Plant Biotechnology 28, 201–210 (2011)
DOI: 10.5511/plantbiotechnology.11.0124a

Original Paper

Novel vector systems to accelerate functional analysis of transcription factors using chimeric repressor gene-silencing technology (CRES-T)

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Received October 25, 2010; accepted January 24, 2011 (Edited by N. Ohtsubo)

Abstract Chimeric repressor gene-silencing technology (CRES-T) is a powerful tool that has recently been developed for the functional analysis of plant transcription factors and for the genetic manipulation of plant traits. For CRES-T, a transcription factor is converted to a strong repressor by fusion with an SRDX repression domain, which is then expressed in plants to induce a loss-of-function phenotype. However, the traditional CRES-T vectors are inconvenient for gene cloning and promoter exchange. In this study, we developed new CRES-T vectors that are efficient and convenient to use by employing the Gateway system, a new vector backbone and a terminator derived from the heat shock protein 18.2 (HSP) gene. Our test experiments revealed that the CRES-T vector containing the Gateway linker sequence within the transcribed region showed reduced efficiency of CRES-T when compared with the traditional CRES-T vector. However, the HSP terminator compensated for the negative effect of the Gateway sequence and improved the efficiency of CRES-T in all cases tested and resulted in the highest efficiency achieved to date. We found that the HSP terminator increased transcription efficiency or transcript stability; in contrast, these factors were negatively affected by the Gateway linker sequence in our vector system. We, therefore, propose that the appropriate CRES-T vector should be chosen depending on situations and purposes.

Key words: Chimeric repressor gene-silencing technology, heat shock protein terminator, Gateway, SRDX, transcription factor.

Transcription factors (TFs) play a key role in the regulation of numerous plant functions and phenotypes by controlling the expression of multiple target genes. Identification of the biological functions of TFs is, therefore, one of the most important subjects in plant science. Genetic modification of TFs has been exploited to manipulate and to introduce valuable traits to plants. However, the high redundancy of gene function often hampers these approaches. Recently, we developed a novel system for gene silencing to overcome this problem that we called chimeric repressor gene-silencing technology (CRES-T). In CRES-T, a chimeric repressor, which was converted from a transcriptional activator to a repressor by fusion to the repression domain, SRDX, is expressed in plants (Hiratsu et al. 2003). SRDX is a modified ERF associated Amphiphilic Repression (EAR) motif found in the SUPERMAN repressor (Hiratsu et al. 2002). The chimeric repressor dominantly represses the expression of target genes, even in the presence of redundant endogenous TFs, resulting in a loss-of-function phenotype of the transcription factor (Ishida et al. 2007; Koyama et al. 2007; Kunieda et al. 2008; Mitsuda et al. 2005). In addition to the analysis of TFs functions, the CRES-T system has been utilized to manipulate traits of agronomically important plants, including those with high polyploidy and limited gene information (Narumi et al. 2011; Shikata and Ohme-Takagi 2008; Shikata et al. 2011). In the traditional CRES-T vector system, the coding sequence (CDS) of a TF without a stop codon has to be cloned into the SmaI site of p35SSRDXG to fuse in frame with SRDX. This is achieved using blunt-end ligation followed by Gateway LR recombination to transfer the transgene cassette into the pBCKH binary vector (Figure 1A; Mitsuda

Abbreviations: AG, AGAMOUS; CDS, coding sequences; CRES-T, chimeric repressor gene-silencing technology; CUC, cup-shaped cotyledon; EAR, ERF associated amphiphilic repression; HSP, heat shock protein 18.2; LUC, luciferase; NOS, nopaline synthase; TF, transcription factor.
This article can be found at http://www.jspcmb.jp/

et al. 2006). However, this traditional CRES-T vector system has several disadvantages. First, it is difficult to change the 35S promoter, which is pre-cloned into p35SSRDXG, to a different promoter. Driving a transgene by a promoter other than 35S is often required to analyze the biological function of the transgene and to express it in a limited tissue(s) where the 35S promoter is not active (Ito et al. 2007; Mitsuda et al. 2007). Second, the blunt-end ligation is inefficient and prevents high-throughput plasmid construction. Third, the activity of the nopaline synthase (NOS) terminator was shown to be low, resulting in reduced efficiency of gene expression (Nagaya et al. 2010). Recently the heat shock protein 18.2 (HSP) terminator was shown to improve the efficiency of gene expression when compared with the NOS terminator (Nagaya et al. 2010).

In this study, we developed new vectors for CRES-T by employing Gateway technology (Hartley et al. 2000; Sasaki et al. 2004) with a different vector backbone and the terminator from the *HSP18.2* gene in various combinations, to improve efficiency and convenience. We found that although the Gateway linker sequence reduced the efficiency of CRES-T in some cases, the HSP terminator compensated for this negative effect and resulted in the highest efficiency of CRES-T ever recorded. We propose that appropriate vectors should be chosen according to requirements.

Materials and methods

Construction of plasmids

The *att*R1-CmR-*ccd*B-*att*R2 fragment, amplified by PCR using attR1f and attR2r primers (Supplemental Table 1), was inserted into the SmaI site of p35SSRDXG (Mitsuda et al. 2006; Figure 1, Table 1) to generate pDEST35SSRDXG (Supplemental Table 2). The HindIII-EcoRI fragment of pDEST35SSRDXG containing the 35S promoter was inserted into the HindIII/EcoRI site of pBCKH, resulting in pDEST_35S_SRDX_BCKH. The *att*R1-CmR-*ccd*B-*att*R2 fragment with stop codons for all three reading frames (3-frame stop codon) was amplified by PCR using attR1f and

