TP901-1 Phage Recombinase Facilitates Genome Engineering in Drosophila melanogaster

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ABSTRACT Molecular biology techniques have a large impact on biomedical research and the availability of diverse tools to perform genome manipulations advances the ease of executing complicated genetic research. Here, we introduce in the fruit fly another such tool by harnessing the phage recombinase TP901-1 to perform site-directed recombination that leads to recombinase-mediated cassette exchange (RMCE). The TP901-1 system complements already existing recombination systems and enhances genome engineering in the fruit fly and other organisms.

KEYWORDS TP901-1 site-directed recombinase cassette exchange CRISPR/Cas9 genome engineering Drosophila melanogaster

Recombinase-mediated cassette exchange (RMCE) is a powerful molecular biology technique that can enhance genome manipulations, including CRISPR/Cas9 genome editing. Efficient RMCE in Drosophila melanogaster has been performed using diverse recombination systems and is an established molecular genetics tool (Horn and Handler 2005; Oberstein et al. 2005; Bateman et al. 2006; Voutev and Mann 2017).

The irreversibility of the site-directed recombination is critical in RMCE because it ensures that once recombination occurs the edited genomic locus is ‘locked’ in a desired state. Heterospecific FRT or lox sites are normally employed to achieve directionality and conditional irreversibility when the Flp/FRT or the Cre/lox systems are used for RMCE in the fruit fly (Horn and Handler 2005; Oberstein et al. 2005). While in the case of serine integrase systems, such as those based on the ΦC31 and Bxb1 recombinases, the conversion of their cognate recognition attB/attP sites to attL/attR sites during recombination (Thorpe and Smith 1998; Ghosh et al. 2003) leads to irrevocable cassette exchange of, for example, attP-flanked loci in Drosophila (Bateman et al. 2006; Voutev and Mann 2017). In order to broaden the spectrum of available irreversible site-directed recombinase systems in the fruit fly, we show that the TP901-1 lactococcal phage recombinase (Christiansen et al. 1996) could perform successful recombination and RMCE by using minimal TP901-1 attB/attP sites.

METHODS AND MATERIALS

The original TP901-1 recombinase cDNA was PCR amplified from plasmid pG35-TP901.2 (a gift from Dr. James Thomson; (Thomson and Ow 2006)) and inserted in vector pRVV210 (Addgene ID#87628; (Voutev and Mann 2017)) in place of the Bxb1 recombinase cDNA, resulting in construct vasa-TP901-1-nos. The fruit fly codon-optimized version of TP901-1 recombinase gene was synthesized based on the analysis of the IDT Codon Optimization Tool (http://www.idtdna.com/CodonOpt; Integrated DNA Technologies Inc., Skokie, IL). The resulting cDNA, TP901-1FlyOpt, was inserted in place of the original cDNA to generate vasa-TP901-1FlyOpt-nos and additional constructs were created through PCR amplification by adding the SV40 large T-antigen NLS sequence (Kalderon et al. 1984) either to the N-terminus or to the C-terminus of TP901-1FlyOpt. A yellow (y+) selectable marker was added to these vectors afterward. Since the most efficient TP901-1FlyOpt recombinase version was the one with an N-terminally tagged NLS, the vector carrying it (pRVV655; Addgene ID#119019) was deposited...
in Addgene, Cambridge, MA. Different fly strains carrying vasa-NLS-TP901-1-flp-Csp-nos were generated by inserting the construct in landing sites ZH-2A, ZH-22A, and ZH-86Fa on chromosomes X, II, and III, respectively (Bischof et al. 2007). These fly strains were deposited in Bloomington Drosophila Stock Center, Bloomington, IN.

The 65 bp minimal TP901-1 recombinase attP site (TCCAGAATTCGTTTGGTACATTGCGAGTTTTTATTTCGTTTATTTCAATTAA-GGTAAACTAAAAAATCCT; (Thomson and Ow 2006)) was introduced in vector pRVV598 (Addgene ID#87629, (Voutev and Mann 2017)) in forward and reverse orientation (Figure 1), flanking a hs-neo cassette (Steller and Pirrotta 1985) and replacing the Bxb1 recombinase attP sites in vector pRVV598 (Voutev and Mann 2017). The hs-neo cassette consists of a heat-shock-protein-70 promoter driving neomycin resistant gene (Steller and Pirrotta 1985) but drug selection was not performed in this study. A loxP site was introduced 5' of this cassette and the resulting vector was used for injection and integration in landing site ZH-51D to subsequently generate a clean allele, TThs-neo.

