

# A Versatile Zero Background T-Vector System for Gene Cloning and Functional Genomics<sup>1</sup>[C][W][OA]

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With the recent availability of complete genomic sequences of many organisms, high-throughput and cost-efficient systems for gene cloning and functional analysis are in great demand. Although site-specific recombination-based cloning systems, such as Gateway cloning technology, are extremely useful for efficient transfer of DNA fragments into multiple destination vectors, the two-step cloning process is time consuming and expensive. Here, we report a zero background TA cloning system that provides simple and high-efficiency direct cloning of PCR-amplified DNA fragments with almost no self-ligation. The improved T-vector system takes advantage of the restriction enzyme *XcmI* to generate a T-overhang after digestion and the negative selection marker gene *ccdB* to eliminate the self-ligation background after transformation. We demonstrate the feasibility and flexibility of the technology by developing a set of transient and stable transformation vectors for constitutive gene expression, gene silencing, protein tagging, protein subcellular localization detection, and promoter fragment activity analysis in plants. Because the system can be easily adapted for developing specialized expression vectors for other organisms, zero background TA provides a general, cost-efficient, and high-throughput platform that complements the Gateway cloning system for gene cloning and functional genomics.

Rapid advances in genome sequencing technologies in the last few years have led to the complete decoding of many complex eukaryotic genomes and have stimulated large-scale analysis of gene functions in sequenced genomes. In general, gene function can be elucidated using a variety of approaches, such as ectopic expression, gene silencing, protein subcellular localization examination, gene expression pattern analysis by promoter activity assay, structure-function analysis, and in vitro or in vivo biochemical assays (Hartley et al., 2000; Curtis and Grossniklaus, 2003; Earley et al., 2006). Typically, all these approaches require the cloning of target genes, mutated fragments, or their promoter fragments into various specialized vectors for subsequent characterization. However, the traditional approach for engineering expression constructs based on the restriction enzyme/ligase cloning method is extremely laborious and time consuming and is often hampered by lack of appropriate restriction sites; thus, the production of constructs is a

significant technical obstacle for large-scale functional gene analysis in plants.

In recent years, the Gateway cloning system from Invitrogen and the Creator cloning system from CLONTECH have been developed to facilitate large-scale production of gene constructs. The recombinational cloning systems are based on a two-step process (Marsischky and LaBaer, 2004). The DNA fragment of interest is first cloned into a general donor plasmid. Subsequently, the DNA fragment flanked by two site-specific recombination sites in the donor vector can be transferred precisely into a variety of expression vectors by site-specific recombination reactions. A great advantage of the recombinational cloning technologies is that once the DNA fragment has been engineered into a donor vector, the transfer of the DNA fragment into an expression destination vector is a simple reaction that requires no traditional restriction enzyme/ligase cloning. The recombinational cloning systems, particularly the Gateway technology, have been widely used in the research community, and many Gateway-compatible open reading frame entry (donor) clone collections and expression vectors have been created for functional genomics in many organisms (Yashiroda et al., 2008), including plants (Karimi et al., 2007b). On the other hand, although extremely useful for the simple and efficient transfer of DNA fragments into multiple expression destination vectors, the usefulness of the Gateway cloning system is rather limited for many projects where only a single expression vector is required for a DNA fragment. The two-step cloning process of the Gateway technology is laborious and time consuming for the production of a

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single expression vector. This is particularly true when a large number of plasmids must be cloned. Although a one-step recombinational cloning method was described to eliminate the production of an entry clone (Fu et al., 2008), the approach is rather limited in scope because long primers containing the specific attachment site (*att*) and two-step PCR are required (Fu et al., 2008).

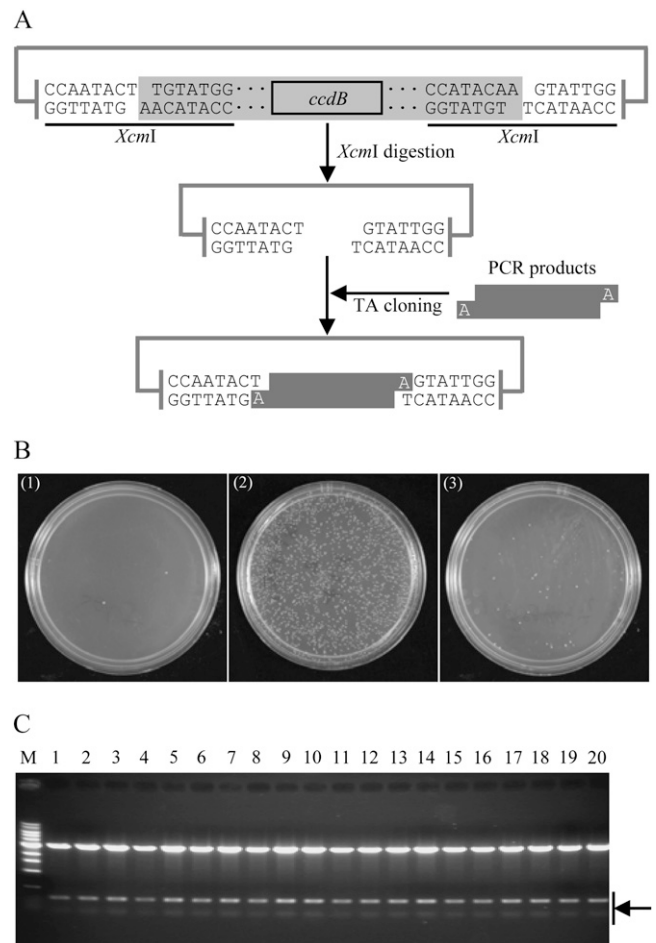
TA cloning is routinely used for cloning of PCR-amplified fragments. This technique exploits the terminal transferase activity of some DNA polymerases that add a 3'-A overhang to each end of the PCR product. PCR products can be easily cloned into a linearized vector with 3'-T overhangs compatible with 3'-A overhangs. Because it is difficult to generate a high-quality TA cloning vector in individual laboratories, many TA cloning kits are available in the market. Many of them use blue/white screening for recombinants, and the DNA fragments can only be cloned into the TA vector provided in the kit. To meet the need for high-throughput cloning of DNA fragments into diverse expression vectors, we have developed a significantly improved TA cloning vector system by taking advantage of the negative selection gene marker *ccdB* to eliminate the self-ligation background after transformation. We refer to this new method as the zero background TA cloning system (ZeBaTA). Numerous cloning tests in our laboratory have shown that ZeBaTA provides very high cloning efficiency with almost no self-ligation. Moreover, the ZeBaTA technology can be flexibly adapted for developing specialized expression vectors allowing single-step assembly of PCR-amplified genes or fragments. We demonstrate the feasibility and flexibility of the technology by developing a set of 12 transient and 12 stable transformation vectors for constitutive gene expression, gene silencing, protein tagging, protein subcellular localization, and promoter fragment activity analysis for rice (*Oryza sativa*) and Arabidopsis (*Arabidopsis thaliana*). Our results suggest that ZeBaTA technology can also be easily used to develop expression vectors for other organisms (e.g. *Escherichia coli*, yeast [*Saccharomyces cerevisiae*], insect, and mammal), thereby providing a novel and general high-throughput platform for functional genomics of target genes.

