A Rice Tc1/Mariner-Like Element Transposes in Yeast

Guojun Yang,a Clifford F. Weil,b and Susan R. Wesslera,1
a Department of Plant Biology, University of Georgia, Athens, Georgia 30602
b Department of Agronomy, Purdue University, West Lafayette, Indiana 47907

The Tc1/mariner transposable element superfamily is widely distributed in animal and plant genomes. However, no active plant element has been previously identified. Nearly identical copies of a rice (Oryza sativa) Tc1/mariner element called Osmar5 in the genome suggested potential activity. Previous studies revealed that Osmar5 encoded a protein that bound specifically to its own ends. In this report, we show that Osmar5 is an active transposable element by demonstrating that expression of its coding sequence in yeast promotes the excision of a nonautonomous Osmar5 element located in a reporter construct. Element excision produces transposon footprints, whereas element reinsertion occurs at TA dinucleotides that were either tightly linked or unlinked to the excision site. Several site-directed mutations in the transposase abolished activity, whereas mutations in the transposase binding site prevented transposition of the nonautonomous element from the reporter construct. This report of an active plant Tc1/mariner in yeast will provide a foundation for future comparative analyses of animal and plant elements in addition to making a new wide host range transposable element available for plant gene tagging.

INTRODUCTION

The Tc1/mariner superfamily contains transposable elements from diverse taxa, including fungi, flies, nematodes, fishes, and mammals (Plasterk and van Luenen, 2002). These elements share three characteristics: a target site duplication (TSD) of the dinucleotide TA, a transposase with a DDE/D catalytic motif (the active site where divalent cations bind), and short terminal inverted repeats (TIRs) of related sequences. Variation in the DDE/D signature led to the placement of elements into six monophyletic groups: DD34E, DD34D, DD37D, DD37E, DD31-33D, and DD35E (Doak et al., 1994; Capy et al., 1998; Robertson et al., 1998; Plasterk et al., 1999; Shao and Tu, 2001). Although two plant Tc1/mariner elements were identified from soybean (Glycine max) (Soymar1) and rice (Oryza sativa) (later named Osmar1), it was not until the design of plant-specific PCR primers that related elements were found to be widespread in plant genomes and to compose a seventh monophyletic group (DD39D) (Jarvik and Lark, 1998; Tarchini et al., 2000; Feschotte and Wessler, 2002; Feschotte et al., 2003; Jacobs et al., 2004).

Mutational analysis of various Tc1/mariner transposases confirmed the critical role of the DDE/D motif and has provided evidence that an intact DNA binding domain (DBD) is also required for activity. Mutations in the DD34E motifs of Tc1 and Tc3 abolished transposase activity in vitro (van Luenen et al., 1994; Vos and Plasterk, 1994). Furthermore, the crystal structure of the Mos1 catalytic domain suggests an interaction between its DD34D motif and divalent cations (Mg2+ or Mn2+) (Richardson et al., 2006). The Tc1/mariner transposases also contain helix-turn-helix (HTH) motifs that are required for its binding to TIRs, the first step of transposition (Lampe et al., 1996; van Pouderoyen et al., 1997; Wang et al., 1999; Auge-Gouillou et al., 2001; Zhang et al., 2001; Izsak et al., 2002; Watkins et al., 2004).

To date, activity has been demonstrated for seven naturally occurring Tc1/mariner elements: Tc1 and Tc3 from Caenorhabditis elegans (Emmons et al., 1983; Collins et al., 1989); Mos1, Mos5, and Himar1 from flies (Bryan et al., 1990; Franz and Savakis, 1991; Robertson and Lampe, 1995), and Impala and F0t1 from the fungus Fusarium oxysporum (Daboussi et al., 1992; Langin et al., 1995). Although superfamily members are widespread in vertebrate genomes, no active elements have been isolated to date. Instead, two active transposases were phylogenetically reconstructed from nonfunctional vertebrate elements: Sleeping Beauty from eight fish species and Frog Prince from Rana p. (frog) (Ivics et al., 1997; Miskey et al., 2003). Both reconstructed elements transpose in a variety of vertebrates, including primates, and, as such, have been developed into valuable tools for human gene discovery (Yant et al., 2000; Davidson et al., 2003; Miskey et al., 2003; Ivics and Izsak, 2004; Dupuy et al., 2005; Starr and Largaespada, 2005).

