Targeted Insertion of the *mPing* Transposable Element

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Class II DNA Transposable Elements (TEs) are moved from one location to another in the genome by the action of transposase proteins that bind to repeat sequences at the ends of the elements. Although the location TE insertion is mostly random, the addition of DNA binding domains to the transposase proteins has allowed for targeted insertion of some elements. In this study, the Gal4 binding domain was added to the transposase proteins, ORF1 and TPase, which mobilize the *mPing* element from rice. The Gal4:TPase construct was capable of increasing the number of *mPing* insertions into the Gal2 and Gal4 promoter sequences in yeast. While this confirms that *mPing* insertion preference can be manipulated, the target specificity is relatively low. Thus, the CRISPR/Cas9 system was tested for its ability to generate targeted insertion of *mPing*. A dCas9:TPase fusion protein had a low transposition rate suggesting that the addition of this large protein disrupts TPase function. Unfortunately, the use of a MS2 binding domain to localize the TPase to the MS2 hairpin containing gRNA failed to produce targeted insertion. Thus, our results suggest that the addition of small DNA binding domain to the N -terminal of TPase is the best strategy for targeted insertion of *mPing*.

Introduction

TEs were discovered in 1948 by Barbara McClintock while studying maize genetics (McClintock 1948; McClintock 1950). Over the ensuing decades, researchers have found that they are present in almost all organisms (Craig 2002). TEs have been divided into superfamilies based on the sequence homology of the encoded transposase proteins. The transposase proteins contain a DDE catalytic domain that is responsible for cutting the element out of the genome and inserting it elsewhere (Yuan and Wessler 2011). The specificity of this reaction is controlled by interaction with terminal inverted repeat sequences that define the ends of the elements (Craig 2002). Another novel characteristic of interest for each element is the insertion site preference. Although their insertion pattern is somewhat random, different elements use different strategies, either inserting near genes, in gene poor regions, or in site specific locations (Craig 1997; Vigdal, Kaufman et al. 2002). In addition to regional preferences for insertion, some elements will only insert into specific local sequences [i.e. TA for *Mariner* elements (Plasterk, Izsvak et al. 1999)].

TEs can disrupt genes and are useful tools for mutagenesis. Traditionally, they have been used as a random gene discovery tools for forward genetic screens. For example, the *Tn5* transposon has been implemented as a mutagenesis tool in bacteria and the *Mutator* element has been used in maize (Goryshin, Jendrisak et al. 2000; Tan, Chen et al. 2011). When using a TE as a mutagenesis tool, the element is allowed to transpose with the intent of disrupting gene sequences or expression. Based on the resulting phenotypes, the disrupted gene's function can be extrapolated. However, the main drawback to using TEs for reverse genetics is that not all mutations produce detectable phenotypic changes. Thus, strategies to use transposable elements for forward genetics are being developed. These strategies are based on fusing a DNA binding domain to a transposase protein in order to induce targeted TE insertion (Colloms and Renault 2013). Studies on the *piggyback* and *Sleeping Beauty* TEs have shown that in some cases, the addition of a DNA binding domain can facilitate insertion of the elements into target regions (Dematteі, Thomas et al. 2010). The first hurdle for this strategy is the fact that addition of the DNA binding domain can inactivate the transposase protein. For example, the transposition rate of the *Tol2* element is significantly impacted by the addition of a binding domain to its transposase (Ammar, Gogol-Doring et al. 2012). The second challenge is to actually change the insertion preference. This is presumably accomplished by the DNA binding domain bringing the targeted DNA sequence into close proximity to the active site of the transposition complex. In one study, researchers fused an adeno-associated virus Rep protein DNA binding domain to the N-terminal of a hyperactive *Sleeping Beauty* transposase and saw no increase in insertions of the element into the target site (Ammar, Gogol-Doring et al. 2012). However, in a study by Maragathavally et al. (2006), a Gal4 DNA binding domain fused with the *Mos1* and *piggyback* transposase proteins produced a 20 fold increase in targeted insertion into a plasmid encoded Gal promoter (Maragathavally, Kaminski et al. 2006). Similarly, researchers found that although the addition of the Gal4 binding domain reduced *Tol2* transposition to 10% of the wild-type, it allowed for a 4 fold increase in insertions near the target site (Ammar, Gogol-Doring et al. 2012). Similarly, the fusion of the Gal4 binding domain to the *Sleeping Beauty* transposase protein resulted in 80% transposition rate and a 15 fold increase in insertions near the plasmid-based target site (Ammar, Gogol-Doring et al. 2012). Other elements that have been shown to produce targeted insertion include the *IS30* element with the addition of cI and Gli1 domains and the *Mos1* element through the addition of the Gal4 binding domain (Szabo, Muller et al. 2003; Maragathavally, Kaminski et al. 2006; Dematteі, Thomas et al. 2010).