## RESULTS

### Construction of the ZeBaTA System

Two different strategies were used to produce T-vectors, i.e. adding a single thymidine at the 3' blunt ends of a linearized vector (Holton and Graham, 1991; Marchuk et al., 1991) and generating single 3'-T overhangs of a linearized vector by restriction endonuclease digestion (Kovalic et al., 1991; Mead et al., 1991; Ichihara and Kurosawa, 1993; Chen et al., 2006a). Although the former has been used to produce commercial cloning kits like the pGEM-T system, we

selected the restriction endonuclease digestion-mediated strategy to develop a TA cloning vector system because this approach is easy to use for individual laboratories. Previous publications have described the use of restriction enzyme *XcmI* (Kovalic et al., 1991; Mead et al., 1991) or *AhdI/Eam1105I* (Ichihara and Kurosawa, 1993; Chen et al., 2006a) to produce intermediate T-vectors. We chose *XcmI* as the digestion enzyme to develop the ZeBaTA cloning system because it had a better digestion efficiency than *AhdI*.



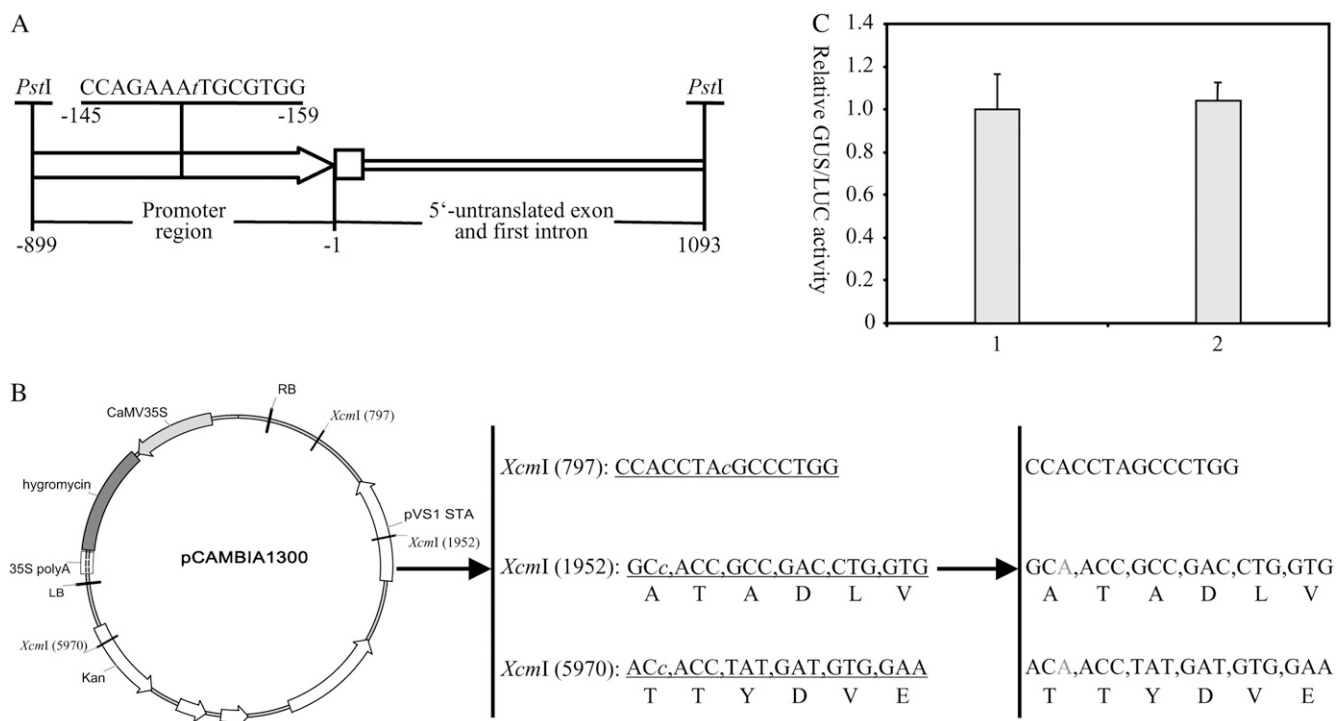
**Figure 1.** Construction of the ZeBaTA system. A, Schematic representation of direct cloning of PCR product using the ZeBaTA vector system. The linker of the vector (in gray) is removed after *XcmI* digestion yielding a linearized T-vector. B, TA cloning tests of the ZeBaTA system. (1) Self-ligation of *XcmI*-digested pGXT using T4 DNA ligase from Promega. (2) Ligation of *XcmI*-digested pGXT with the PCR product of the rice blast fungus *M. oryzae* gene *MGG\_07986.5* using T4 DNA ligase from Promega. (3) Ligation of *XcmI*-digested pGXT with the PCR product of *MGG\_07986.5* using T4 DNA ligase from USB Corporation. C, Samples of restriction digestion analysis of the randomly selected colonies derived from ligation of *XcmI*-digested pGXT with the PCR product of *MGG\_07986.5* using T4 DNA ligase from Promega. pGXT contains two *Bam*HI recognition sites outside the two *XcmI* recognition sites (Supplemental Fig. S1), and *MGG\_07986.5* contains one internal *Bam*HI site. All samples (lanes 1–20) digested by *Bam*HI released two bands as expected. M, 1-kb DNA ladder.