The 61 bp minimal TP901-1 recombinase attB site (CTGATA-TTGCACAACAGATACATCAATGGAATTGCGTTTGCATTGTTTGTGGC; (Thomson and Ow 2006)) was introduced in vector pRVV601 (Addgene ID#87632; (Voutev and Mann 2017)) in forward and reverse orientation, flanking an MCS and replacing the Bxb1 recombinase attB sites in vector pRVV601, resulting in an TP901-1 attB RMCE vector (pRVV610; Addgene ID#119018). Shorter TP901-1 attB/attP pairs have successfully been used for recombination in bacteria (Breüner et al. 2001; Stoll et al. 2002) but since the recombination efficiency of TP901-1 in the fruit fly was not very high (see below), we refrained from testing shorter TP901-1 attB/attP sites than the ones described by Thomson and Ow 2006.

The TP901-1 ubi-GFP RMCE vector (Figure 1) was created by PCR amplifying a ubi-GFP cassette and placing it in the MCS of pRVV610. Plasmid DNA, maps, and complete vector sequences of the above and other useful TP901-1 system vectors are made available at Addgene (www.addgene.org); Addgene IDs: 119014-119019.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS AND DISCUSSION

Similar to our previous RMCE technique studies (Voutev and Mann 2017; Voutev and Mann 2018), we introduced in landing site ZH-51D a hs-neo cassette flanked by minimal inverted TP901-1 attP sites (Figure 1; Methods and Materials), using standard ΦC31-mediated integration (Bischof et al. 2007). A loxP site that we positioned upstream of the hs-neo cassette allowed us to excise all intervening vector sequences (Bischof et al. 2007) and to create a clean TP901-1 attP-flanked hs-neo allele that we called TThs-neo (Figure 1).

Next, we generated a fly strain that expressed the TP901-1 phage recombinase in the fly germ line by placing its cDNA under the control of the vasa promoter and nanos 3'UTR (vasa-TP901-1-nos) and introduced this construct into landing site ZH-86Fa (Bischof et al. 2007) through ΦC31-mediated transgenesis.

We also created a RMCE vector that contains ubiquitin-GFP (ubi-GFP) cassette flanked by minimal inverted TP901-1 recombinase attB sites (Figure 1; Methods and Materials). The TP901-1 ubi-GFP RMCE vector is marked by white (+w) (Figure 1) that allows the differentiation between RMCE and integration events in our experimental design.

Previously (Voutev and Mann 2018), we found that the ΦC31 integrase performs most efficient recombination when it is expressed in the fly germ line in an established strain that contains two copies of the transgene driving its expression (most likely because of increased dosage of the recombinase). Analogously, we decided to test if the TP901-1 recombinase could perform RMCE when the transgene vasa-TP901-1-nos [ZH-86Fa] is homozygous. We established a strain, TThs-neo/Cyo; vasa-TP901-1-nos [ZH-86Fa], and injected embryos laid by it with the TP901-1 ubi-GFP RMCE vector at 250 ng/ml concentration. We raised the resulting larvae at 25° and crossed individual fertile adults to yw flies. Surprisingly, these injections did not lead to any RMCE or integration events of the TP901-1 ubi-GFP RMCE vector even though we injected more than 1000 embryos that produced ~350 fertile adults.