The availability of sequence from most of the genomes of two subspecies of rice, indica and japonica, facilitated a computer-assisted survey that identified 34 Tc1/mariner elements belonging to 25 subfamilies (Feschotte et al., 2003). Seven of the 34 elements (Osmar1A, Osmar5A, Osmar5Bi, Osmar9A, Osmar15Bi, Osmar17A, and Osmar19) encode potentially functional transposases with no interrupting stop codons. Among these, Osmar5 was chosen as the best candidate for an active element because virtually identical copies were present in japonica (one copy) and indica (two copies; one full length and one truncated) at different genomic loci. In a previous study, binding of the Osmar5 transposase to its TIRs was demonstrated in a yeast one-hybrid assay
in which the protein bound specifically to copies of the TIR on a reporter construct. Specific binding was also demonstrated in vitro using a fusion protein synthesized in *Escherichia coli* and DNA fragments from the ends of *Osmar5*. The first 206 residues of *Osmar5* transposase, which contain two HTH motifs (Figure 1), were shown to bind specifically to two sequence motifs that comprised a 17-bp region of the TIR (called Box1 and Box2; Figure 1). An additional copy of the 17-bp binding site adjacent to the 3′ TIR also binds transposase (Feschotte et al., 2005).

In this study, we have again used a yeast assay, but here to test for *Osmar5* transposition, including excision and reinsertion. We turned to a yeast assay for two reasons. First, previous studies indicated that transposition of *Tc1/mariner* elements (e.g., *Himar1*, *Mos1*, and *Tc1*) could occur without host-specific factors (Lampe et al., 1996; Vos et al., 1996; Tosi and Beverley, 2000). The second reason for turning to yeast is that it was shown previously to support transposition of the maize *Ac* and *Ds* elements (Weil and Kunze, 2000). Here, we report that the rice *Osmar5* element transposes in the budding yeast *Saccharomyces cerevisiae*. Analysis of transposon footprints at the excision site suggests a model for how the transposase cleaves this site to promote element transposition. In addition, new insertions of *Osmar5* into TA dinucleotides were detected in the vector and in yeast chromosomes. Finally, transposition was reduced or prevented by mutation of the DD39D catalytic domain and by either deletion of the transposase DBD or mutation of the TIR binding site.

**RESULTS**

**Yeast Transposition Assay**

A yeast assay was devised to determine whether *Osmar5* encoded an active transposase and, if so, the features of excision and reinsertion. The assay involved two constructs, one encoding the transposase source and the other a reporter for excision. The transposase source, *pOsm5Tp*, has *Osmar5* coding sequence (Figure 1) fused to the inducible *gal1* promoter and contains *his3* as a selectable marker (Figure 2). The reporter construct, *pOsm5NA*, contains a nonautonomous *Osmar5* element (*Osmar5NA*) (Figure 1) inserted in the 5′ untranslated region (5′ UTR) of an *ade2* reporter gene with *ura3* as a selectable marker (Figure 2). To prevent the repair of excision sites by the very efficient yeast homologous recombination system, a haploid yeast strain was used as recipient (DG2523; see Methods) in addition to including ARS1/CEN4 in the plasmid reporter construct (*pOsm5NA*), so that it was maintained as a single copy in yeast (Falcon and Aris, 2003).

Transformants containing both plasmids were selected on plates containing 2% galactose and 1% raffinose but lacking histidine and uracil. Colonies were picked from plates containing the double transformants, and *ADE2* revertants were selected based on growth on agar plates without adenine. Excision events were confirmed by PCR amplification of the *ade2* 5′ UTR and subsequent sequencing (Figure 2, see primer location). Finally, as a control, we used plasmid pRS413, which is identical to *pOsm5Tp* except that it lacks the *Pgal1-Osmar5* transposase gene.