The schematic illustration of the improved T-vector system for PCR-amplified gene/fragment cloning is shown in Figure 1A. A pair of *XcmI* recognition sites, CCAATACT/TGTATGG, was introduced in the vectors, which allowed the generation of a single thymidine residue at both 3' ends of the vector when digested with *XcmI*. To eliminate the potential self-ligation due to incomplete *XcmI* digestion of the vector, the *ccdB* gene (Bernard and Couturier, 1992; Miki et al., 1992), which inhibits growth of *E. coli* strains by expressing a protein to interfere with its DNA gyrase, was introduced between the two *XcmI* sites. Hence, any self-ligation transformants containing the *ccdB* gene will be eliminated. To test the cloning efficiency of the T-vector system, an intermediate vector pGXT was generated based on the backbone of the pGEM-T easy vector. After *XcmI* digestion, ligation reactions of the resulting T-vector alone and T-vector with the PCR-amplified product of the rice blast fungus *Magnaporthe oryzae* gene MGG\_07986.5 were set up following the standard protocol of the Promega pGEM-T easy vector system. Transformation tests showed that ligation of the T-vector with the MGG\_07986.5 fragments yielded a large number of colonies, whereas ligation of the T-vector alone yielded only a few colonies (Fig. 1B). Restriction digestion screening confirmed that the plasmids yielded from ligation of the

T-vector with the PCR product were true recombinants (Fig. 1C). To establish a general guide for consistently successful cloning, several factors, such as *XcmI* over-digestion for generating a T-vector, insert-to-vector molar ratios, and different T4 DNA ligases, were tested to determine their effect on cloning efficiency. Surprisingly, we observed that T4 DNA ligases could have a significant impact on cloning efficiency. Ligations using Promega T4 DNA ligase, the same product used by the pGEM-T easy vector system, consistently gave very high cloning efficiency with almost no self-ligation background. However, regular T4 DNA ligases from USB Corporation usually gave very low ligation efficiency for this TA cloning system (Fig. 1B). Although the ligation efficiencies were a little higher at insert-to-vector molar ratios of 4:1 to 8:1 with the T-vector generated by standard digestion, ligations from vectors with 10- or 20-fold overdigestion and ligations with insert-to-vector molar ratios of 1:1, 4:1, 8:1, and 12:1 all yielded good cloning efficiency when Promega T4 DNA ligase was used (data not shown).

#### Set of Expression ZeBaTA Vectors for Plants

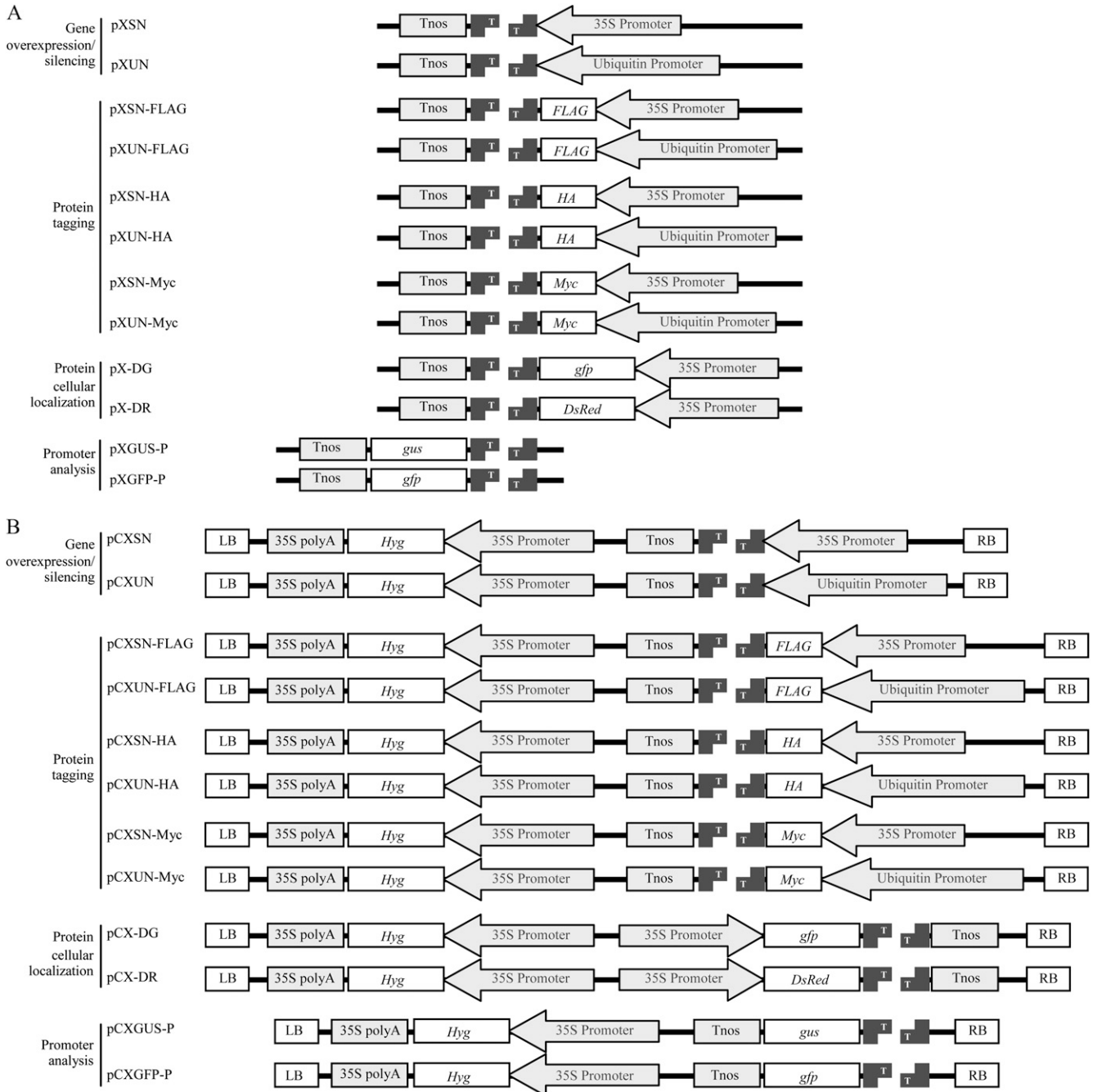
Using ZeBaTA, we developed a set of transient and stable expression vectors for different applications in both dicot and monocot plants. The backbone of all



**Figure 2.** Site-specific mutagenesis of the maize ubiquitin-1 promoter (A) and the backbone of the binary vector pCambia1300 (B) in which three *XcmI* recognition sites were deleted. The nucleotides represented in lowercase italic letters are the positions where deletions or mutations were made. Kan, Kanamycin resistance gene; LB, T-DNA left border; RB, T-DNA right border. C, Comparison of the levels of GUS expression mediated by the original and modified maize ubiquitin-1 promoter in transiently transfected rice protoplasts. GUS activities are represented as a ratio of relative GUS/LUC. The experiment was repeated three times with similar results. 1, Protoplast sample transfected with pUbiGUS; 2, protoplast sample transfected with pXUN-GUS.

transient expression vectors is derived from pBlue-script II KS (www.stratagene.com), a high-copy-number cloning vector that can facilitate the isolation of a large amount of plasmid DNA for transient expression. The backbone of all stable expression vectors is derived from pCAMBIA1300 (www.cambia.org), an *Agrobacterium tumefaciens* binary vector widely used

for transformation in both dicot and monocot plants. Two different promoters, a cauliflower mosaic virus 35S promoter (Odell et al., 1985) and a maize (*Zea mays*) ubiquitin-1 promoter (Christensen et al., 1992) were used to drive expression of genes of interest in dicots and monocots, respectively. The 35S promoter is more efficient in dicots, whereas the maize ubiquitin-1



