistil, Seeds of 5-day after pollination (DAP), 10 DAP seeds, 25 DAP embryos and 25 DAP endosperm. As 3 out of 70 genes showed non-pollen-specific expression by microarray data (Figure 3), these three genes were excluded for further verification by the RNA_Seq data. The verification analysis showed that, for all the 67 genes, the highest level of expression abundance was observed in the anthers (Figure 4). Further analysis showed that the expression signal of 97% genes in anthers is at least 5 times higher than the expression levels in the remaining tissues. The remaining 2 genes showed the expression level in anthers with 2.6 (LOC_Os02g04210) and 2.9 (LOC_Os12g23170) times higher than in the other tissues, respectively (Figure 4). Thus, the RNA_Seq data further confirmed the pollen-specific expression of these 67 genes.

Construction of binary vector carrying the promoter::GFP cassette by using the novel T-vector

To further verify the pollen-specific expression of these candidate genes, we randomly selected one gene for promoter activity analysis. The selected gene is with the locus name LOC_Os10g35930. The 1,492 base pairs of DNA sequence upstream of start codon of the gene was amplified from the rice genome by PCR as shown in Figure 2. After verification by sequencing, the PCR fragment was directly ligated to the T-vector prepared by AhdI digestion of pDsTGFP (Figure 2). After transformation, we have obtained a total of 9 colonies and five of them were randomly selected for plasmid DNA preparation and verification. Sequencing analysis showed that two colonies contained the insertion from the PCR fragment with correct orientation. One of the two colonies was selected for Agrobacterium-mediated genetic transformation to generate transgenic rice plants carrying the promoter::GFP cassette.

The gene LOC_Os10g35930 was expressed at the mature stage of panicle development

As the gene LOC_Os10g35930 was selected as a candidate gene to further study its expression profiling by surveying its promoter activity, its expression patterns was also investigated in more details. The achieved data from the MPSS database showed that the gene LOC_Os10g35930 has the highest expression signal at the last stage of inflorescence development, where most of pollens are in the mature stage (Figure 5A). We then analyzed the microarray data with GEO accession number GSE6893, where a total of 15 tissues were collected for expression analysis. The data showed that the gene LOC_Os10g35930 has the highest expression level at the last stage of inflorescence development, where most of pollens are in the mature stage (Figure 5B). The second microarray data consists of three tissues including seedling, pollens at anthesis and sperms at anthesis. The highest expression level was observed in the pollens at anthesis (Figure 5C). The third microarray data contained 8 samples and 5 of them were from pollens. The gene LOC_Os10g35930 showed the highest expression level in both mature and germinated pollens (Figure 5D). Besides both the MPSS and microarray data, the RNA_Seq data (http://mpss.udel.edu/rice_RNAseq) was also employed for the
expression verification (Figure 5E). Based on the data, the gene exhibited the strongest expression signal at the post-emergence inflorescence (Figure 5E). At this stage of inflorescence, most of the pollens were at the mature stage. Finally, the quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to verify the expression of this gene. The total RNA samples were prepared from a total of 11 tissues and were then submitted to qRT-PCR analysis (Figure 5F). The analysis showed that the gene was mainly expressed at the opening panicles and flowering panicles, where majority of pollens were at the mature stage. Thus, our qRT-PCR data further confirmed the expression of this gene in the late stage of panicle development.

The promoter activity in transgenic rice plants carrying the promoter::GFP cassette

The novel T-vector is suitable for cloning PCR products amplified by Taq DNA polymerase which can catalyze the addition of an adenine residue to the 3'-end of its PCR fragment. If a PCR is carried out using other DNA polymerases, for example, Phi DNA polymerase, the PCR products are required to be treated with Taq DNA polymerase to add A-overhang in both
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A large table showing gene expression data with columns for different conditions and rows for different genes. The table is color-coded to represent different levels of expression. The figure on the right shows a heatmap with a color scale indicating expression levels.
Figure 3. Expression patterns of pollen-specific genes by two sets of microarray data. The normalized microarray expression values in callus cells for the dataset GSE279881 and in seedlings for the dataset GSE17002 were set as controls, respectively. Their values were set as “1” and all other values were calculated by comparing with the control values. Then, all the values are log-transformed (base 2 for simplicity) and the resulted log2 values were used for the heat mapping. Samples were labelled as below: 1, callus cells; 2, leaves; 3, roots; 4, unineurine microspores; 5, bicalcar pollens; 6, tricellular pollens; 7, mature pollen grains; 8, germinated pollen grains; I, seedlings; II, pollens at anthesis; III, sperms at anthesis. Red, black, and green colors indicated that transformed expression values were >0, =0, and <0, respectively, in the matrix. The red stars indicate the genes with inconsistent expression patterns when compared with the values from the MPSS dataset.