The element used in this study, *mPing*, is a non-autonomous miniature inverted repeat TE discovered in rice (Jiang, Bao et al. 2003; Kikuchi, Terauchi et al. 2003; Nakazaki, Okumoto et al. 2003). The 430 bp element is a member of the *PIF/Harbinger* superfamily (Zhang, Jiang et al. 2004; Grzebelus, Lasota et al. 2007) and is highly active in some rice lines (Naito, Cho et al. 2006; Naito, Zhang et al. 2009; Naito, Mondee et al. 2014). The element requires both the ORF1 and TPase proteins from either the autonomous *Ping* or *Pong* elements for mobilization (Yang, Zhang et al. 2007; Hancock, Zhang et al. 2010). These two proteins have separate functions with the ORF1 protein functioning as a DNA binding domain while TPase contains the DDE catalytic domain that is responsible for DNA cleavage during transposition (Sinzelle, Kapitonov et al. 2008; Hancock, Zhang et al. 2010). *mPing* and its relatives have a strict target site requirement of either TTA or TAA (Zhang, Jiang et al. 2004). This element also preferentially inserts into regions 2,500 bp upstream or downstream of genes in plants (Hancock, Zhang et al. 2011). Our goal was to determine if addition of a DNA binding domain to either the ORF1 or TPase proteins would result in targeted insertion of the *mPing* element.

In addition to testing the well characterized Gal4 binding domain, we also tested the CRISPR/Cas9 system which relies on a guide RNA (gRNA) sequence to determine sequence specificity (Carroll 2012) for its ability to direct *mPing* insertion. This system is very versatile because the binding site is easily changed by altering the short gRNA sequence. Importantly, a nuclease-null version of the Cas9 protein (dCas9) has been used to target other fusion proteins to specific sequences *in vivo* (Gilbert, Larson et al. 2013; Maeder, Linder et al. 2013; Mali, Aach et al. 2013; Perez-Pinera, Kocak et al. 2013). We hypothesized that fusing the dCas9 protein to the *mPing* transposition complex might be able to recruit gRNA-specified DNA sequences to the transposition complex, promoting insertion into the targeted region. We tested this by directly fusing dCas9 to the TPase protein and using an MS2 domain that bound to an MS2 hairpin loop added to the gRNA (Konermann, Brigham et al. 2015).

Methods

Yeast Genotypes

Saccharomyces cerevisiae strains used in this study were developed previously (Gilbert, Bridges et al. 2015). The genotype of the CB101 strain is: *MATa ade2Δ::hphMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lys2Δ::ADE2*.*

Constructs

The *Pong TPase LA* and *ORF1SC1 (ORF1 Shuffle NLS)* genes were described previously (Hancock, Zhang et al. 2010; Payero, Outten et al. 2016) and were Gateway cloned into the pAG413 Gal, pAG415 Gal [gifts from Susan Lindquist (Addgene plasmids # 14141 and # 14145)], and pDEST32 (Invitrogen) vectors. To create the control plasmids, the Gal4 DNA binding domain was cleaved out of the pDEST32 plasmids using *Hind*III and *Not*I and replaced with the following linker sequence

5' –GAATCAAGGCTAGAAAGACTGGAACAGCTATTTCTACTGA TTTTTCCTCGAGAAGACCTTGACATGATTTTGAAAATGGATTC TTTACAGGATATAAAAGCATTGTTAACAGGATTATTT – 3'.