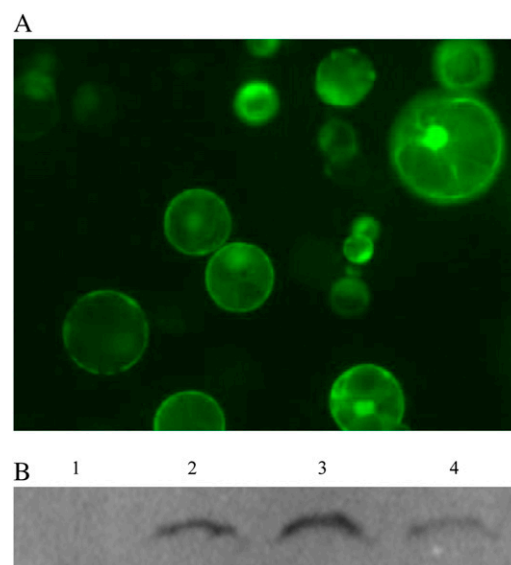
**Figure 3.** ZeBaTA-based expression vectors for gene overexpression/silencing, protein tagging, protein subcellular localization, and promoter analysis in plants. A, Schematic structures of the transient expression vectors generated by *XcmI* digestion. B, Schematic structures of the *Agrobacterium*-mediated stable transformation vectors generated by *XcmI* digestion. LB, T-DNA left border; RB, T-DNA right border.

promoter is more efficient in monocots (Christensen et al., 1992). The original maize ubiquitin-1 promoter and pCAMBIA1300 vector, however, contain one and three *XcmI* recognition sites, respectively (Fig. 2, A and B). To facilitate the construction of the ZeBaTA-based expression vectors, the *XcmI* recognition sites of the maize ubiquitin-1 promoter and pCAMBIA1300 vector were eliminated by site-specific deletion or site-specific mutation (Fig. 2, A and C). The designed expression vectors were all engineered with the cassette of the *XcmI-ccdB-XcmI* fragment (Supplemental Fig. S1). Figure 3, A and B, illustrates the structural maps of the 12 transient and 12 stable transformation T-vectors. All vectors have been tested for cloning at least one time, and the results showed that these ZeBaTA expression vectors, including those binary vectors that are relatively large in size (>10 kb), consistently yielded high cloning efficiency (Supplemental Fig. S2).

Because the maize ubiquitin-1 promoter used in this system was modified to block its original *XcmI* recognition site by deleting a single base (Fig. 2A) at the position of nucleotide -480, a *gus* gene (Jefferson et al., 1987) was amplified by PCR and then cloned into the *XcmI*-digested pXUN vector to produce an expression construct to test the expression activity of the modified maize ubiquitin-1 promoter. The derived construct pXUN-GUS and a control construct pUbi-GUS (Chen et al., 2006b), of which a *gus* gene is driven by the original maize ubiquitin-1 promoter, were tested transiently in the transfected rice protoplasts. Transient expression assays showed that the levels of GUS activity in rice protoplasts transfected with these two constructs were similar (Fig. 2C), indicating that the deletion of nucleotide -480 does not affect the activity of the maize ubiquitin-1 promoter.

#### Testing of Tagged Protein Expression

Epitope tagging is a widely used method for the rapid and effective characterization, purification, and *in vivo* localization of the protein products of cloned genes. To facilitate gene cloning for epitope tagging in plants, a total of 12 epitope-tagging vectors (Fig. 3, A and B) were constructed using ZeBaTA. These vectors contain a 35S promoter or a maize ubiquitin-1 promoter, allowing direct cloning of genes of interest into expression vectors to express a translational fusion of target protein with three commonly used epitope tags in plants (i.e. FLAG, HA, or Myc; Earley et al., 2006). To determine the feasibility of this epitope-tagging system, a *gfp* gene was cloned into the pXUN-HA vector to fuse with the HA tag. The resulting expression construct pXUN-HA-GFP was transiently expressed in rice protoplasts. As shown in Figure 4, A and B, protoplasts transfected with pXUN-HA-GFP showed strong GFP fluorescence, and HA-tagged GFP protein was detected in protein extracts of transfected protoplasts but not in the nontransfected control, demonstrating the potential application of this