**Excision of Osmar5NA**

Double transformants containing *pOsm5Tp* (or control plasmid pRS413) and *pOsm5NA* were streaked onto plates lacking adenine to select for *ADE2* revertants. Many *ADE2* revertant colonies were obtained for *pOsm5Tp*, but none were obtained for control plasmid pRS413 (Figure 3A). Plasmid DNA was prepared from *ADE2* revertants, and excision of the *Osmar5NA* element from the reporter construct was confirmed by PCR amplification using primers flanking the element insertion site on *pOsm5NA* (Figure 3B). Sequencing of this locus from independent *ADE2* revertants revealed that excision of *Osmar5NA* was accompanied by the formation of many and diverse transposon footprints (Figure 3C). Compared with this locus in the original plasmid (Figure 3C, *pOsm5NA*, boxed region), all but one of the plasmids from *ADE2* revertant colonies had the TA duplication intact but also contained between one and seven additional nucleotides that appeared to be derived from the ends of *Osmar5NA*. For all of these excision events, none had what would be equivalent to a precise excision, that being the removal of the entire element and one copy of the dinucleotide TA from the TSD (see Discussion).

**Reinsertion of Osmar5NA**

Transposition involves both excision and reinsertion of the excised element into a new locus. To understand the fate of the excised *Osmar5NA*, DNA extracted from eight independent *ADE2* revertants was used for DNA gel blot analysis. To this end, the DNAs were digested with *DraI* (which does not cut in *Osmar5NA*), and the resultant DNA gel blot was probed with labeled *Osmar5NA* (Figure 4).

Compared with the plasmid control (Figure 4, *pOsm5NA*), new bands were visualized in samples 1, 4, 5, and 8, suggesting insertion of *Osmar5NA* at new loci. However, because samples...
2, 3, 6, and 7 contained a single band that comigrated with the plasmid control, as does one of the two bands in sample 1, we reexamined the presumptive excision sites in these strains. For each strain, sequenced PCR products revealed a transposon footprint in place of the Osmar5NA element (data not shown). Based on these results, we hypothesized that in each strain, the Osmar5NA element had transposed to new sites in the pOsm5NA vector. To test this hypothesis, DNAs isolated from each strain were used to transform E. coli and recover their plasmids. Because the DNA samples contained both pOsm5Tp and pOsm5NA, PCR amplification of the ade2 5′ UTRs of the recovered plasmids was performed to screen for plasmids containing the ade2 gene (in the plasmid derivatives of pOsm5NA) (Figure 5A).

Reinsertion sites of Osmar5NA in the excision derivatives of pOsm5NA (called pOsm5NA-d) were analyzed by comparing their restriction digestion patterns with those of control plasmids after digestion with \( \text{Dra I} \) (Figure 5B) and \( \text{Nde I} \) (Figure 5C). Four of the eight plasmids (Figures 5B and 5C, lanes 1, 2, 5, and 6) have an altered pattern from that of pWL89A (otherwise identical to pOsm5NA except lacking Osmar5NA), suggesting that Osmar5NA had reinserted into the plasmid after excision. The putative insertion sites in pOsm5NA-d plasmids were approximated by analysis of the restriction digests with \( \text{Dra I} \) and \( \text{Nde I} \) (data not shown). Once the approximate location of the reinserted element was known, sequencing primers were designed to determine precise insertion sites of Osmar5NA in the vector (Figure 5D). All four had inserted at TA dinucleotides and generated TSDs upon insertion (Figure 5E). The fact that all insertion sites were intergenic suggests that the majority of insertions may have been eliminated by selection for plasmid functions.

The remaining four plasmids (Figures 5B and 5C, lanes 3, 4, 7, and 8) have an identical pattern to that of pWL89A, indicating the absence of Osmar5NA in the vector and the possibility that the element had transposed into a yeast chromosome. For these strains, insertion sites in the yeast genome were determined by performing inverse PCR with primers located near the Osmar5NA termini, with their 3′ ends to be extended outward into presumed flanking yeast genomic DNA (see Methods). PCR products were successfully obtained for two samples (lanes 4 and 8 in Figure 4; data not shown), and BLAST searches of the resultant sequences.
led to the identification of insertion sites of Osmar5NA in the yeast genome (Figure 5E).