The hCas9 and hCas9 D10A plasmids [Addgene plasmids #41815 and #41816 (Mali, Yang et al. 2013)] were PCR amplified using the following primers, Cas9 KpnI IF For and Cas9 XbaI IF Rev (Table 1). The amplified producs was then cut using *Kpn*I-HF and *Xba*I enzymes and In-Fusion (Takara) cloned into digested pDONR *Pong* TPase LA plasmid. To make the dCas9 version, QuikChange site-directed mutagenesis was performed on the hCas9 D10A plasmid with the following primers: QC Cas9 H840A For and QC Cas9 H840A Rev (Table 1). Gateway cloning was used to transfer Cas9 constructs into pAG415 Gal-ccdb [a gift from Susan Lindquist (Addgene plasmid # 14145)].

Bridge fusion PCR (Mehta and Singh 1999) was used to join an MS2-NLS domain, PCR amplified from a synthesized gBlock (IDT, sequence available upon request) with the MS2-NLS attb For and MS2-NLS TPase Rev primers, with a *Pong* TPase LA T2A ORF1SC1 ONE construct amplified with the TPase MS2 For and Pong ORF1 Rev attb primers (Table 1). Purified PCR product was Gateway cloned into the pDONR vector before transferring to the pAG423 Gal-ccdb vector [a gift from Susan Lindquist (Addgene plasmid # 14149)].

The CANI gRNA2.0 gene was synthesized by IDT (sequence available upon request) and amplified using SNR52 Promoter C For primer and sgRNA Flank R primer (Table 1). The amplicon was ligated into the *Cla*I and *Bsr*GI sites of p426-SNR52p-gRNA.CAN1.Y-SUP4t [Addgene plasmid #43803 (DiCarlo, Norville et al. 2013)].

Transposition Assay

The transposition rate was determined using the previously developed yeast transposition assay (Gilbert, Bridges et al. 2015; Payero, Outten et al. 2016). Briefly, constructs were transformed into yeast using the LiAc/single stranded carrier DNA/PEG method (Schiestl and Gietz 1989). Cultures were grown for one day at 30ºC in liquid media and then 100 µl was plated onto selective galactose media (CSM-His-Leu-Ura-Ade) and incubated for 10 days at 30ºC. A 10^{-4} dilution of the culture was plated onto YPD media to determine the titer. The transposition rate was calculated as the number of *ADE2* revertant cells per million cells plated. For each experiment, the average and standard error of 6 replicates were calculated. Selected colonies were treated with zymolyase (Zymo Research) and screened for *mPing* excision with ADE2-CF and ADE2-CR primers (Table 1).

Detecting *mPing* Insertions

CB101 *ADE2* revertant colonies were treated with zymolyase (Zymo Research) and screened with the Gal2P For, pAG Gal For, or Gal10P For primers in conjunction with the *mPing* 41 Rev and *mPing* 403 For primers (Table 1). *ADE2* revertant yeast colonies were cultured several times in CSM-Ade media and then individual colonies were screened for loss of the ORF1 and TPase plasmids on CSM-His and CSM-Leu. They were then treated with zymolyase (Zymo Research) and screened for *mPing* by PCR with *mPing* TTA For and Rev primers (Table 1). The *mPing* positive colonies were screened for genomic Gal2 promoter insertion using Gal2P Flank For and *mPing* 41 Rev and *mPing* 403 For primers. The PCR products from positive clones were sequenced after cloning into pJet1.2 (Thermo Scientific).

Table 1. Primers used in this study.