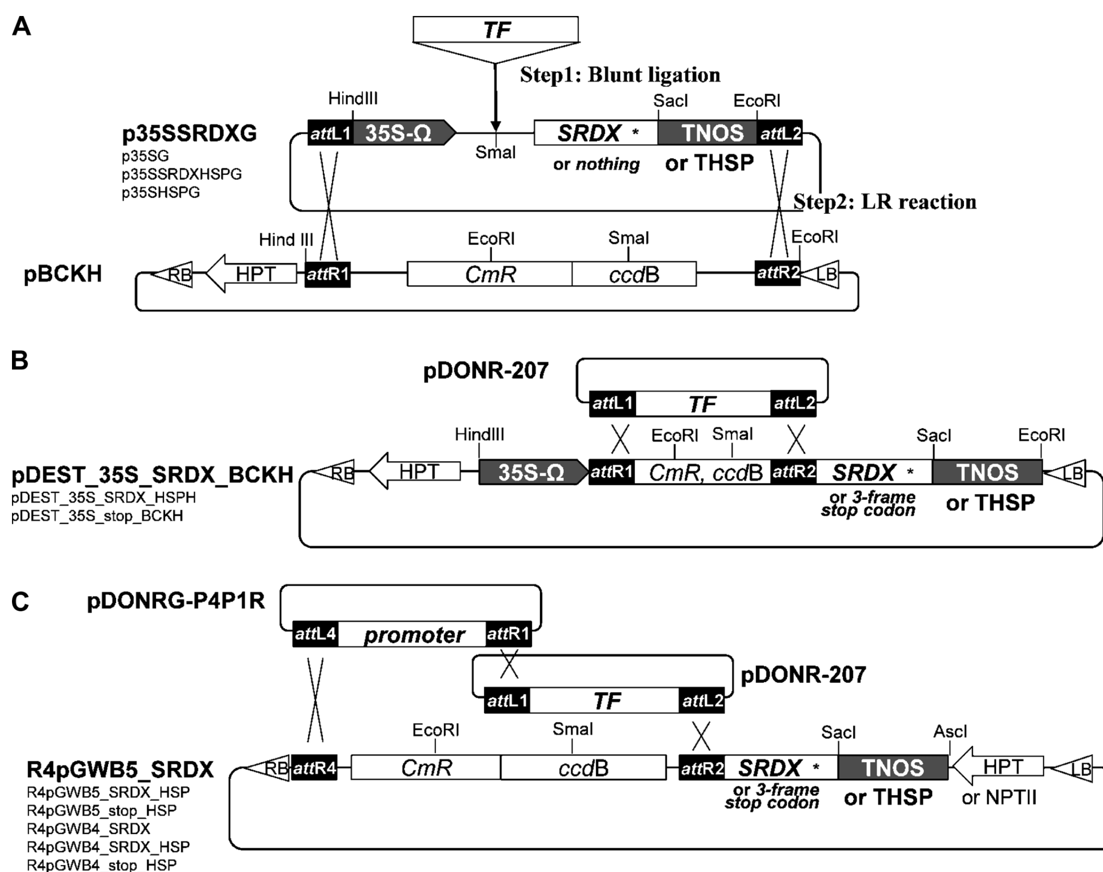

Figure 1. Overview of CRES-T vector systems and their derivatives. (A) Traditional vector system. Step1: The coding sequence of a PCR-amplified TF without a stop codon is cloned via blunt-end ligation into the SmaI site of p35SSRDXG to fuse with SRDX. Step2: The transgene cassette on the entry clone is transferred into pBCKH by an LR reaction. (B) 35S-driven Gateway vector system (pDEST_35S_SRDX_BCKH series). The TF entry clone is constructed by a BP reaction between pDONR-207 and a PCR-amplified coding sequence of a TF without a stop codon with *att*B1 and *att*B2 sequences, and inserted into pDEST_35S_SRDX_BCKH by an LR reaction. (C) Multisite Gateway vector system (R4pGWB-SRDX series). The entry clone of the promoter is constructed by a BP reaction between pDONRG-P4P1R and a PCR-amplified promoter with *att*B4 and *att*B1r sequences. The promoter and TF are inserted into R4pGWB5_SRDX by an LR reaction. 35S, cauliflower mosaic virus 35S promoter; Ω, the translational enhancer sequence from tobacco mosaic virus; TNOS, nopaline synthase terminator; THSP, *Arabidopsis HSP18.2* terminator; HPT, hygromycin B phosphotransferase; RB, right border; LB, left border; CmR, chloramphenicol-resistant marker; *ccd*B, negative selection marker.