**Mutagenesis Analysis of Osmar5 Transposase and Transposon TIRs**

In a previous study, the putative transposase peptide sequences for 34 Osmar elements were aligned with that of Soymar1 to identify conserved residues (Feschotte et al., 2003). Highly conserved sites include Met-220, which is located at the junction of the DBD and the catalytic domain, and the predicted DD39D motif (Asp-242, Asp-405). Interestingly, Asp-400, which is 34 residues from Asp-365, is also well conserved (94% identity). To evaluate the importance of these conserved sites for transposition, site-directed mutagenesis was performed. Mutation of Met-220 to Ile and Asp-242, Asp-400, Asp-405 to His abolished activity, as no ADE2 revertants were obtained in the excision assay (Figure 6). However, mutation of Asp-365 to His reduced the ADE2 revertant frequency to approximately one-fourth ($0.40 \times 10^{-6}$/cell) of that of intact Osmar5 transposase ($1.51 \times 10^{-6}$/cell). These results suggest that the putative DD39D motif, as well as the conserved Met-220 and Asp-400 motifs, are important for efficient transposition activity.

To test whether interaction between Osmar5 TIRs and transposase DBDs is required for transposition, site-directed mutagenesis of Osmar5NA was performed so that the TIRs contained mutations in the strictly conserved (>99% identity among 34 Osmar elements) terminal sequence CTCCCTCC as well as in the two previously identified motif boxes of the TIRs (Figure 6) (Feschotte et al., 2005). When a derivative of pOsm5NA containing mutated Osmar5NA TIRs was used in the excision assay with pOsm5Tp, no ADE2 revertants were obtained, indicating that transposition of Osmar5 requires correct TIR sequences. Similarly, no ADE2 revertants were obtained when the DBD of Osmar5 transposase was deleted (Figure 6). These results suggest that both functional TIRs and transposase DBDs are required for transposition.

**DISCUSSION**

The Tc1/mariner superfamily is widespread and well characterized in eukaryotic genomes. However, although it is also widespread in the genomes of flowering plants, no active elements have been reported. In this study, we demonstrate that the rice Osmar5...
element encodes a transposase that catalyzes the excision and reinsertion of a nonautonomous derivative element in yeast. Because the catalytic domains of plant Tc1/mariner elements form a distinct monophyletic clade, it was of interest to initiate a comparative analysis of the catalytic properties of plant and animal elements. In addition, as discussed in more detail below, Tc1/mariner elements are thought to furnish the transposase for the movement of the nonautonomous Stowaway miniature inverted-repeat transposable elements (MITEs) (Feschotte and Mouches, 2000; Feschotte et al., 2003). Stowaway MITEs are present in thousands of copies in the genomes of many plant species, where they are particularly enriched in the noncoding regions of genes (Bureau and Wessler, 1994; Turcotte et al., 2001; Schenke et al., 2003). To date, no actively transposing Stowaway elements have been identified. As such, the availability of an active plant Tc1/mariner element provides an opportunity to analyze the amplification of Stowaway MITEs and their contribution to the evolution of plant genomes.

**Tc1/Mariner Element Transposition: Plants versus Animals**

A transposition mechanism for Tc1/mariner elements was originally proposed based on in vivo and in vitro analysis of Tc3 from *C. elegans* (van Luenen et al., 1994), whereby transposition occurs in several steps: (1) transposase binds to the element TIR through its bipartite DBD; (2) the catalytic domain mediates element excision by cleavage at two sites, two nucleotides inside the 5'9 ends and precisely at the 3'9 junction between the TSD and

---

**Figure 5.** Reintegration Sites of Osmar5NA.

(A) Scheme of plasmid rescue from ADE2 revertant genomic DNA. Yeast genomic DNA was extracted from ADE2 revertants and used to transform *E. coli* (see text for details). The small gray and black circles represent pOsm5NA and pOsm5TP, respectively.

(B) Agarose gel analysis of DraI digestion of the recovered pOsm5NA derivative plasmids from (A). DNA size markers are shown at left.

(C) NdeI digestion of the plasmids used for (B).

(D) Insertion sites in pOsm5NA derivatives (pOsm5NA-d); pWL89A lacks Osmar5NA. Note that Osmar5NA has a NdeI site but not a DraI site.