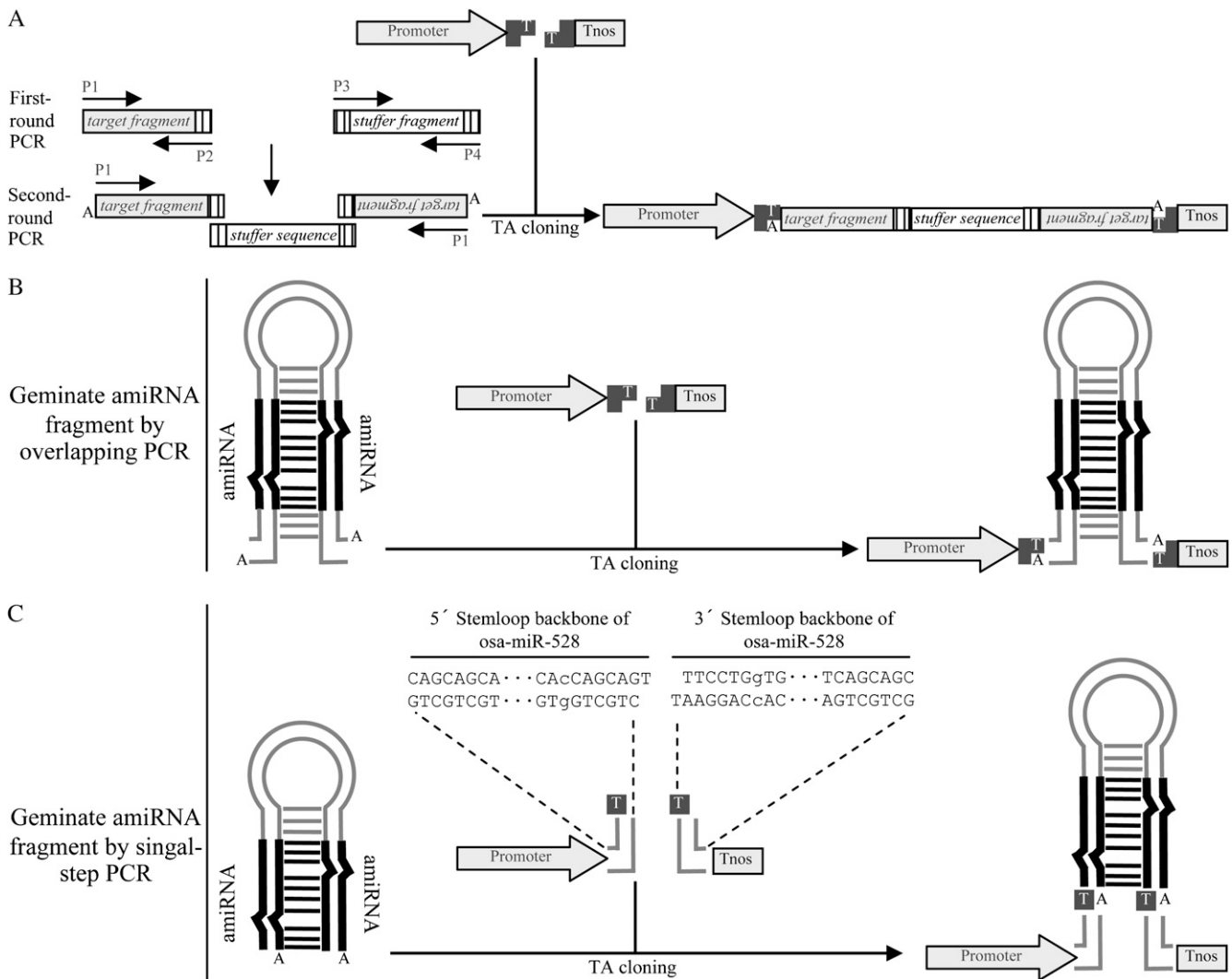


**Figure 4.** Transient expression and protein-tagging detection of the ZeBaTA vectors in rice protoplasts. A, Fluorescence microscopy of the expression of HA-tagged GFP in rice protoplasts. B, Detection of HA-tagged GFP by western blot. Lane 1, Nontransfected control protoplast sample; lanes 2 to 4, independent protoplast samples transfected with pXUN-HA-GFP. [See online article for color version of this figure.]

system for functional study of target proteins in plants.

#### Gene Silencing by Hairpin RNAi or Artificial MicroRNA

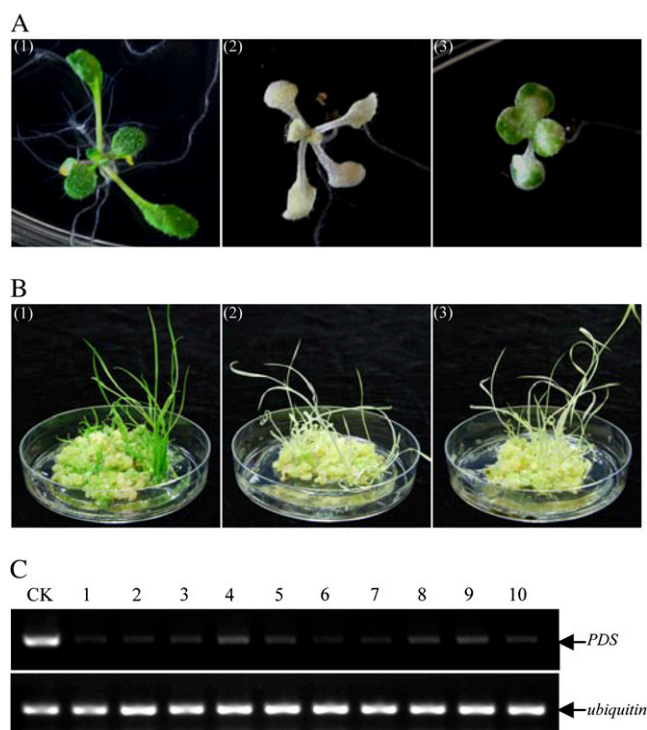
In plants, a typical and efficient approach to induce gene silencing is to use an inverted-repeat construct to express hairpin RNA (hpRNA; Waterhouse et al., 1998; Smith et al., 2000). However, a major limitation of the hpRNA interference (hpRNAi) approach for high-throughput gene functional analysis is the cumbersome cloning procedure for generating hpRNAi constructs (Helliwell and Waterhouse, 2003). The generation of a hpRNAi construct using conventional restriction enzyme digestion and DNA ligation methods usually requires several cloning steps. Although Gateway cloning technology has been adapted to generate hpRNAi constructs (Helliwell and Waterhouse, 2003; Miki and Shimamoto, 2004), it still requires two cloning steps. With the ZeBaTA system, hpRNAi constructs can be made by a single-step cloning procedure (Fig. 5A). Instead of making an inverted-repeat cassette by DNA recombination techniques, we designed a new approach to assemble the hpRNAi cassette by overlapping PCR. Briefly, a target fragment with an additional 3'-terminal sequence complementary to both the 5'- and 3'-terminal ends of a designed spacer fragment is amplified as a first step. The overlapping fragments are then fused together in a subsequent PCR reaction, and the resulting inverted-repeat is cloned directly into a ZeBaTA expression vector (Fig. 5A). To test the feasibility of this approach, an RNAi construct



**Figure 5.** Schematic illustration of the construction of hpRNAi or amiRNA constructs by single-step cloning. **A**, Generation of hpRNAi constructs by overlapping PCR approach. The target gene fragment and the stuffer sequence fragment are amplified in the first-round PCR. Primers P2, P3, and P4 introduce complementary adapters (indicated by vertically lined boxes) to the amplified fragments. The two amplified fragments are fused together as an inverted-repeat cassette in the second-round PCR by using single P1 primer. The resulting fragment is then directly cloned into the plant expression T-vector. **B**, Generation of amiRNA constructs by overlapping PCR approach. **C**, Generation of amiRNA constructs for rice genes by single-step PCR. The expression vectors pXUN-osaMIR528 and pXUN-osaMIR528 were preassembled with 5' and 3' stemloop backbone sequences of a rice miRNA precursor Osa-MIR-528 (Warthmann et al., 2008). Thus, making amiRNA constructs for rice target genes only requires an amiRNA-amiRNA\* fragment generated from single-step PCR. The nucleotides represented in lowercase letters are the positions where mutations were made to introduce two *XcmI* recognition sites.

was generated by overlapping PCR in which the sense and antisense 217-bp fragments of the Arabidopsis phytoene desaturase gene (*PDS*) were separated by a 420-bp stuffer fragment derived from the *gus* gene. The resulting fragment was cloned into the pCXSN vector (Fig. 3B) to generate the expression construct pCXSN-atPDS-RNAi. The RNAi construct was introduced into Arabidopsis by the floral-dip method. Over 80% of transgenic plants had a clear albino phenotype (Fig. 6A), a typical visible phenotype caused by silencing of the *PDS* gene (Guo et al., 2003; Miki and Shimamoto, 2004).