attR2r_stop primers and was inserted into the SmaI site of p35SG (which was generated by removing SRDX from p35SSRDXG) to generate pDEST_35S_stop_BCKH. The fragment containing the HSP terminator (Nagaya et al. 2010) was subcloned into the SacI/EcoRI sites of p35SSRDXG and p35SG to generate p35SSRDXHSPG and p35SHSPG, respectively. The CmR-*ccd*B-*att*R2 fragment was amplified by PCR using EcoRI-CmR and attR2r primers and was inserted into the SmaI site of p35SSRDXHSPG to generate p35SCmRattR2SRDXHSP. The EcoRI fragment of p35SCmRattR2SRDXHSP was subcloned into the EcoRI site of pDEST_35S_SRDX_BCKH to generate pDEST_35S_SRDX_HSPH. The HindIII-35S-AtADH 5′UTR-SmaI fragment was digested from pRI 101-An DNA (Takara Bio, Japan) and subcloned into the HindIII/SmaI sites of p35SSRDXG and pDEST_35S_SRDX_BCKH to generate p35AASRDXG and pDEST_35AA_ccdBSXH, respectively. The *att*R1-CmR-*ccd*B fragment, amplified by PCR using attR1 and ccdBR primers, was inserted into the SmaI site of pDEST_35AA_ccdBSXH to generate pDEST_35AA_SRDX_BCKH. The synthesized SRDX fragment or the 3-frame stop codon was inserted into the Eco47III site of R4pGWB501 and R4pGWB401 (Nakagawa et al. 2008) to construct R4pGWB5_SRDX, R4pGWB5_stop, R4pGWB4_SRDX and R4pGWB4_stop, respectively. The NOS terminator located between SacI and AscI sites in these vectors was substituted with the HSP terminator, resulting in R4pGWB_SRDX_HSPs and R4pGWB_stops_HSPs. pDONRG-P4P1R was generated by the insertion of a BspHI fragment containing the gentamicin-resistance gene derived from pDONR207 (Life Technologies, USA) into pDONR-P4P1R (Life Technologies) digested by BspHI. pGWB501 and pGWB401 (Nakagawa et al. 2007b) were digested by SacI and AscI and then recircularized using a Blunting kit (Takara Bio, Japan) to generate pBCSH and pBCSK, respectively. The construction of pBCKH-NOS, pBCKH-NOS-CUC2SRDX and pBCKH-NOS-AGSRDX was previously described as 35S::CUC1SRDX, 35S::CUC2SRDX and 35S::AGSRDX, respectively (Hiratsu et al. 2003; Mitsuda et al. 2006). The coding regions of *CUC1*, *CUC2* and *AG* were cloned into the SmaI site of p35SSRDXHSPG and p35AASRDXG as described previously (Mitsuda et al. 2006). The coding sequence of luciferase (LUC) was amplified by PCR from p35S-GAL4-TATA-LUC (Hiratsu et al. 2002) using LUCF and LUCR primers and cloned into the SmaI site of p35SG. Different *CUC1* fragments, amplified by appropriate primer sets, were also cloned into the SmaI site of p35SSRDXG or p35SG (Supplemental Table 3). The transgene cassette was transferred into the destination vector pBCKH or pBCSH by the Gateway LR reaction (Life technologies). The *att*B4-35S-omega-*att*B1R, *att*B1-LUC-*att*B2 and *att*B1-CUC1SRDX-*att*B2 fragments, amplified by PCR using appropriate primers, were cloned into pDONRG-P4P1R or pDONR207 by the Gateway BP reaction (Life technologies). Preparation of the conventional entry clones for *CUC1*, *CUC2* and *AG* was described by Mitsuda et al. (2010). The content of each entry clone was introduced into pDEST_35S_SRDX/stop_BCKHs, pDEST_35S_SRDX_HSPH, pDEST_35AA_SRDX_BCKH, R4pGWB_SRDXs and R4pGWB_SRDX_HSP using the Gateway LR reaction (Life technologies). pBCKH-NOS was digested by HindIII to remove the *att*B1 sequence, followed by recircularization. The *att*B2 sequence was also removed from the resulting plasmid by EcoRI digestion, followed by recircularization to generate pBCKH-Δatt.

Transformation and genomic PCR

Arabidopsis thaliana ecotype Col-0 was transformed by the above-listed constructs, as described previously (Hiratsu et al. 2003). To confirm the genomic integration of transgenes, we extracted genomic DNA from 3 pBCKH-NOS, 36 pDEST-

Table 1. Vectors for CRES-T and expression of transgene

Name	Promoter	Enhancer	Tag	Terminator	Plant marker	Bacterial marker	Back bone	Cloning method
pBCKH	—	—	—	—	Hm	Km	pBIG	LR reaction
pBCSH	—	—	—	—	Hm	Spe	pPZP	LR reaction
pBCSK	—	—	—	—	Km	Spe	pPZP	LR reaction
p35SSRDXG	35S	Ω	SRDX	NOS	—	Amp	pUC	SmaI ligation
p35SG	35S	Ω	—	NOS	—	Amp	pUC	SmaI ligation
p35SSRDXHSPG	35S	Ω	SRDX	HSP	—	Amp	pUC	SmaI ligation
p35SHSPG	35S	Ω	—	HSP	—	Amp	pUC	SmaI ligation
p35AASRDXG	35S	ADH	SRDX	NOS	—	Amp	pUC	SmaI ligation
pDEST_35S_SRDX_BCKH	35S	Ω	SRDX	NOS	Hm	Km	pBIG	LR reaction
pDEST_35S_SRDX_HSPH	35S	Ω	SRDX	HSP	Hm	Km	pBIG	LR reaction
pDEST_35AA_SRDX_BCKH	35S	ADH	SRDX	NOS	Hm	Km	pBIG	LR reaction
pDEST_35S_3fstop_BCKH	35S	Ω	stop	NOS	Hm	Km	pBIG	LR reaction
R4pGWB4_SRDX	—	—	SRDX	NOS	Km	Spe	pPZP	MultiSite LR
R4pGWB4_SRDX _HSP	—	—	SRDX	HSP	Km	Spe	pPZP	MultiSite LR
R4pGWB4_stop _HSP	—	—	stop	HSP	Km	Spe	pPZP	MultiSite LR
R4pGWB5_SRDX	—	—	SRDX	NOS	Hm	Spe	pPZP	MultiSite LR
R4pGWB5_SRDX _HSP	—	—	SRDX	HSP	Hm	Spe	pPZP	MultiSite LR
R4pGWB5_stop _HSP	—	—	stop	HSP	Hm	Spe	pPZP	MultiSite LR
pDONRG_P4P1R	—	—	—	—	—	Gm	pDONR	BP reaction

35S, cauliflower mosaic virus 35S promoter; Ω, the translational enhancer sequence of tobacco mosaic virus; stop, 3-frame stop codons; NOS, a nopaline synthase terminator; HSP, *Arabidopsis* HSP18.2 terminator; ADH, *Arabidopsis* alcohol dehydrogenase 5′-untranslated region; Hm, hygromycin; Km, Kanamycin; Spe, Spectinomycin; Gm, Gentamicin.