(E) Insertion sites of ADE2 revertants in either the plasmid vector or yeast genomic DNA. Accession numbers of yeast genomic DNA are shown at right.
the element ends (Figure 7); cleavage results in excision sites (and excised elements) with two-nucleotide protruding 3’ ends; (3) excised elements exist as free circular intermediates that target TA dinucleotides for insertion; (4) the 3’ hydroxyl group initiates nucleophilic attack at a TA dinucleotide, producing a staggered cut; (5) element integration is accompanied by DNA synthesis, which repairs the gaps and generates the TSD; and (6) host repair of the excision site, creating transposon footprints. This model was also shown to hold for Tc1 and Himar1 (Radice and Emmons, 1993; Lipkow et al., 2004).

Consistent with the transposition mechanism proposed for Tc3, Tc1, and Himar1, Osmar5 transposase binds specifically to its TIR through the N-terminal binding domain, as demonstrated previously (Feschotte et al., 2005). An interaction between DBD and TIRs is further supported in this study by the failure of TIR mutations and a DBD deletion to mediate transposition in yeast.

The most significant contribution of this study with regard to the mechanism of transposition of a plant Tc1/mariner element comes from the analysis of the transposon footprints. As mentioned above, transposase endonuclease activity mediates cleavage of the element from the donor site. Like animal Tc1/mariner elements, Osmar5 transposase appears to cut several nucleotides within the element’s 5’ end. This view is supported by the composition of footprints generated by Osmar5 excision (Figures 3C and 7). Specifically, the nucleotides located between the remaining TSDs are identical to nucleotides at the element ends. By comparison with the Tc3 footprints and its deduced

**Figure 6.** Mutations Introduced in the Transposase and TIR and Their Effect on Transposition.

Vectors containing the intact Osmar5 transposase gene (wild type) and its mutated forms were cotransformed with pOsma5NA, and double transformants were selected for ADE2 reversion. mTIR, mutated TIR of Osma5NA; DBD deletion, DNA binding domain deletion; M220→I, Met at position 220 mutated to Ile; D242→H, Asp at position 242 mutated to His; D365→H, Asp at position 365 mutated to His; D400→H, Asp at position 405 mutated to His; D405→H, Asp at position 405 mutated to His. Standard errors for six independent events are shown. The nucleotide changes in the Osma5NA TIRs are shown in lowercase letters. Dots represent omitted internal sequences of Osma5NA. Previously identified DNA binding motifs are shown in boxes.

**Figure 7.** Representative Footprints for Tc3, Mos1, Minos, and Osma5NA.

Donor sites are shown as double stranded, but footprints are shown as single strands (top strand). TSDs are shown in red. Lowercase letters indicate residues retained from transposon ends. Arrows indicate proven (Tc3 and Mos1) or predicted (Minos and Osma5NA) excision cleavage sites. Based on Bryan et al. (1990), van Luenen et al. (1994), Arca et al. (1997), and Zagoraiou et al. (2001).
mechanism, we propose that the Osmar5 transposase cleaves four nucleotides within the element’s two 5' ends, and, at its 3' ends, precisely at the TSD/element junction. As such, both the excised element and the excision site would contain 3' overhangs of four nucleotides, thus accounting for the number and composition of nucleotides between the TSDs.

Variation in the 5' cleavage site has been observed for Tc1/mariner transposases. For example, the transposon from Mos1, Sleeping Beauty, and Frog Prince cleave three nucleotides within the element ends (Dawson and Finnegan, 2003; Miskey et al., 2003; Yant and Kay, 2003). Interestingly, the putative site of Osmar5 cleavage, four nucleotides from the element ends, has also been observed for the Drosophila Minos element (Figure 7), a distantly related member of the Tc1/mariner superfamily (belonging to the DD34E group) (Arca et al., 1997; Zagoraiou et al., 2001).