Name	Sequence (5'- 3')
ADE2-CF	GGGTTTTCCATTCGTCTTAAGTCGA GGAC
ADE2-CR	CATTTCCACACCAAATATACCACAA CCGGGA
mPing TTA For Short	CATGATTGTGAGGTCTGTTAGGCCA GTCACAATGGCTAGTGTC
mPing TTA Rev Short	GTAAGAAAACACTAAACCGTTAAG GCCAGTCACAATGGGGGTTTC
pAG Galp Flank For	GCCTACATACCTCGCTCTGC
pAG Galp Flank Rev	ATCAAAAATCATCGCTTCGC
Gal2p Flank For	CGACAAGATGCCAAGCTGTA
Gal2p Flank Rev	CTCACCGGCTTTCAATTCAT
Gal10p Flank For	ACCCAAGTTCCACTCACGAC
mPing_403_For	CGTGCAATGACACTAGCCAT
mPing_41_Rev	TGCATGACACACCAGTGAAA
Cas9 Kpnl IF For	CGTGGTACCATGGACAA-GAAGTACTCCATTGGG
Cas9 *Xba*I IF Rev	CGTTCTAGACACCTTCCTCTTCTTCT TGGG
QC Cas9 H840A For	CTCCGACTACGACGTGGATGCCATC GTGCCCCAGTCTTT
QC Cas9 H840A Rev	AAAGACTGGGGCACGATGGCATCCA CGTCGTAGTCGGAG
MS2-NLS attb For	GGGGACAAGTTTGTACAAAAAAGCA GATGGCTTCAAACTTTACTCAGTTCG TG
MS2-NLS TPase Rev	GCTAAACTCTGCATGGATCCAGCGG CCGCC
TPase MS2 For	GCGGCCGCTGGATCCATGCAGAGTT TAGCCATCTCTCTA
Pong ORF1 Rev attb	GGGGACCACTTTGTACAAGAAAGCT GGGTCTTAGTCAGCAAATAACTTTTC CTCCA
SNR52 Promoter C For	TCACACCCTACAATGTTCTGTTCA
sgRNA Flank R	ACTTCAGGTTGTCTAACTCCTTCC

Results and Discussion

Small domains fused to Transposase maintains transposition activity

Our first goal was to determine how the addition of DNA binding domains affected ORF1 (ORF1SC1) or TPase function. We first made N -terminal Gal4 DNA Binding Domain (DBD) fusion protein constructs and the appropriate control plasmids lacking the Gal4 DBD (pDES32Δ Gal). Figure 1A shows the results of transposition assays using a plasmid version of the *mPing*:*ade2* reporter. The result indicate that the addition of the 148 amino acid Gal4 DBD to *Pong* TPase (pDEST32) did not decrease the *mPing* transposition rate significantly compared to the control (pDEST32Δ Gal). However, when the Gal4 DBD was added to ORF1SC1 version of ORF1 (pDEST32), the *mPing* transposition rate was drastically reduced. This suggests that the addition of the DBD is interfering with the folding or activity of the ORF1 protein. The fact that transposition rates for the controls are different (pDEST32Δ *ORF1SC1* was lower than pDEST32Δ *Pong TPase LA*) is likely a result of the fact that different promoters were used to drive expression of the ORFSC1 and TPase proteins in the different experiments (ADH1 promoter in pDEST32 and Gal2 promoter in pAG413).