NOS, 9 pBCSH-NOS and 12 R4pGWB-NOS plants that displayed the wild-type-like phenotype. We then performed genomic PCR analysis using 35S and SRDX primers to detect transgenes, and PP2AA3F and PP2AA3R primers for internal control (Supplemental Table 1).

RNA analysis

Total RNA was isolated from seedlings of 7-day-old T1 plants grown on a hygromycin-containing medium and was treated with DNase I, as described previously (Shikata et al. 2009). Quantitative real-time reverse transcription-PCR (RT-PCR) was performed as described previously (Mitsuda et al. 2005) with appropriate primers (Supplemental Table 1).

Measurement of luciferase activity

7-day-old T1 plants were used to measure luciferase activity as described previously (Fujimoto et al. 2000). Relative luciferase activity was normalized to protein concentration in the lysate, which was quantified using a protein assay kit (Bio-Rad, USA) following the manufacturer's instructions. Bovine serum albumin was used to prepare a standard curve and absorbance at 595 nm was measured using an Infinite 200 plate reader (TECAN, Switzerland).

Results

The Gateway system occasionally reduced the efficiency of CRES-T

To construct plasmids in a more convenient and high-throughput manner, we developed two kinds of new Gateway vectors, the pDEST_35S_SRDX_BCKH series and the R4pGWB-SRDX series. These vectors adopt a single or a multisite Gateway system that contain an *att*R1-*att*R2 or an *att*R4-*att*R2 Gateway cassette followed by the coding sequence of SRDX to which a TF CDS without stop codon or both promoter and CDS are cloned by LR recombination from entry clone(s) generated from pDONR207 or from pDONR207 and pDONRG-P4P1R, respectively (Figure 1, Table 1). The resultant chimeric repressor would contain an extra 10 or 12 amino acids derived from Gateway linker sequences between the TF and SRDX (Figure 2A). These vectors are intended for the construction of high-throughput CRES-T plasmids driven by 35S or other favorable promoters. We analyzed whether the extra *att*B sequences in Gateway vectors affect the efficiency of CRES-T by employing CUP-SHAPED COTYLEDON1 (CUC1), a NAC TF, as a model. *CUC1* and its paralogous gene, *CUC2*, are involved in the formation of shoot apical meristem and organ separation (Aida et al. 1997, 1999; Takada et al. 2001). *cuc1 cuc2* double mutants exhibit cup-shaped cotyledons in which two cotyledons are fused at the margin without shoot formation (Aida et al. 1997). Previously, we reported that *35S:CUC1SRDX* induced *cuc1 cuc2*-like cup-shaped cotyledon in about 10% of T1 plants using the traditional vector system (Hiratsu et al. 2003). We classified the phenotypes of *35S:CUC1SRDX* into five groups; *cuc1 cuc2*-like, cup-shaped, heart-shaped, *stm*-like and wild-type-like (Supplemental Figure 1). We found that the frequency of *cuc1 cuc2*-like and cup-shaped phenotypes in the transgenic plants expressing the

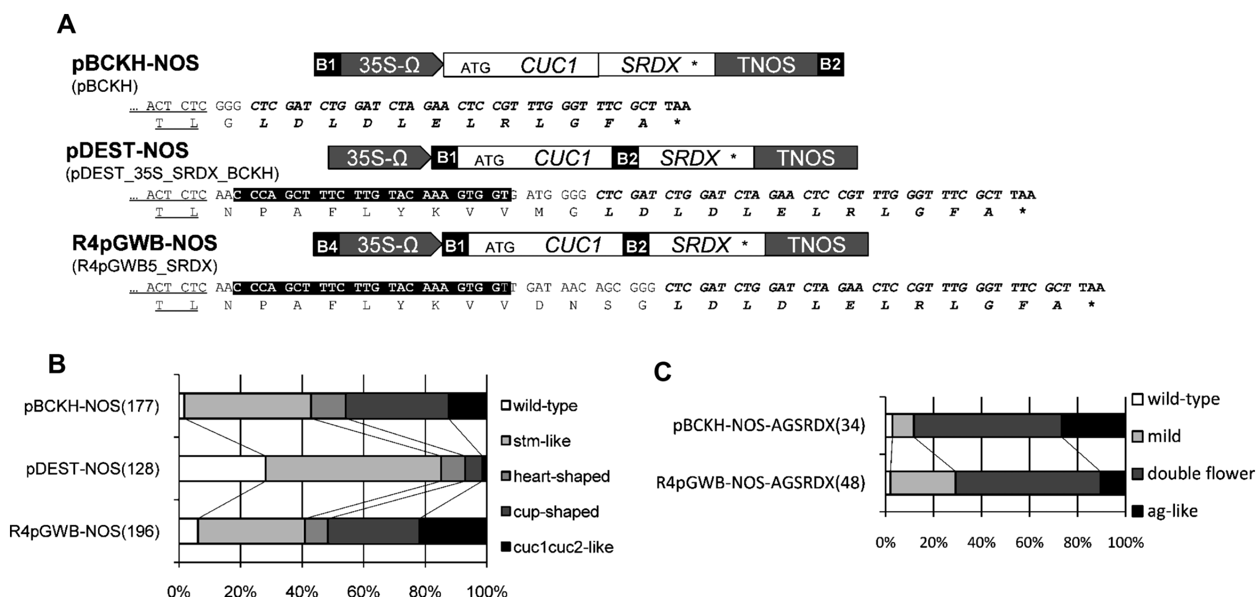

Figure 2. The efficiency of CRES-T in the new Gateway vectors. (A) Schematic representation of *35S:CUC1SRDX* constructs using pBCKH/p35SSRDXG (pBCKH-NOS), pDEST_35S_SRDX_BCKH (pDEST-NOS) and R4pGWB5-SRDX (R4pGWB-NOS), showing the DNA sequences and the amino-acid sequences around SRDX. The filled boxes indicate *att*B1 (B1), *att*B2 (B2) and *att*B4 (B4) sequences. Underlined and italic sequences and asterisk indicate CUC1, SRDX and stop codon, respectively. (B) The frequency of each *35S:CUC1SRDX* phenotype class. (C) The frequency of each *35S:AGSRDX* phenotype class from pBCKH/p35SSRDXG (top) and R4pGWB5-SRDX (bottom). Phenotype class was defined as indicated in Supplemental figures 1 and 2. The number of 10-day-old T1 plants examined is given in parentheses in each case.