doi:10.1371/journal.pone.0107328.g003

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better understand the regulatory mechanism of pollen development. Wei et al. (2010) revealed 25,062 pollen-preferential transcripts by investigating the expression profiles in three vegetative tissues and 5 pollen-related samples [40]. Genome-wide expression patterns among seedling, pollens and sperms at anthesis revealed 3,045 pollen-/sperm-specific genes [41]. In both cases, only one or three no-pollen tissues were selected for comparative expression analysis. Thus, some pollen-specific genes might be wrongly identified when these genes would be expressed in other vegetative tissues which were not be surveyed. Therefore, pollen-specific genes should be identified by using more vegetative tissues as references for more accurate evaluation. In this study, a total of 1,013 pollen-specific genes were identified by using the MPSS database. These genes were further verified in their expression microarray and RNA-Seq datasets and 67 highly expressed pollen-specific genes were selected for further analysis (Figures 3 and 4). As these pollen-specific genes were initially identified by using the MPSS database, where only the expression data from mature pollens are available, young pollen-specific genes were not included. Thus, more pollen-specific genes with high expression level should be identified if expression data from young pollens are available in the MPSS database.

A stage-specific/enriched gene is expressed only in a specific stage or shows significant higher abundance in the stage, for example, in the unicellular stage, of pollen development. The identification of the stage-specific/enriched genes should benefit to better understand the regulatory mechanism of pollen development. Wei et al. (2010) identified 2,203 stage-enriched transcripts [40]. Another report showed that three transcriptomes of egg cells, sperm cells and pollen vegetative cells are highly divergent and about ¼ of those genes were differentially expressed in these cell types [42]. Russell et al. (2012) also identified 1,668 sperm cell specific genes [41]. In this study, among 67 pollen-specific genes, most of them showed expression in the whole pollen development stages with the highest abundance in mature or germinated pollen grains and the expression level in sperms was obviously reduced (Figure 3). Our data suggested that most of mature pollen specific genes might play roles not only in mature pollens but also in their tube germination.

Materials and Methods

Plant materials and growth conditions

The japonica rice variety Nipponbare plants (Oryza sativa L.) were used for all DNA and RNA preparation as well as Agrobacterium-mediated genetic transformation in this study. The rice seeds were germinated in water at 37°C for three days and were then planted in pots. All the plants were grown in greenhouse under natural light and temperature conditions.

Preparation of DNA and total RNA samples

Leaves from two-week old plants were collected and were then frozen in liquid nitrogen for DNA isolation as described by Dellaporta et al. (1983) [46]. For total RNA isolation, a total of 11 different stages of rice tissues were collected and these tissues were listed as below: (1) two-week old leaves, (2) two-month old leaves,
(3) two-week old roots, (4) two-month old roots, (5) 0–5 cm long panicles, (6) 5–10 cm long panicles, (7) more than 10 cm long panicles, (8) booting panicles, (9) flowering panicles, (10) milky seeds and (11) mature seeds. Total RNA samples were isolated using a QIAGEN RNeasy Mini kit. The total RNA quality was analysed by Nanodrop reading and only the total RNA samples with A$_{260}$/A$_{280}$ = 1.8 were used for further experiments.

Figure 4. Expression heat map of pollen-specific genes by the RNA_Seq data. In the RNA_Seq dataset, a total of 10 different developmental stages of tissues were collected for the expression analysis. These tissues were labelled as below: 1, shoots; 2, leaves-20 days; 3, pre-emergence inflorescence; 4, post-emergence inflorescence; 5, anther; 6, pistil; 7, seed-5 days after pollination (DAP); 8, seed-10DAP; 9, embryo-25DAP; 10, endosperm-25 DAP. The normalized expression values were directly used to generate heat map. The values “0” indicated that no expression signal was detected for these genes in the corresponding tissues. The larger the expression values are, the stronger the genes show their expression. The prefix “LOC_” in locus names is omitted for convenience in this figure. doi:10.1371/journal.pone.0107328.g004