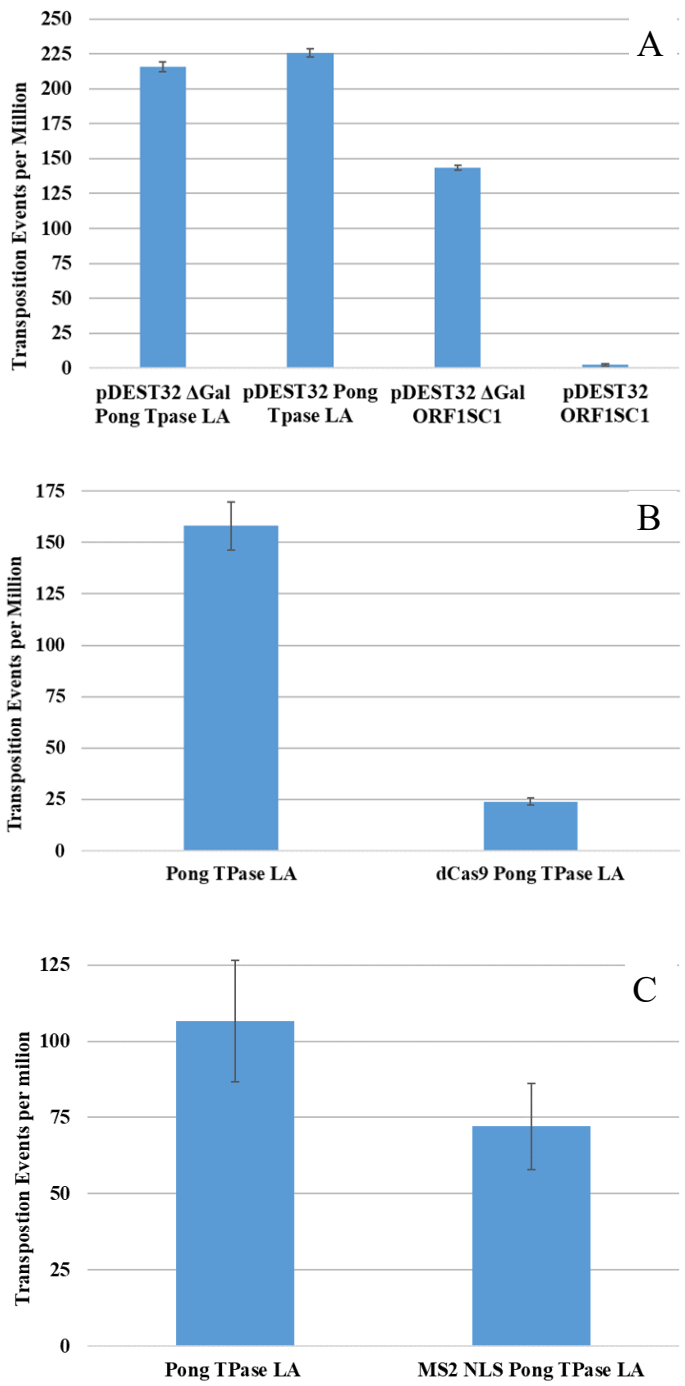

Figure 1. Transposition rates of *Pong* fusion proteins. Histograms illustrating the average transposition rate and standard error of six replicates. Experiments include comparison of (A) control (pDEST32Δ Gal) to Gal4 DBD fusions (pDEST32), (B) control (*Pong* TPase LA) to a dCas9:TPase fusion (dCas9:*Pong* TPase LA), and (C) control (*Pong* TPase LA) to a MS2 NLS:TPase fusion (MS2 NLS *Pong* TPase LA).

Similarly, we tested if direct fusion of the 1381 amino acid dCas9 protein to the N-terminal of TPase would affect transposition rates. Figure 1B shows that the dCas9 TPase fusion protein produced very low transposition compared with the control. This indicates that the addition of larger protein fusions may disrupt the folding of the TPase protein or its ability to form functional transposition complexes with ORF1 and the *mPing* element. In comparison, addition of the 160 amino acid MS NLS domain (Johansson, Liljas et al. 1997) to TPase had no significant effect on transposition rates (Figure 1C).