35S-driven CRES-T construct for CUC1 from the pDEST_35S_SRDX_BCKH vector, referred to as “pDEST-NOS”, was lower than that from the traditional vector system, referred to as “pBCKH-NOS” (Figure 2B). On the other hand, R4pGWB5_SRDX, referred to as “R4pGWB-NOS”, exhibited a similar frequency for each group of phenotypes as “pBCKH-NOS” (Figure 2B). Another experiment to evaluate the new CRES-T vectors was performed using AGAMOUS (AG), a C-class MADS box transcription factor that is involved in stamen and carpel identity (Yanofsky et al. 1990). T1 plants harboring *35S:AG-SRDX* had *ag*-like flowers, such that sepals and petals were repeatedly produced as a result of substitution of stamen and carpel by petal and another flower, respectively (Mitsuda et al. 2006; Supplemental Figure 2A). For R4pGWB5_SRDX (R4pGWB-NOS-AGSRDX), the frequency of *ag*-like flowers was comparable to that using the traditional vector system pBCKH-NOS-AGSRDX (Figure 2C). These results suggest that, depending on specific vectors, the extra *att*B sequences derived from the Gateway reaction occasionally reduce the efficiency of CRES-T when compared with the traditional vector system.

The attB sequence in the transcribed region reduced the efficiency of CRES-T

To reveal a cause of the negative effect of the extra *att*B sequences described above, we generated a series of modified *35S:CUC1SRDX* constructs. Because the sequence context around the initiation codon is known to affect translational efficiency (Satoh et al. 2004), we first prepared pDEST-ATG, which has an additional start codon between the translational enhancer and the *att*B1 of pDEST-NOS to make the distance between the translational enhancer and the initiation codon equal to that in the traditional vector system (Figure 3A). The resultant pDEST-ATG plants did not have an increased frequency of the cup-shaped phenotype, thus the difference in distance between the translational enhancer and the initiation codon did not appear to be the cause of the negative effect (Figure 3B). Next, we analyzed whether the extra peptide translated from the *att*B2 sequence between TF and SRDX is a cause of the negative effect by generating three different constructs, pDEST-attB2frame2, pDEST-attB2frame3 and pDEST-SRDXstop-att, in which the reading frame of the *att*B2 sequence was different from that of pDEST-NOS in the

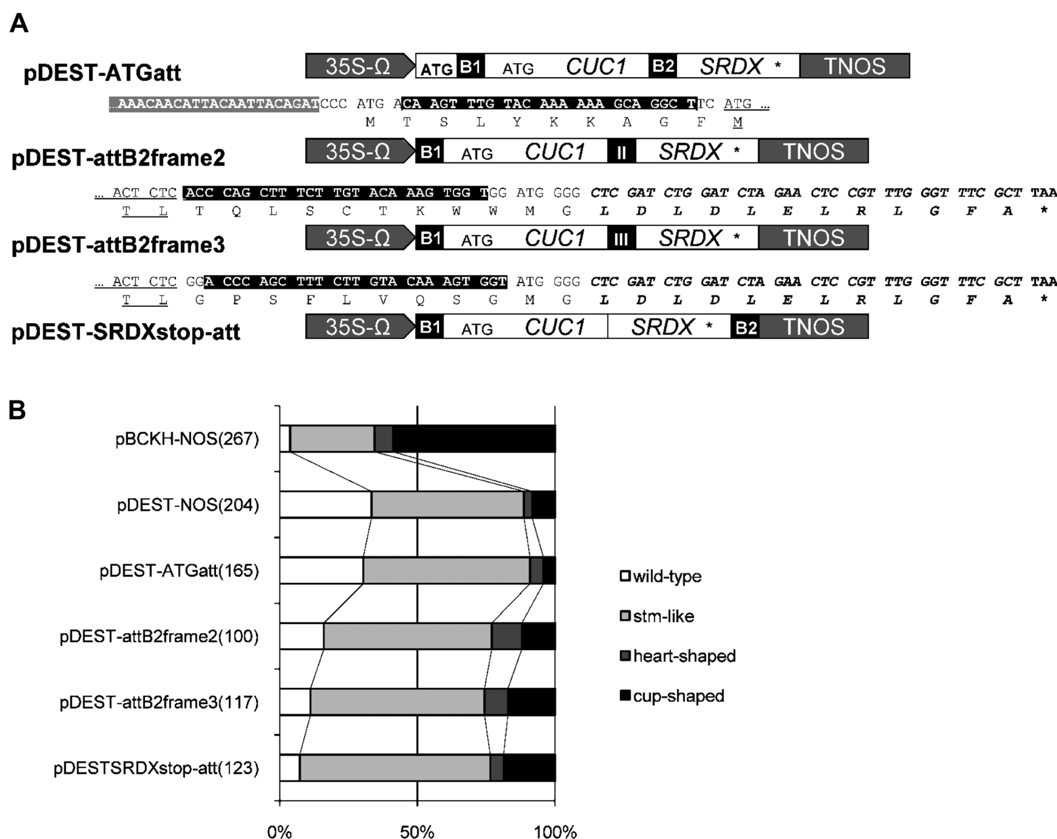

Figure 3. The efficiency of CRES-T in the context of different *att*B sequences. (A) Schematic representation of *35S:CUC1SRDX* constructs showing the DNA sequences and the amino-acid sequences around the ATG codon (top) and the SRDX (center, bottom). The filled boxes indicate *att*B1 (B1) and *att*B2 with different reading frames (B2, II and III). The underlined and italic sequences and asterisk indicate CUC1, SRDX and stop codon, respectively. A gray box in the DNA sequence indicates omega sequence. (B) The frequency of each phenotype class by *35S:CUC1SRDX*. Phenotype class was defined as indicated in Supplemental figure 1, and “cup-shaped” includes *cuc1 cuc2*-like phenotype in this case. The number of 10-day-old T1 plants examined is given in parentheses in each case.