Since the TP901-1 recombinase has been shown to function in vitro and in other eukaryotic systems (Stoll et al. 2002; Thomson and Ow 2006), it was unlikely that it required additional factors to perform recombination in the fruit fly. However, the optimal temperature for TP901-1 function is 30-35° (Stoll et al. 2002), which is above the standard temperatures for fly husbandry and is closer to temperatures normally used in fly heat-shocks. To test if higher temperatures would improve TP901-1 recombination performance, we injected 800 TTThs-neo/Cyo; vasa-TP901-1-nos [ZH-86Fa] embryos, with TP901-1 ubi-GFP RMCE vector and raised the resulting larvae and crossed the adults to yw flies at 29.5°. This experiment also did not lead to any positive RMCE or integration events.
The above results suggested that there might be some other reason why the TP901-1 recombinase does not function in flies. Since we have previously used the same vector backbone and drivers to express the Bxb1 recombinase in Drosophila (Voutev and Mann 2017), we reasoned that the problem did not lie in the expression levels driven by the vasa promoter and the nos 3'UTR. Therefore, we looked more closely at the original cDNA of TP901-1 and found that it uses unfavorable codons for the fruit fly (e.g., 22 ACU (Thr), 36 AAA (Lys), 12 UUA (Leu), 12 UCA (Ser), 11 AGA (Arg)). Therefore, we codon-optimized the TP901-1 recombinase cDNA to favor translation in the fruit fly (Methods and Materials) and created a new construct (vasa-TP901-1FlyOpt-nos) that we introduced in landing site ZH-86Fa. In addition, we tested if adding the SV40 large T-antigen nuclear localization signal (Kalderon et al. 1984) to either the N- or the C-terminus would improve the function of the TP901-1 recombinase in flies. We created two additional vectors and transgenes: vasa-NLS-TP901-1FlyOpt-nos [ZH-86Fa] and vasa-TP901-1FlyOpt-NLS-nos [ZH-86Fa]. The strains carrying these transgenes did not exhibit any noticeable toxicity to the flies.

We tested each of the optimized TP901-1 recombinase transgenes by injecting with the TP901-1 ubi-GFP RMCE vector ~400 embryos produced by each of the following strains: TThs-neo/CyO; vasa-TP901-1FlyOpt-nos [ZH-86Fa], TThs-neo/CyO; vasa-NLS-TP901-1FlyOpt-nos [ZH-86Fa] and TThs-neo/CyO; vasa-TP901-1FlyOpt-NLS-nos [ZH-86Fa]. We performed these experiments at standard temperature, 25°C. The optimization of the TP901-1 recombinase improved its performance because each of the injection schemes produced integration events through recombination between one of the TP901-1 attB/attP pairs (GFP-positive, w+ flies). However, no full RMCE events (GFP-positive, w- flies) were observed. NLS-TP901-1FlyOpt was the most efficient recombinase and produced 4.3% (n = 117) G0 flies that segregated progeny with integration events. TP901-1FlyOpt and TP901-1FlyOpt-NLS were less efficient and produced 1.2% (n = 83) and 1% (n = 97) G0s, respectively, which segregated progeny with integration events.

Previously, we showed that during cassette exchange, integration events catalyzed by either the PhiC31 or the Bxb1 integrases could be converted to complete RMCE events through intramolecular recombination between intact attB/attP pairs left in the genome (Voutev and Mann 2017; Voutev and Mann 2018). Similarly, we wanted to test if the optimized TP901-1 recombinase could perform intramolecular excisions. We maintained the TThs-neo, ubi-GFP alleles (Figure 1), resulting from the integration events, in the background of optimized TP901-1 recombinase in three versions: TThs-neo, ubi-GFP/CyO; vasa-TP901-1FlyOpt-nos/MKRS, TThs-neo, ubi-GFP/CyO; vasa-NLS-TP901-1FlyOpt-nos/MKRS and TThs-neo, ubi-GFP/CyO; vasa-TP901-1FlyOpt-NLS-nos/MKRS. We crossed males with these genotypes to yw females and asked if, and at what rate, intramolecular excision could be observed in the resulting non-CyO progeny by absence of the w+ marker in the eyes. All three optimized versions of the TP901-1 recombinase catalyzed intramolecular recombination (Figure 1) and NLS-TP901-1FlyOpt outperformed the other two versions since the males carrying it produced 14/100 w- GFP-positive flies with complete RMCE. The other two optimized versions of TP901-1 catalyzed intramolecular recombination at equal rates since males in each case produced 7/100 flies that were w- GFP-positive. We sequence-verified four of these recombination events to confirm that the RMCE occurred correctly. As expected, we found that the ubi-GFP cassette was inserted in either forward or reverse orientation.