mPing insertion can be directed to Gal promoter sequences

To determine if addition of the Gal4 DBD altered the insertion site preference, we analyzed 96 *ADE2* revertant colonies from the transposition assays with and without the Gal4 DBD. Each colony results from a single transposition event, thus, each colony represents a single insertion site. We determined the number of *mPing* insertions that occurred in the Gal2 promoter (encoded on both the plasmid and genome) and the Gal10 promoter (genome only) region which contain four and three of the Gal4 DBD recognition sequences [CGG-N_{11}-CCG (Traven, Jelicic et al. 2006)] respectively. PCR using a primer flanking the promoter regions and two primers directed out of each end of *mPing* showed that the control *Pong* TPase produced 5/96 *mPing* insertions in the plasmid copies of the Gal2 promoter and no insertions in the genomic Gal2 or Gal10 promoter regions (Figure 2). In contrast, the addition of the Gal4 DBD to *Pong* TPase increased the plasmid Gal2 promoter insertions to 11/96, the genomic Gal2 promoter insertions to 3/96, and the Gal10 promoter insertions to 1/96 (Figure 2). Thus, from this survey of a small number of possible target sites we observed an approximately 5 fold increase in Gal promoter insertions. In addition, analysis of the *ADE2* revertant colonies resulting from the Gal4 DBD ORF1SC1 protein resulted in 30/96 insertions into the plasmid copy of the Gal2 gene, 1/96 insertions into the genomic Gal2 promoter region, and no insertions into the Gal10 promoter region. Thus, despite that fact that this construct produces low number of transposition events, they appear to have a very high rate of targeted insertion.

Together these results indicate that *mPing* preferentially inserts into plasmid DNA under the conditions used for these experiments. While the modified ORF1SC1 construct produced a much higher rate of targeted insertion of *mPing* into Gal4 recognition sequences, the modified *Pong* TPase LA construct retained a much higher transposition rate and still increased the number of insertions into the Gal4 recognition sequences when compared with the control. Thus, we decided to focus our efforts on the TPase fusion protein because of its higher transposition efficiency.

We next sequenced some of the *mPing* insertions into Gal promoters to determine how close they are to the Gal4 target sequences. Cloning and sequencing six selected insertion events from pDEST32 *Pong* TPase yeast showed that these insertions were all within 177 bp of a Gal4 recognition sequence. The average insertion distance was 63 bp away and one insertion was in a Gal4 recognition site (Figure 3). The fact that the insertions are not in a single location is consistent with the Gal4 DBD recruiting the target site to the transposition complex, but then allowing insertion into a nearby site.

Cas9 DNA binding is not sufficient to direct _mPing_ insertion

Analysis of the *ADE2* revertant colonies resulting from the dCas9-TPase fusion proteins together with a CAN1 specific gRNA (DiCarlo, Norville et al. 2013) showed that there was no increase in canavanine resistant colonies resulting from *mPing* insertion compared to controls (data not shown). This result indicated that targeted insertion of *mPing* into the *CAN1* gene was not occurring. We then tested the MS2-TPase fusion together with a gRNA with two MS2 hairpin-binding sites (gRNA 2.0) (Konermann, Brigham et al. 2015) and dCas9. Once again, this combination of proteins did not increase canavanine resistant colonies above background levels. This is despite the fact that we confirmed that the gRNA2.0 was capable of directing a control Cas9 to the target successfully (Figure 4a) and the MS2-TPase fusion was capable of mobilizing *mPing* (Figure 4b). Together these results suggests that the excessive number of components in the CRISPR/Cas9 complex decreases the overall probability of successfully capturing the single target genomic sequence. Our results showing that Cas9 is not a viable option for targeted insertion is strikingly similar to what was recently reported for targeted insertion of the *L1* retrotransposon (Ade, Derbes et al. 2018).

Conclusions

These results show the feasibility of targeted insertion of the *mPing* element. This is made possible by the addition of a relatively small DNA binding protein to the N-terminal of the *Pong* TPase protein. While we