Although our study provides evidence for the importance of the Osmar5 DD39D motif in the transposition reaction (Figure 6), we were surprised to find that mutation of the second Asp residue (Asp-365) did not completely abolish transposition activity. This could be explained by one of two possibilities: (1) the Asp-365-to-His mutation does not completely disrupt the reaction center, because His may act like a cation and the role of the mutated Asp residue may be compensated by another nearby Asp residue (Asp-375, present in all 34 Osmar elements in the rice genome); (2) the DD39D motif may not accurately reflect the reaction center of the plant elements, as its significance was based on sequence conservation rather than functional criteria. In fact, comparison of the rice transposases and that of Soyamar1 revealed five conserved Asp residues (Asp-242, -365, -375, -400, and -405) and two conserved Glu residues (Glu-243 and Glu-261) in the presumed catalytic domain. The fact that mutation of Asp-400, which is not part of the DD39D motif, completely abolished transposition activity supports the view that the exact components of the catalytic motif in plant transposases remain to be defined further.

Although flowering plants are rich in Tc1/mariner elements, it is not known whether they have a preference, like the maize Ac and other hAT elements (Chen et al., 1987; Moreno et al., 1992; Tower et al., 1993), to transpose into linked sites. Local transposition has been demonstrated for other Tc1/mariner elements (e.g., Sleeping Beauty) (Luo et al., 1998; Fischer et al., 2001). In this study, insertion of Osmar5NA was documented to both linked (reporter pOsm5NA) and unlinked (yeast chromosome) sites. Four of the eight excised Osmar5NA elements (independent events) inserted into sites in the reporter plasmid. In addition, this number is probably a considerable underestimate, as only insertions between plasmid genes were recovered in this assay because of a requirement for several plasmid functions. However, although these data strongly suggest a preference for local transposition of Osmar5NA, target selection in the yeast assay may have been influenced by the location of Osmar5NA on a plasmid. This ambiguity can be addressed in future experiments by analyzing transposition from an Osmar5NA reporter construct that is integrated into the yeast chromosome.

The extreme evolutionary distances involved can also complicate conclusions drawn from the analyses of plant transposases in yeast. For example, it is important to understand whether the observed events are attributable to the properties of the transposase or to the yeast host, or both. In this regard, comparison of the footprints generated by two plant transposases (Ac and Osmar5) in yeast is informative. Footprints generated by Ac and Osmar5 are markedly different (Weil and Kunze, 2000; Yu et al., 2004). The Ac transposase, in either a yeast or a plant host, generates footprints with deletions in the TSD and some that extend into flanking sequences. In addition, nucleotides are not retained from the element ends (Baran et al., 1992; Bancroft and Dean, 1993; Rinheart et al., 1997; Weil and Kunze, 2000). By contrast, the majority of footprints generated by Osmar5 (and other Tc1/mariner elements) contain intact TSDs and nucleotides from the element ends. This difference can be explained by the different transposition mechanisms of Ac/DS and Tc1/mariner elements. The prevailing model for Ac transposition hypothesizes that the transposase cleaves in the TSD and at the element boundary and that the resultant repair of excision sites produces footprints with inverted repeat structures (Peacock et al., 1984; Kunze and Weil, 2002). By contrast, as discussed above, Tc1/mariner elements have been shown to cleave within the element, and the Osmar5 footprints in yeast are consistent with previously described mechanisms, although transposition activity of Osmar5 in the rice genome has yet to be demonstrated. Together, these data indicate that the very different plant transposases require no host-specific factors, and as such, yeast is an excellent system in which to study diverse transposition mechanisms.

Stowaway MITEs and Osmar Elements

In a previous study, computer-assisted analysis of rice genomic sequence led to the identification of >34 Osmar elements and >22,000 Stowaway MITEs (Feschotte et al., 2003). Several lines of evidence had suggested that Tc1/mariner elements were the source of transposase for the nonautonomous Stowaway elements (Feschotte and Mouches, 2000; Turcotte and Bureau, 2002; Feschotte et al., 2003). Specifically, they have related TIRs and the same TA dinucleotide TSD. For this reason, it was surprising that none of the Stowaway elements in the rice genome were derived from the Osmar elements by deletion (Feschotte et al., 2003). Thus, to understand Stowaway amplification in plant genomes, it will be necessary to establish functional connections between Stowaway MITEs and plant Tc1/mariner elements. As such, this study provides two important starting points. First, it demonstrates that at least one Osmar element, Osmar5, is active. Second, demonstration of Osmar transposition in yeast provides a valuable assay system to screen for functional partners between Osmar elements and rice Stowaway elements. Without extensive sequence similarity between presumed autonomous elements (the Osmar elements) and nonautonomous partners (the Stowaway elements), it may be necessary to test many, perhaps dozens, of combinations of Osmar and Stowaway pairs to establish functional connections. The assay system described in this study would be ideal for such large-scale screening, with yeast serving as a living test tube in which the relationships among Osmar and Stowaway elements can be dissected to understand the spread of these important elements throughout plant genomes.
METHODS