The successful replacement of the hs-neo cassette with ubi-GFP through two-step TP901-1FlyOpt-catalyzed recombination (Figure 1) marks the introduction of yet another site-directed recombinase system in the repertoire of Drosophila melanogaster molecular genetics tools. Despite the fact that TP901-1FlyOpt was not observed to catalyze complete RMCE events in one step and required a second intramolecular excision step, it is nevertheless useful in genome engineering of loci where multiple irreversible recombinase systems are required. For example, three genome elements of interest could be engineered on the same chromosome arm using three different recombinase systems (PhiC31, Bxb1, or TP901-1) and studied simultaneously (Figure 2). These genome elements could be different ‘shadow’ enhancers, paralogous genes, exons, promoters, 3’UTRs, insulators, snRNAs, etc., that are positioned too far from each other to be studied on a single platform for cassette exchange and too close to each other to be easily recombined with each other. The resulting triple platform for cassette exchange would allow thorough combinatorial investigation of different versions of these genome elements. In addition, since the FLP/FRT

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**Figure 2** Schematicized example of three engineered platforms for cassette exchange on chromosome 3R, based on the Bxb1, PhiC31 and TP901-1 recombinase systems. Platforms are generated on the genetic background of FRT82B in order to perform clonal analysis by using the FLP/FRT recombination system. Recognition sites for the KD recombinase (represented with black triangles) and the B2 recombinase (represented with yellow triangles) or other site-directed recombinases could be introduced to flank particular sequences in order to conditionally excise or invert specific alleles. The Bxb1 attP/attB sites are represented with dark/light blue triangles, respectively; the PhiC31 attP/attB sites are represented with maroon/gray triangles, respectively; the TP901-1 attP/attB sites are represented with dark/light green, respectively.
system is normally used for clonal analysis in the fruit fly (Xu and Rubin 1993; Lee and Luo 1999), which renders it unsuitable to use for creation of platforms for cassette exchange if subsequent clonal analysis of the generated alleles is required, the engineered triple platform for cassette exchange could easily be combined with standard FRT sites (Xu and Rubin 1993) that are positioned close to the centromere (Figure 2). Also, sites for other available reversible recombination systems, such as those of the KD, B2, B3 or R recombinases (Nern et al. 2011) could be introduced in desired manner within the replacement cassettes in order to create conditional alleles where a specific studied genome element could be inverted or removed upon expression of the corresponding recombinase depending on the directionality of the introduced cognate sites (Figure 2).

Usage of irreversible recombinase systems (ΦC31, Bxb1, TP901-1) for creation of platforms for cassette exchange prevents aberrant reversion of alleles during the RMCE steps and the respective recombinases could be expressed again in the background of such alleles for different purposes. Reversible recombinase systems, on the other hand, are very suitable for excision/inversion of cassettes in post-engineering allele-manipulation steps and generation of stochastic clones. Alles containing reversible recombinase sites are not suitable for subsequent expression of the corresponding recombinase for other genetic purposes as chromosomal rearrangements may occur. For example, engineered alleles generated through RMCE by using the ΦC31, Bxb1, or TP901-1 systems could be readily combined with the Flybow (Hadjieconomou et al. 2011) and dBrainbow (Hampel et al. 2011) cell labeling techniques, which are based on the Fp/FRT and Cre/lox recombination systems, respectively, without concerns that aberrant recombination might occur.

The introduction of the TP901-1 recombinase system as a genome engineering tool in the fruit fly could facilitate combinatorial genomic research where creation of multiple platforms for cassette exchange (Figure 2) could allow generation of allelic series that greatly augments the research value of a particular genetic study. Similar optimizations of the TP901-1 recombinase could also be done for other organisms in order to render the system useful in diverse species.

ACKNOWLEDGMENTS

Many thanks to James G. Thomson for providing us with the TP901-1 recombinase cDNA (Thomson and Ow 2006); to Lalani Venkatasubramanian for technical assistance; to Rainbow Transgenic Flies Inc. for fly injections. RV is a Leukemia and Lymphoma Society fellow. This work was supported by NIH grant R35GM118336 awarded to RSM.