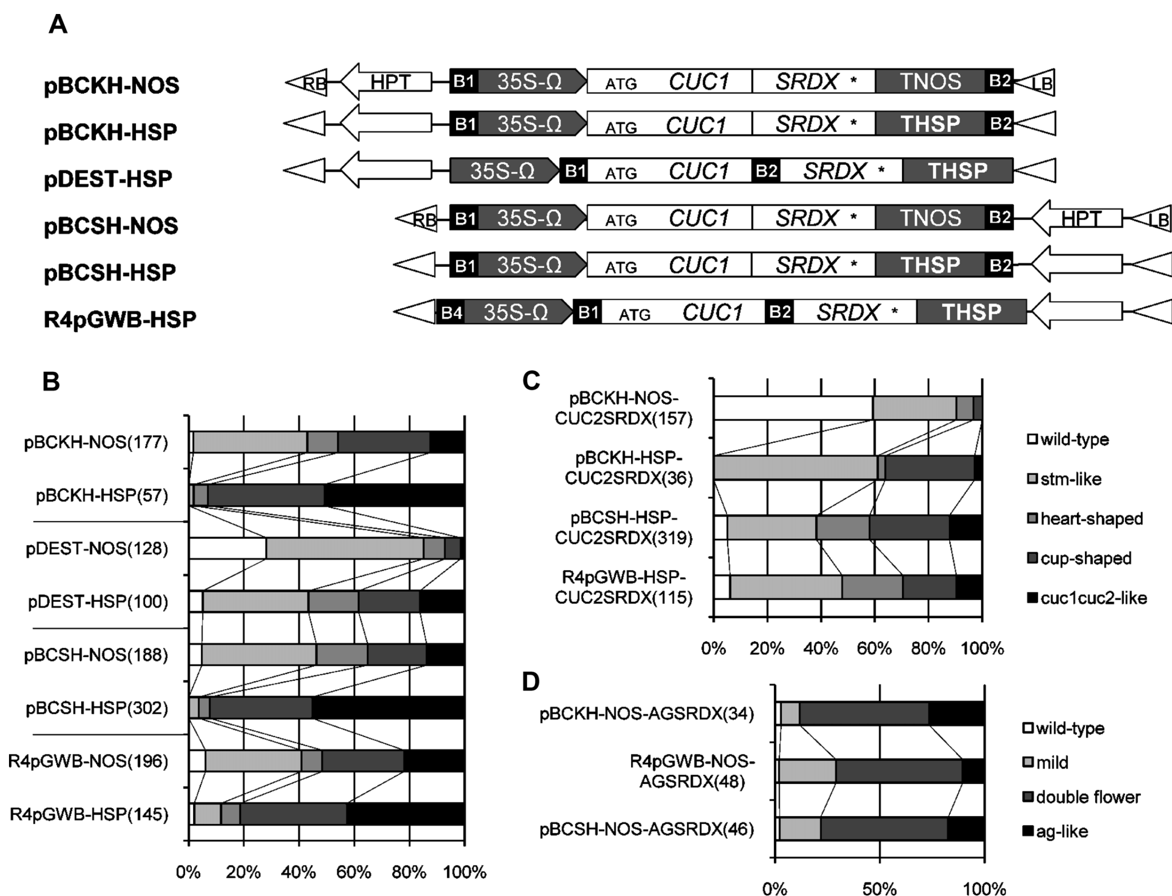

Figure 4. The efficiency of CRES-T with the HSP terminator. (A) Schematic representation of *35S:CUC1SRDX* constructs using pBCKH/p35SSRDXG (pBCKH-NOS), pBCKH/p35SSRDXHSPG (pBCKH-HSP), pDEST_35S_SRDX_HSPH (pDEST-HSP), pBCSH/p35SSRDXG (pBCSH-NOS), pBCSH/p35SSRDXHSPG (pBCSH-HSP), and R4pGWB5_SRDX_HSP (R4pGWB-HSP). (B) The frequency of each *35S:CUC1SRDX* phenotype class. (C) The frequency of each *35S:CUC2SRDX* phenotype class constructed by insertion of *CUC2* instead of *CUC1* in (A). (D) The frequency of each *35S:AGSRDX* phenotype class constructed by insertion of *AG* instead of *CUC1* in (A). Phenotype class was defined as indicated in Supplemental figures 1 and 2. The number of T1 plants examined is given in parentheses in each case. The pBCKH-NOS, pDEST-NOS, R4pGWB-NOS, pBCKH-NOS-AGSRDX and R4pGWB-NOS-AGSRDX data are also shown in Figure 2.

first two constructs and the *att*B2 sequence is not translated in the third construct (Figure 3A). We found that none of these constructs significantly improved the efficiency of CRES-T (Figure 3B). We further found that a construct without *att*B sequences also did not improve the efficiency of CRES-T (Supplemental Figure 3), suggesting that the *att*B sequence within the transcribed region reduced the efficiency of CRES-T even if the sequence is not translated.