PCR amplification of bridge fragments from the rice genome for the construction of the T-vector pDsTGFP

In order to introduce two AhdI restriction sites into the pCAMBIA 1300 vector, two DNA fragments were amplified from the rice genome using two primer sets as described in Table S1. One of the fragments (425 bp) matches the region from 77322nd–77746th of the rice BAC clone OSJNBb0049H14 and another fragment (900 bp) is from the region 76044th–76943rd of the rice
PCR amplification was carried out in 25 μl of reaction mixtures with 50 ng of genomic DNA, 200 μM of each of dNTPs, 0.5 μM each of primers, 2.5 mM MgCl₂, 1 unit of DNA Taq polymerase from QIAGEN, and buffer provided by the supplier. The temperature profile started at 94°C for 5 min and followed by 30 cycles at 94°C for 40 s, 55°C–65°C for 40 s (depending on the Tm value of primers) and 72°C for 1 min. The reaction was terminated at 72°C for 10 min. After amplification, the two fragments were purified from agarose gel and were then cloned into pGEM-T Easy Vector from Promega. These two fragments were then subcloned into the GFP containing binary vector pCAMBIA 1300 by multiple steps of enzyme digestion and ligation.
Expression datasets used in this study for expression verification of pollen-specific genes

The MPSS dataset was downloaded from the website https://mpss.udel.edu/rice/mpss_index.php [39]. Three microarray datasets were downloaded from the GEO datasets [36] (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE6893, GSE17002 and GSE27988. The RNA_Seq dataset was downloaded from the MSU rice genome annotation database [47] (http://rice.plantbiology.msu.edu/index.shtml).

Expression analysis by qRT-PCR

The primer sequences used for qRT-PCR were selected by the Applied Biosystems Primer Express software and were listed in Table S1. An eEF-1a gene from the rice genome was used as an internal control to normalize the amplification data. Their primer sequences were also listed in Table S1. A total of 11 RNA samples from different tissues were submitted to the first-strand cDNA synthesis using an Invitrogen kit. The cDNA first strand was used as templates for qRT-PCR analyses. The reactions were carried out using the AB power SYBR Green PCR Master mix kit (Applied Biosystems, P/N 4367659) according to the manufacturer’s protocol. The threshold cycle (Ct) value was automatically calculated based on the fluorescence of SYBR Green I dye in every cycle, which was monitored by the ABI 7900 system software. The mRNA relative amount was calculated by \( \Delta \Delta Ct \) according to our previous description [48]. The value was used to evaluate the expression profiling of a gene.

The construction of the promoter::GFP cassette and Agrobacterium-mediated transformation

The gene LOC_Os10g35930 shows pollen-specific expression and its promoter was selected as an example to construct the promoter::GFP cassette using the novel T-vector pDsTGFP. A 1,492 bp of promoter fragment was amplified by PCR using the promoter-specific primer set as listed in Table S1. After purification from agarose gel and sequencing verification, the promoter fragment was directly ligated to the T-overhang pDsTGFP. The ligation was then used for transformation into the competent E. coli DH5a cells by the heat-shock method. Plasmid DNA samples were prepared using QIAGEN Plasmid Mini Kit. After verification and orientation by PCR and sequencing, the plasmid DNA was then introduced into Agrobacterium by electroporation using GIBCOBRL Cell-Porator. Embryonic calli were induced from mature rice embryos and were used for Agrobacterium-mediated genetic transformation according to the description by Hiei et al (1994) [49].

Fluorescence microscopy for GFP signal detection

A Nikon microscope with epifluorescence was used to detect the promoter-driven GFP expression using a B1E filter (excitation 470...
Sequencing of DNA fragments

DNA sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing kit according to the instruction from the supplier. The PTC200 (MJ Research, Inc.) thermocycler was used for temperature profiling according to the ABI cycle sequencing protocol: 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. The sequencing samples were purified using the Agencourt CleanSEQ sequencing reaction clean-up system (Beckman Coulter) and was then analyzed using an ABI 377 automatic sequencer.

 Supporting Information

Table S1 Primer sequences used for PCR, sequencing and qRT-PCR.

Author Contributions

Conceived and designed the experiments: SYJ SR. Performed the experiments: SYJ JV YB. Analyzed the data: SYJ. Contributed reagents/materials/analysis tools: SYJ JV YB. Contributed to the writing of the manuscript: SYJ SR.

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