LITERATURE CITED

Bateman, J., A. M. Lee, and C. T. Wu, 2006 Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. Genetics 173: 769–777. https://doi.org/10.1534/genetics.106.056945
Bischof, J., R. K. Maeda, M. Hediger, F. Karch, and K. Basler, 2007 An optimized transgenesis system for Drosophila using gmr-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA 104: 3312–3317. https://doi.org/10.1073/pnas.0611511104
Brüner, A., L. Brondsted, and K. Hammer, 2001 Resolvase-like recombination performed by the TP901-1 integrase. Microbiology 147: 2051–2063. https://doi.org/10.1099/00221287-147-8-2051
Christianisen, B., L. Brondsted, F. K. Vogensen, and K. Hammer, 1996 A resolvase-like protein is required for the site-specific integration of the temperate lactococcal bacteriophage TP901-1. J. Bacteriol. 178: 5164–5173. https://doi.org/10.1128/jb.178.15.5164-5173.1996
Ghosh, P., A. I. Kim, and G. F. Hatfull, 2003 The orientation of mycobacteriophage BxB1 integration is solely dependent on the central dinucleotide of attP and attB. Mol. Cell 12: 1101–1111. https://doi.org/10.1016/S1097-2765(03)00444-1
Hadjieconomou, D., S. Rotkopf, C. Alexandre, D. M. Bell, B. J. Dickson et al., 2011 Flybow: genetic multicolor cell labeling for neural circuit analysis in Drosophila melanogaster. Nat. Methods 8: 260–266. https://doi.org/10.1038/nmeth.1567
Hampel, S., P. Chung, C. E. McKellar, D. Hall, L. L. Looger et al., 2011 Drosophila Brainbow: a recombinase-based fluorescence labeling technique to subdivide neural expression patterns. Nat. Methods 8: 253–259 (errata: Nat. Methods 9: 929; 12: 893). https://doi.org/10.1038/nmeth.1566
Horn, C., and A. M. Handler, 2005 Site-specific genomic tagging in Drosophila. Proc. Natl. Acad. Sci. USA 102: 12483–12488. https://doi.org/10.1073/pnas.0504305102
Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith, 1984 A short amino acid sequence able to specify nuclear location. Cell 39: 499–509. https://doi.org/10.1016/0092-8674(84)90457-4
Lee, T., and L. Luo, 1999 Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22: 451–461. https://doi.org/10.1016/S0896-6273(00)80701-1
Nern, A., B. D. Pleifker, K. Svoboda, and G. M. Rubin, 2011 Multiple new site-specific recombinases for use in manipulating animal genomes. Proc. Natl. Acad. Sci. USA 108: 14198–14203. https://doi.org/10.1073/pnas.1016722108
Obeserstein, A., A. Pare, L. Kaplan, and S. Small, 2005 Site-specific transgenesis by Cre-mediated recombination in Drosophila. Nat. Methods 2: 583–585. https://doi.org/10.1038/nmeth775
Steller, H., and V. Pirotta, 1985 A transposable P vector that confers selectable G418 resistance to Drosophila larvae. EMBO J. 4: 167–171. https://doi.org/10.1002/j.1460-2075.1985.tb02332.x
Stoll, S. M., D. S. Ginsburg, and M. P. Calos, 2002 Phage TP901-1 site-specific integrase functions in human cells. J. Bacteriol. 184: 3657–3663. https://doi.org/10.1128/JB.184.13.3657-3663.2002
Thomson, J. G., and D. W. Ow, 2006 Site-specific recombination systems for the genetic manipulation of eukaryotic genomes. Genesis 44: 465–476. https://doi.org/10.1002/dvg.20237
Thorpe, H. M., and C. M. Smith, 1998 In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl. Acad. Sci. USA 95: 5505–5510. https://doi.org/10.1073/pnas.95.10.5505
Voutev, R., and R. S. Mann, 2017 Bxb1 phage recombinase assists genome engineering in Drosophila melanogaster. Biotechniques 62: 37–38. https://doi.org/10.2144/000114494
Voutev, R., and R. S. Mann, 2018 Robust PhiC31-Mediated Genome Engineering in Drosophila melanogaster Using Minimal attP/attB Phage Sites. G3 (Bethesda) 8: 1399–1402. https://doi.org/10.1534/g3.118.2000051
Xu, T., and G. M. Rubin, 1993 Analysis of genetic mosaics in developing and adult Drosophila tissues. Development 117: 1223–1237.