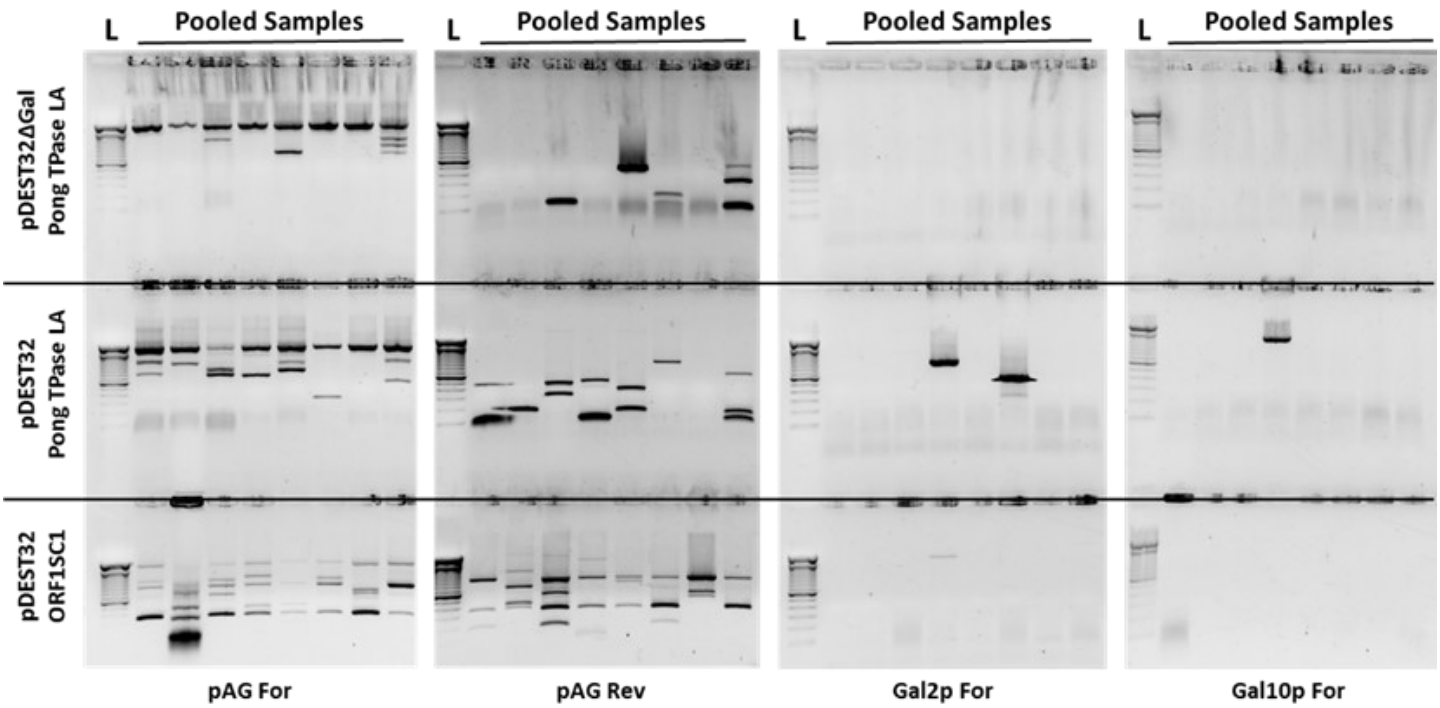

Figure 2. Electrophoresis gels of PCR from 96 (8 pools of 12) *ADE2* revertant colonies obtained from control (pDEST32Δ Gal) and Gal4 DBD fusions (pDEST32). Amplifications were performed with primers facing out from the *mPing* element and a primer adjacent to Gal promoter regions: (A) pAG For, (B) pAG Rev, (C) Gal 2P For, and (D) Gal 10P For. Each band represents a unique insertion into the target region. L= DNA Ladder.

Figure 3 - Sequence of selected *mPing* insertion sites. DNA maps indicating the location of six *mPing* insertion events induced by pDEST32 *Pong* TPase into the Gal10 and Gal2 promoters. The *mPing* insertions are shown in red and the Gal4 binding sites are indicated in dark blue. The Gal2 and Gal10 genes are shown in green.

were only able to successfully use the Gal4 DBD, it is also feasible that other small DNA binding proteins could produce targeted transposition. The finding that localizing the TPase protein to the dCas9 or gRNA components did not induce targeted insertion is disappointing. However, there may be other versatile DNA binding domains that will be more applicable in the future.

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