An HSP terminator dramatically improved the efficiency of CRES-T

The terminator of the *Arabidopsis* gene, *HSP18.2*, can increase the amount of mRNA and protein produced by a transgene (Nagaya et al. 2010); therefore, we employed the HSP terminator instead of the NOS terminator. To examine the effect of the HSP terminator on CRES-T, *CUC1*, *AG* and *CUC2* were used (Figure 4A). We found that the frequency of the severe CUC1SRDX phenotype increased in pBCKH-HSP, pDEST-HSP and R4pGWB-HSP plants compared with that in pBCKH-NOS, pDEST-NOS and R4pGWB-NOS plants, respectively (Figure 4B). The efficiency in pDEST-HSP plants was comparable to that in pBCKH-NOS plants, indicating that the HSP terminator compensated for the negative effect of the *att*B sequences. The HSP terminator was also able to increase the cup-shaped phenotype about 3-fold, even in CUC2SRDX, which the traditional vector system with the NOS terminator had induced at a very low efficiency (Figure 4C).

We also employed a recently-developed translational enhancer, a 5′-untranslated region of the *Arabidopsis* alcohol dehydrogenase gene (*AtADH*5′UTR), which can induce a 90-fold higher GUS activity compared with activity without a translational enhancer (Sugio et al. 2008). We found, however, that the efficiency of CRES-T was comparable to using the Ω sequence of tobacco mosaic virus (Supplemental Figure 4).

Vector backbone influences the negative effect of attB sequences

The R4pGWB series, which has a different vector

backbone from the pBCKH and pDEST series, always shows a higher efficiency when compared with the pDEST series; therefore, we generated a pBCSH series to examine the effect of vector backbone using a modified pGWB501 vector (Nakagawa et al. 2007). The pBCSH series has almost the same backbone as the R4pGWB series, but it has the *att*R1-*att*R2 single Gateway cassette in the T-DNA region, to which the entire transgene cassette consisting of promoter, gene and terminator is transferred, as in the traditional vector system (Figure 1, Table 1). We found that no significant improvement of CRES-T efficiency was observed in the pBCSH series when compared with the pBCKH and R4pGWB series (Figure 4B, C, D). Furthermore, it should be noted that the transgene was detected in all tested wild-type-like pBCKH-NOS, pDEST-NOS, pBCSH-NOS and R4pGWB-NOS plants, indicating that they have not lost their transgenes. These data suggest that the differences of vector backbones described in this study are not involved in the efficiency of CRES-T itself and support the notion that the negative effect on CRES-T efficiency of the *att*B sequences in the transcribed region is not observed in all vector systems.

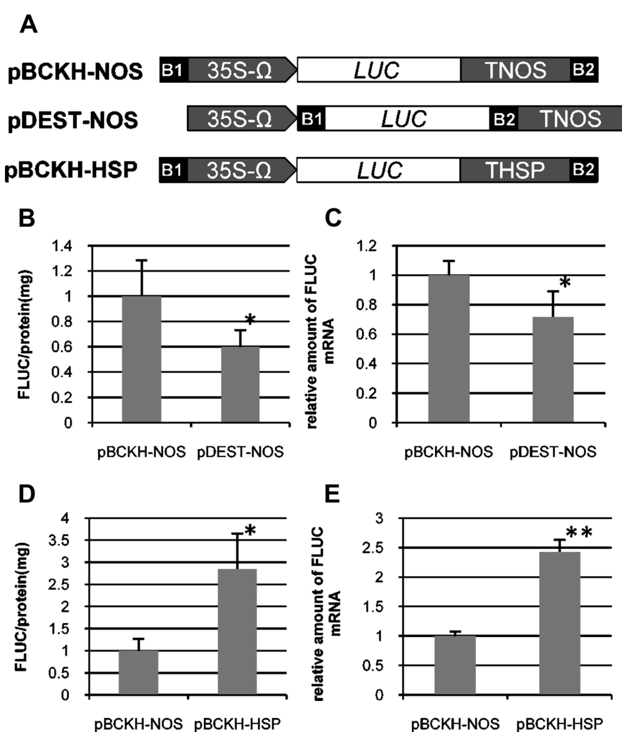

Figure 5. Gene expression and activity of luciferase in the novel Gateway vector. (A) Schematic representation of *35S:LUC* constructs using pBCKH/p35SG (top), pDEST_35S_stop_BCKH (center) and pBCKH/p35SHSPG (bottom). The filled boxes indicate *att*B1 (B1) and *att*B2 (B2). (B, D) Relative firefly luciferase (FLUC) activities are expressed per milligram of protein. (C, E) RT-PCR quantification of *FLUC* mRNA relative to that of *UBIQUITIN 1*. Average of LUC activity and mRNA level among 19-50 of T1 plants is shown. Error bars represent standard deviation (B, C, n=4; D, E, n=3). Significant differences: * $P<0.05$, ** $P<0.01$.

The efficiency of transcription is affected negatively by the attB sequences and positively by the HSP terminator

To analyze how *att*B sequences and the HSP terminator affect the efficiency of CRES-T, we prepared constructs in which the firefly luciferase (LUC) gene was introduced into pDEST_35S_stop_BCKH (pDEST-NOS-LUC), pBCKH/p35SHSPG (pBCKH-HSP-LUC) and into the traditional vector (pBCKH-NOS-LUC). We then analyzed LUC activity in transgenic plants. We found that the LUC activity was lower in pDEST-NOS-LUC plants, while it was higher in pBCKH-HSP-LUC plants compared with that of plants expressing the LUC reporter gene by the traditional pBCKH-NOS-LUC vector system, suggesting that both the negative and positive effects of the *att*B sequences and the HSP terminator are not limited to CRES-T (Figure 5). The amount of transcript in pDEST-NOS-LUC plants was reduced compared with that in pBCKH-NOS-LUC plants, while that in pBCKH-HSP-LUC plants was higher (Figure 5). These results suggest that the efficiency of transcription is affected negatively by *att*B sequences and positively by the HSP terminator.