Recently, the artificial microRNA (amiRNA) approach has been introduced for highly specific gene silencing in both dicot and monocot plants (Niu et al., 2006; Schwab et al., 2006; Ossowski et al., 2008; Warthmann et al., 2008). Typically, the amiRNA is generated by site-directed mutagenesis on precursors of endogenous miRNAs to exchange the natural miRNA sequences with those of amiRNAs using overlapping PCR (Ossowski et al., 2008). The same ZeBaTA-based vector system developed for ectopic gene expression and hpRNAi can also be used for making amiRNA expression constructs by simple TA



**Figure 6.** Silencing of the *PDS* gene in Arabidopsis and rice by the ZeBaTA-based hpRNAi or amiRNA approaches. A, Arabidopsis plants transformed with the hpRNAi construct pCXSN-atPDS-RNAi showing the *PDS* silencing albino phenotype. (1) Control plant; (2 and 3) two examples of transgenic Arabidopsis plants. B, Rice plants transformed with the amiRNA vectors showing the albino phenotype. (1) Control plant; (2) example of pCXUN-amiPDS-transformed plants; and (3) example of pCXUN528-PDS-transformed plants. C, RT-PCR analysis of *PDS* suppression transgenic rice plants. Five independent primary plants (1, 2, 3, 4, and 5) transformed with pCXUN-amiPDS and five independent primary plants (6, 7, 8, 9, and 10) transformed with pCXUN528-PDS were selected for the analysis. CK, Wild-type Nipponbare plant used as the control.

cloning (Fig. 5B), thus bypassing the time-consuming two-step procedure for the regular restriction enzyme digestion-mediated cloning or the Gateway cloning (Ossowski et al., 2008). We further developed a ZeBaTA-amiRNA system to simplify the generation of rice amiRNA constructs because our lab is focusing on rice functional genomics. The new ZeBaTA-amiRNA vector was designed based on the stemloop backbone derived from Osa-MIR528, an endogenous rice miRNA precursor that has been used to efficiently express amiRNAs for highly specific silencing of targeted genes in rice (Warthmann et al., 2008). By site-directed mutagenesis of a single base on the 5' and 3' stemloop backbones of Osa-MIR528, respectively, a cassette of 5' Osa-MIR528 stemloop backbone-*XcmI*-*ccdB*-*XcmI*-3' Osa-MIR528 was assembled and cloned into the expression vectors where the expression of amiRNA is under the control of the maize ubiquitin-1 promoter. Figure 5C illustrates the structural maps of the Osa-MIR528-based vectors pXUN-osaMIR528 and pCXUN-osaMIR528. The vectors allow for high-throughput generation

of rice amiRNA constructs by cloning the amiRNA-amiRNA\* fragment generated from a single-step PCR into the ZeBaTA vector with the preassembled Osa-MIR528 stemloop backbone (Fig. 5C; Supplemental Fig. S3), thus avoiding the time-consuming overlapping PCR. The modified vector was evaluated by expression of the amiRNA for silencing of the *OsPDS* gene. The two constructs pCXUN-amiPDS and pCXUN528-PDS, which contain original or modified Osa-MIR528 stemloop backbone with amiRNA sequence targeting *OsPDS*, respectively, were introduced into rice cv Nipponbare by *Agrobacterium*-mediated transformation. Consistent with a previous study (Warthmann et al., 2008), 70.1% of the primary transgenic lines transformed with pCXUN-amiPDS had a bleaching *PDS* silencing phenotype (Fig. 6B; Table I). Similarly, 77.1% of the primary transgenic lines transformed with pCXUN528-PDS had the same albino phenotype, suggesting that the mutagenesis on the Osa-MIR528 stemloop backbone does not affect the biogenesis of the amiRNA for silencing of the *PDS* gene.

#### Protein Subcellular Localization/Colocalization and Promoter Activity Assay

To investigate the subcellular localization or colocalization of particular proteins, a set of ZeBaTA vectors (i.e. pXDG, pXDR, pCXDG, and pCXDR) was devised for transient or stable expression of protein fusions with GFP or red fluorescent protein. The vectors contain a 35S promoter-driven *gfp* or *DsRed* cassette that has been used to visualize protein localization in both dicot and monocot plants (Goodin et al., 2002; Chen et al., 2006b). As shown in Figure 3, A and B, PCR products of genes of interest can be simply engineered into the vectors to fuse with the *gfp* or *DsRed* gene. To confirm whether the vectors can be used for detecting protein localization in plant cells, the rice *Spin1* gene encoding a putative RNA-binding protein previously shown to be nuclear targeted (Vega-Sánchez et al., 2008) was cloned into vectors pXDG and pXDR to fuse in-frame with *gfp* and *DsRed*, respectively. Transient expression of the constructs pXDG-Spin1 and pXDR-Spin1 in rice protoplasts demonstrated that the GFP- and DsRed-SPIN1 fusion proteins were targeted to the nuclear region as predicted (Fig. 7A).

For promoter activity assays, two reporters, *gus* and *gfp*, were used for constructing pXGUS-P/pCXGUS-P and pXGFP-P/pCXGFP-P, respectively. The linear T-vectors of pXGUS-P/pCXGUS-P or pXGFP-P/

**Table 1.** *PDS* silencing frequency of transgenic rice mediated by the ZeBaTA-amiRNA system

amiRNA Vector	Total Independent Transformants	Albino Phenotype	Efficiency
			%
pCXUN-amiPDS	55	39	70.1
pCXUN528-PDS	35	27	77.1

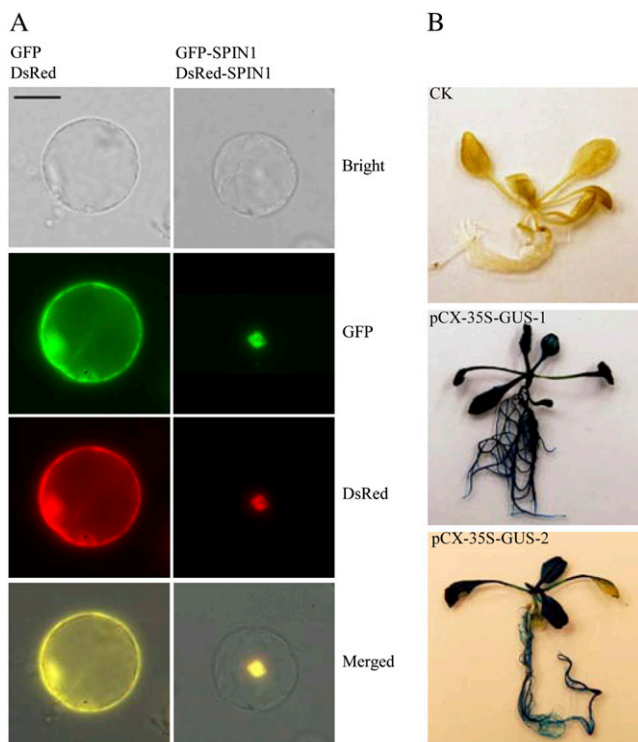
pCXGFP-P (Fig. 3, A and B) allow direct cloning of PCR-amplified promoter fragments located in front of the reporter genes. As proof of concept, the 35S promoter was cloned into pCXGUS-P to drive expression of the reporter gene *gus*. Arabidopsis plants stably transformed with the construct pCX-35S-GUS showed constitutive GUS expression in the whole plants (Fig. 7B), confirming the feasibility of the system for assaying promoter activity.