Yeast Strain and Plasmid Construction

Excision assays were performed after transformation of the yeast haploid strain DG2523 (MATalpha ura3-167 trp1-hisG leu2-hisG his3-de200 ade2-hisG) (obtained from David Garfinkel). The plasmid containing the Osma5 transposase, pOsm5Tp, was constructed from plasmid pRS416 (New England Biolabs) as follows. First, the gal1 promoter was inserted between the SacI and NotI sites, and the ctc1 terminator was inserted into the KpnI site (resulting in plasmid pRS416-gal1). Then, the fragment between SacI and Nael from pRS416-gal1 was cloned into the corresponding sites in plasmid pRS413 (New England Biolabs), resulting in plasmid pRS413-gal1. Finally, the coding sequence of the Osma5 transposase (previously described by Feschotte et al., 2005) was cloned between the BamHI and EcoRI sites (downstream of the gal1 promoter) of pRS413-gal1, resulting in plasmid pOsm5Tp. The reporter plasmid containing the nonautonomous Osma5 element, pOsm5NA, was constructed as follows. First, Osma5NA was constructed using PCR and rice (Oryza sativa) genomic DNA from cv Nipponbare to amplify sequences from the ends of Osma5 (562 and 319 bp from the 5’ and 3’ ends, respectively) and joining the resultant PCR products with a linker sequence (available upon request). The combined fragment of 950 bp (including TA at both ends) was inserted into the Xhol site of pWL89A (Yu et al., 2004), resulting in plasmid pOsm5NA. The orientation of Osma5NA is insertion opposite that of ade2 transcription (the other orientation results in leaky expression of Ade2).

Yeast Transformation and ADE2 Revertant Selection

Transformation reactions (50 μL of competent cells, 5.8 μL of 5 mg/mL denatured salmon sperm DNA, 1 μL [-200 ng] each of plasmids pOsm5Tp and pOsm5NA, and 400 μL of 50% PEG-3500 buffer [Gietz and Woods, 2002]) were incubated at 42°C for 45 min. Cells were collected and plated on plates containing complete supplement mixture (CSM) (Q-BIOgene), 2% galactose, and 1% raffinose but lacking histidine and uracil. Colonies appeared after 3 to 4 d of incubation at 30°C and were grown to saturation at room temperature (~10 d). ADE2 revertants were selected from the double transformants by streaking colonies onto CSM plates containing 2% galactose and 1% raffinose but lacking adenine. To calculate excision frequency, colonies from plates lacking histidine and uracil were picked into 50 μL of water, of which 49 μL was plated onto CSM plates containing 2% galactose but lacking adenine and 1 μL was used for 10^2 or 10^3 dilutions. Of the diluted yeast cell suspension, 49 μL was plated on YPD (yeast extract/peptone/dextrose) or CSM plates lacking histidine and uracil to calculate the total number of live yeast cells in the cell suspension. The revertant frequency was calculated as the number of ADE2 revertants per cell.

Footprint Analysis

ADE2 revertant colonies were cultured in YPD liquid medium overnight or in CSM drops outside medium lacking adenine for 2 to 3 d. Plasmid DNA was extracted using the E.Z.N.A. yeast plasmid kit (Bio-Tek). PCR primers used to detect the excision of Osma5NA on pOsm5NA were 5’-CTGAC-AATGTGACTCTTTGTTGCGAGGCTACGAAC-3’ and 5’-TGAAAAAGGAG-GCCATTAACGTGCTACGGAGC-3’. PCR products were sequenced directly.