Discussion

The negative effect of extra attB sequences in the transcribed region

We have described here the development of new CRES-T vectors that incorporate the single or multisite Gateway system to improve CRES-T efficiency and cloning convenience. We found, however, that *att*B sequences in the transcribed region of the pDEST series reduced the efficiency of CRES-T, even if the sequences were not translated, when compared with the traditional pBCKH series, which does not have *att*B sequence in the transcribed region. We further found that this negative effect was not limited to the application of CRES-T and was probably due to the reduced efficiency of transcription and/or the stability of transcript (Figure 5). On the other hand, such a negative effect was not observed for the multisite Gateway vector R4pGWB series, which has *att*B sequences in the transcribed region, suggesting that the differences in vector systems and/or *att*B sequences between the pDEST and R4pGWB series may be involved in the negative effect of the *att*B sequences. Although studies in several eukaryotes have reported that the additional sequences from the Gateway system did not interfere with the biological activity of a cloned fragment, including subcellular localization or tissue specificity, there has been no description about any effect on gene expression (Curtis and Grossniklaus 2003; Nakagawa et al. 2007a, b; Roure et al. 2007; Yahata et al. 2005). Our results demonstrated that the Gateway linker sequences did not

interfere with expected biological function, but could reduce gene expression depending on vector systems.

The differential effects of vector backbone

As shown in this study, the difference of vector backbone does not affect the efficiency of CRES-T but appears to affect the negative effect of *att*B sequences in the transcribed region. The two major differences in the vector backbones of the pBCKH/pDEST and R4pGWB series are that the transgene cassette is located at the left-border (LB) side or the right-border (RB) side of the T-DNA region, respectively, and that the replication origin in *Agrobacterium* is RK2 (Becker 1990) or pVS1 (Hajdukiewicz et al. 1994), respectively. In many plants, including tobacco and cotton, T-DNA inserted into the genome was often truncated to the internal border sequences on the LB side (Rossi et al. 1996; Zhang et al. 2008) and, therefore, it is considered preferable that the transgene cassette is placed on the RB side. However, large-scale examination revealed that such deletion was rarely observed in *Arabidopsis* (Brunaud et al. 2002). In our experiment, correct transgene integration was also detected, even in the wild-type-like plants, suggesting that the difference of transgene cassette location in the T-DNA region did not affect CRES-T efficiency. On the other hand, the replication origin might affect the efficiency of CRES-T if the copy number of the plasmid in *Agrobacterium* could be correlated with the number of integration events in the plant genome. However, these differences cannot explain the difference between the negative effects of the *att*B sequences in the pDEST and R4pGWB series. Further investigation will be required to reveal the effect of vector backbone.

The effect of a translational enhancer

The 5′-untranslated region (5′-UTR) is important for the translation of the downstream gene; GUS activity in tobacco was previously shown to increase 16- to 18-fold by the insertion of an Ω sequence from tobacco mosaic virus (Gallie et al. 1989). The tobacco *ADH* 5′-UTR is as effective as the Ω sequence as a translational enhancer in tobacco and *Arabidopsis*, and is also effective in chrysanthemum and torenia, in which the Ω sequence is not effective (Aida et al. 2008; Satoh et al. 2004). Similarly, the 5′-UTR of the *Arabidopsis ADH* homolog (*AtADH* 5′-UTR) was also effective in dicots (Sugio et al. 2008). Our results indicated that although the effect of the *AtADH* 5′-UTR on the efficiency of CRES-T did not exceed that of the Ω sequence in *Arabidopsis*, it may improve the efficiency of CRES-T in other dicots where the Ω sequence is less effective.

The effect of the HSP terminator

The transcriptional terminator can influence the level of gene expression (Ingelbrecht et al. 1989). Mitsuhara et al. (1996) reported that the 35S terminator has a higher efficiency than the NOS terminator, which is predominantly used for plant-transformation vectors. The HSP terminator is also more effective than the 35S terminator at increasing transgene protein accumulation in combination with various promoters (Nagaya et al. 2010). Here we demonstrated that the HSP terminator dramatically improved the efficiency of CRES-T to induce loss-of-function phenotypes in all vector systems examined, probably due to increased protein accumulation of the chimeric repressor (Figure 5). These data are consistent with the lower efficiency of CRES-T observed in the pDEST series, suggesting that the efficiency of CRES-T is sensitive to the level of chimeric repressor protein. The HSP terminator is also expected to be effective for other applications in which the level of protein is important.

How to use the new CRES-T vectors

We have described the development of a new series of CRES-T vectors, which can be applied to a variety of experiments and purposes. To obtain the highest efficiency of CRES-T, we recommend the traditional vector system with the HSP terminator, despite the cloning difficulties. However, if the chimeric repressor induces a severe growth defect, including lethality, it might be better to use "weaker" vectors, such as the pDEST series with the NOS terminator that should induce a milder phenotype. For high-throughput plasmid construction of 35S-driven CRES-T, pDEST_35S_SRDX_HSPs is useful and can induce CRES-T phenotypes to a similar efficiency as the traditional vector system with the NOS terminator. Multisite Gateway vectors, R4pGWB_SRDX_HSPs, are convenient for plasmid construction with various combinations of promoters and TFs. Because our new Gateway vectors are compatible with many other Gateway vectors, the new vector systems developed in this study should accelerate more flexible applications of CRES-T to a wide range of plant species by using preexisting common entry clones.

Acknowledgements

The authors thank Y. Kimura, N. Ujiie, M. Watanabe, A. Kuwazawa and Y. Takiguchi for their skilled technical assistance. This work was supported by the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry from the Bio-oriented Technology Research Advancement Institution.

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