## DISCUSSION

With the rapid development of the next-generation sequencing technology, more plant genomes will be sequenced in the near future. How to rapidly determine the function of the identified genes on a large scale is a daunting challenge. The ability to efficiently make constructs to transiently and stably express specific genes in cells, tissues, or whole plants is a fundamental aspect and bottle neck of plant functional genomics research. Traditionally, the cloning vectors

for plant research carry a multicloning site (MCS) within their target gene expression cassettes. The restriction sites in the MCS are rather limited, making cloning of most target genes difficult. Although TA cloning vectors have been widely used for cloning of PCR-amplified fragments, the system has not yet been incorporated in the cloning vectors for transient and stable expression of target genes because of the technical challenge of generating low-background TA cloning vectors. The Gateway system has been a popular choice for generating various constructs because it allows the gene of interest to be easily cloned into specifically designed plasmids without DNA restriction digestions. The two-step cloning and expensive reagents, however, make the Gateway system impractical for large-scale cloning in most individual laboratories when the entry clone collections are not available. The ZeBaTA system described here overcomes the limitations of both the TA and Gateway cloning systems. After two *XcmI* recognition sites have been introduced into the MCS, any PCR fragments with a T-overhang can be easily cloned into a ZeBaTA vector. With the introduction of the negative selection marker gene *ccdB* between the two *XcmI* sites, any self-ligation transformants are eliminated. Using this technology, we constructed a set of 12 transient and 12 stable transformation vectors for plant gene expression studies and tested the vectors in rice or Arabidopsis in our laboratories. These vectors can be used in a wide range of functional genomics projects in plants and will be distributed to the research community upon request.

Under certain conditions, cloning with T-vectors generated by digestion with *AhdI* or *XcmI* gave low efficiency and the T residue of the insert-vector junction in the recombinant clones is often missing (Mead et al., 1991; Chen et al., 2006a). Chen et al. (2006a) speculated that this may be due to the presence of unknown factors that, during digestion and preparation of the T-vectors, influence the stability of 3'-T overhangs. In this study, we found that the main factor affecting successful cloning is the use of an appropriate T4 DNA ligase. We tested the Promega T4 DNA ligase, which is included in the pGEM-T easy vector system, and the T4 DNA ligase from USB Corporation. The ligations using Promega T4 DNA ligase consistently gave a very high cloning efficiency; most of the ligations using USB Corporation T4 DNA ligases yielded low efficiency. Many of the recombinant plasmids from the latter ligations missed a T residue in the insert-vector junction, consistent with observations by Mead et al. (1991) and Chen et al. (2006a). The T residue is missing mainly because regular commercial T4 DNA ligases contain exonuclease activities that can remove the 3'-T tails from the vector, as reported in the technical manual of the pGEM-T and pGEM-T easy vector systems (<http://www.promega.com/tbs/tm042/tm042.pdf>); removal of the 3'-T tails from the vector results in very low cloning efficiency. When the Promega T4 DNA ligase was used for ligation, we



**Figure 7.** Protein subcellular localization and promoter activity analysis using the ZeBaTA vectors. A, Fluorescence microscopy of the coexpression of GFP and DsRed, or GFP-SPIN1 and DsRed-SPIN1 fusions in rice protoplasts. Scale bar = 20  $\mu$ m. The RNA binding nuclear protein SPIN1 was used as a tester (Vega-Sánchez et al., 2008). B, GUS staining of Arabidopsis transformed with pCX-35S-GUS, where the 35S promoter was cloned into the vector pCXGUS-P to test the system. CK, Plant transformed with control vector pCambia1300 ([www.cambia.org](http://www.cambia.org)); pCX-35S-GUS-1 and pCX-35S-GUS-2, two independent primary transgenic plants.



never observed the absence of the T residue in the recombinant plasmids (data not shown), suggesting that the 3'-T overhangs of the T-vectors generated by *XcmI* digestion were stable. Our cloning tests also indicated that the size of T-vectors, vector overdigestion, and the insert-to-vector molar ratios had a minimal impact on cloning efficiency.

In recent years, Gateway cloning technology has become increasingly popular for functional genomics in plants (Karimi et al., 2002, 2005, 2007a, 2007b; Curtis and Grossniklaus, 2003; Earley et al., 2006; Hilson, 2006; Himmelbach et al., 2007; Lei et al., 2007). In comparison to the Gateway system, ZeBaTA offers several significant advantages. First, it allows simple and highly efficient one-step cloning for PCR-amplified genes/fragments of interest into a specialized ZeBaTA expression vector. Second, the ZeBaTA constructs have a short vector-insert junction with only 8 bp (CCANNNNN), whereas the constructs generated by Gateway recombination contain a longer *att* linker sequence with 25 bp or even up to 125 bp (Lei et al., 2007). For example, protein-tagging constructs generated by Gateway cloning technology usually result in a long linker with at least eight or sometimes up to 20 amino acids (Dubin et al., 2008) between the tag and the protein that could interfere with protein function or expression. In contrast, ZeBaTA-based constructs for protein tagging result in a short linker with only three amino acids (Supplemental Fig. S1), thereby reducing the potential for interference with the tag-protein fusion. Last, the *XcmI* recognition sequences CCANNNNN/NNNNTGG, where N can be any pyrimidine or purine, is more flexible for designing special vectors for high-throughput functional analysis.

In this article, we demonstrate the flexibility of this system by developing a ZeBaTA-amiRNA vector for rice functional genomics (Fig. 5C; Supplemental Fig. S3). With the introduction of a single base mutation of GCA → CCA and TGC → TGG on the 5' and 3' stem-loop backbones of an endogenous rice miRNA precursor Osa-MIR528 (Warthmann et al., 2008), respectively, the modified vector allows for generating an expression T-vector with preassembled 5' and 3' Osa-MIR528 stemloop backbone sequences. Thus, amiRNA expression constructs can be simply generated by cloning an amiRNA-amiRNA\* fragment generated from a single-step PCR. Prediction of the original and modified Osa-MIR528 stemloop gave exactly the same secondary structures (Supplemental Fig. S4), suggesting that the modification of the stem-loop backbone did not affect the structural features of the miRNA precursor. Further stable transformation using vectors based on the original or modified stemloop backbones gave a similar high efficiency for targeted *OsPDS* silencing, indicating that without changing structural features, the modified precursor stemloop backbone can functionally express a miRNA to trigger targeted gene silencing. Although we did not apply the approach to other miRNA precursors, our

results suggest that this approach should be amenable to different amiRNA systems in other plants.