Genomic DNA Gel Blot Analysis

Genomic DNA (100 ng) from ADE2 revertants was extracted using the E.Z.N.A. yeast DNA kit (Bio-Tek), digested with Dral, and resolved on an agarose gel (1%). DNA was blotted onto a Hybond N+ nylon membrane (Amersham Biosciences) using capillary transfer in 20 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate). Probes were prepared with the DECA prime II kit (Ambion) using Osma5NA as template. Hybridization and washing conditions were as described by the supplier (ULTRAhyb ultrasensitive hybridization buffer; Ambion).

Plasmid Recovery from ADE2 Revertant Genomic DNA

Genomic DNA (30 to 100 ng) from ADE2 revertants was transformed into Escherichia coli competent cells (Invitrogen), and transformants were selected on Luria-Bertani plates with carbenicillin (50 mg/L). Plasmid DNA was extracted from transformant colonies. Because there were two plasmids in the genomic DNA samples, PCR amplification of the ade2 5’ UTR was used to identify strains containing pOsm5NA-d.

Mutagenesis of Osma5 Transposase and TIRs

To delete the DBD of Osma5 transposase, a BamHI site was created using site-directed mutagenesis at the junction of the DBD and the catalytic domain (primer 5’-AGAGAAAAAGCAGTGTCAGTTGATCTAT-GCTAGATCCGACCA-3’) so that a BamHI fragment (with the DBD) could be removed and the remaining plasmid could be self-ligated. Site-directed mutagenesis of transposase sites Met-220, Asp-242, Asp-365, Asp-400, and Asp-405 was performed with the QuikChange multi-site-directed mutagenesis kit (Stratagene) using primers 5’-GGCTGCACTG-GTTGTTTCTCTATAGTGACCAATCTTTGGTATCTAGTTGTTTT-3’ and 5’-CCAATCCCGGGATGATTTGATCTACATGCTTTCGTTTGTTCT-3’, respectively. Primers for mutagenesis of Osma5NA TIRs were 5’-AAAAAAGAAGGAAGTCGAGTACCTGATGCGTGTGATCACAAACCGGTGTT-3’ and 5’-TGAGAAAATTATCCACATCATGAAAAACTGCGAGTTCAATGCATCAC-3’. All plasmids were sequenced to confirm the presence of the targeted mutation. ADE2 revertant frequencies were calculated for all mutant constructs.

Inverse PCR

Genomic DNA from ADE2 revertants (~100 ng) was digested with Dral, purified (with a PCR purification kit [Qiagen]), and ligated with T4 DNA ligase (in 35 μL at 25°C for 3 h, then overnight at 6°C). Ligation products (5 μL) were amplified with primers 5’-GGCAGCTCTTTTTTCTGTT-CACCTGCGATGTACC-3’ and 5’-TGAGATCGATGCTAACATGCTCGAGACTAATGCTG-3’ and either Pfu DNA polymerase (Stratagene) or Phusion DNA polymerase (New England Biolabs) using the same cycling conditions for both enzymes (98°C for 45 s; 35 cycles of 98°C for 45 s, 58°C for 45 s, and 72°C for 2 min; and 72°C for 10 min). PCR products were sequenced directly, and the resultant sequences were used as queries for BLAST searches to determine Osma5NA insertion sites.

Accession Numbers

The GenBank accession numbers for Osma5 used in this study are AP008207 and AP003294.

ACKNOWLEDGMENTS

We thank David J. Garfinkel and Abram Gabriel for yeast strains, plasmids, and technical assistance. We also thank Ryan Peeler, Cedric Feschotte, Mark Osterland, Tianle Chen, Nathan Hancock, Feng Zhang, and technical assistance. We also thank Ryan Peeler, Cedric Feschotte, Mark Osterland, Tianle Chen, Nathan Hancock, Feng Zhang,
and Dawn Holligan for technical assistance and helpful discussions. This study was supported by grants from the National Institutes of Health and the University of Georgia Research Foundation to S.R.W.

Received July 14, 2006; revised August 21, 2006; accepted September 22, 2006; published October 13, 2006.

REFERENCES