## CONCLUSION

We developed the novel and versatile ZeBaTA cloning system for plant functional genomics studies. The newly developed 12 transient and 12 stable expression T-vectors in this study allow for direct cloning of PCR-amplified genes/fragments of interest for constitutive expression, gene silencing, protein tagging, protein subcellular localization detection, and promoter activity analysis in plants. We have demonstrated the usefulness of the system in rice and Arabidopsis. The ZeBaTA system can be easily adapted for the generation of more specialized expression vectors for other plants, as well as for *E. coli*, yeast, fungus, insect, and mammalian systems. In addition, the use of the basic ZeBaTA vector for PCR-fragment cloning will save a considerable amount of money for a laboratory to purchase commercial TA cloning kits. Therefore, our ZeBaTA system provides a general and cost-efficient molecular cloning platform for all molecular laboratories.

## MATERIALS AND METHODS

### ZeBaTA Vector Construction and Propagation

Standard molecular manipulations (Sambrook and Russell, 2001) were performed to make all the constructs. Details of plasmid construction are available in Supplemental Materials and Methods S1. The ZeBaTA vectors carry the *ccdB* gene, which inhibits growth of most *Escherichia coli* strains by expressing the CcdB protein to interfere with their DNA gyrase. All ZeBaTA vectors were propagated in the *E. coli* strain DB3.1, which contains a specific mutation in the *gyrase* gene, and thus insensitive to CcdB toxic protein (Hartley et al., 2000).

### TA Cloning with ZeBaTA Vectors

The ZeBaTA vectors were digested with *XcmI* (New England Biolabs). PCR products were amplified using either a proofreading Pfu DNA polymerase (Stratagene) followed by the A-addition procedure (technical manual of pGEM-T and pGEM-T easy vector systems; <http://www.promega.com/tbs/tm042/tm042.pdf>) or a Platinum DNA polymerase (Invitrogen). The digested ZeBaTA plasmids and the PCR-amplified products were purified from agarose gels after electrophoresis using the Qiagen MinElute gel extraction kit (Qiagen), and product concentrations were then measured using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). T4 DNA ligases from Promega (catalog no. M1804; Promega), and USB Corporation (no. 70005) were tested for TA cloning efficiency. For ZeBaTA cloning, the ligation was carried out using the T4 DNA ligase from Promega (catalog no. M180; Promega) in a total volume of 10  $\mu$ L containing 50 ng of T-vector and the corresponding volume of PCR product with a standard insert-to-vector molar ratio around 6:1. The ligation reaction mixture was transformed into *E. coli* strain DH 10B by electroporation. All recombinant plasmids identified from individual *E. coli* colonies were verified by sequencing.

### Rice Protoplast Transient Expression

Rice (*Oryza sativa*) protoplasts were isolated from 2-week-old seedlings of rice cv Nipponbare. Protoplast preparation and transfection were carried out by following previously described procedures (Chen et al., 2006b). After transfection, protoplasts were incubated in darkness for about 16 h at room temperature.

## GUS, Western-Blot, and Fluorescence Microscopy Assays

GUS, western-blot, and fluorescence microscopy assays of transiently transfected rice protoplast samples were carried out following previously described procedures (Chen et al., 2006b). For GUS assays, protoplasts were cotransfected with the GUS construct and a 35S-LUC construct as the internal control. GUS assays were performed using 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG; Sigma) as the substrate according to Jefferson et al. (1987). LUC assays were performed by using the Promega luciferase assay system (Promega). The GUS activity in each sample was expressed relative to LUC activity to normalize data for variation in transfection efficiency and cell viability. For western-blot analysis, total protein isolated from transfected protoplast samples was subjected to dodecyl sulfate-PAGE. A rat monoclonal anti-HA antibody (Roche Molecular Biochemicals) was used to detect the HA-tagged GFP protein in protoplasts. Fluorescence microscopy was performed with a Nikon Eclipse 80i fluorescence microscope (Nikon). Excitation and emission filters Ex450-490/BA520-560 and Ex540-580/BA600-660 were used for GFP and DsRed, respectively.

## Plant Transformation and Analysis

For *Arabidopsis thaliana* transformation, the ZeBaTA vectors were first introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *Arabidopsis* cv Col-0 plants were transformed via the floral-dip method as described by Clough and Bent (1998). *Arabidopsis* seeds obtained after transformation were plated on one-half-strength Murashige and Skoog medium containing 30 mg L<sup>-1</sup> hygromycin and 100 mg L<sup>-1</sup> carbenicillin for selection. GUS histochemical staining was carried out as described by Jefferson et al. (1987).

For rice transformation, the ZeBaTA vectors were introduced into *A. tumefaciens* LBA4404 by electroporation. Calli of rice cv Nipponbare were used for transformation via the *Agrobacterium*-mediated procedure as described previously (Hiei et al., 1994; Yin and Wang, 2000). Semiquantitative reverse transcription (RT)-PCR was used to examine the level of RNA accumulation of control plants and transgenic albino plants. Total RNA was isolated from leaf tissues by using TRIzol reagent (Invitrogen) and was treated with RNase-free DNase I (Ambion) to remove DNA contamination. One microgram of DNase I-treated total RNA was subjected to RT using the Promega RT system (Promega). RT-PCR was carried out under standard conditions with specific primers for *OsPDS* (Miki and Shimamoto, 2004), and the internal control *ubiquitin* genes.

Sequence data from this article can be found in the GenBank data library under accession numbers FJ905202 to FJ905213 (for pXSN, pXUN, pXSN-FLAG, pXUN-FLAG, pXSN-HA, pXUN-HA, pXSN-Myc, pXUN-Myc, pX-DG, pX-DR, pXGUS-P, and pXGFP-P, respectively), and FJ905214 to FJ905225 (for pCXS, pCXUN, pCXS-FLAG, pCXUN-FLAG, pCXS-HA, pCXUN-HA, pCXS-Myc, pCXUN-Myc, pCX-DG, pCX-DR, pCXGUS-P, and pCXGFP-P, respectively).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Sequence structure of the *XcmI-cdB-XcmI* cassette in ZeBaTA vectors.

**Supplemental Figure S2.** TA cloning test of ZeBaTA-based *A. tumefaciens* binary vector.

**Supplemental Figure S3.** Schematic representation of single-step PCR generation of amiRNA construct for gene silencing in rice.

**Supplemental Figure S4.** Predicted secondary structure of the mutated *osa-MIR528* stemloop.

**Supplemental Table S1.** Primers used in this study.

**Supplemental Materials and Methods S1.** Construction of ZeBaTA vectors and plant expression vectors for proof of concept testing.

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