Development 138, 5451-5458 (2011) doi:10.1242/dev.066498

pTransgenesis: a cross-species, modular transgenesis resource

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SUMMARY

As studies aim increasingly to understand key, evolutionarily conserved properties of biological systems, the ability to move transgenesis experiments efficiently between organisms becomes essential. DNA constructions used in transgenesis usually contain four elements, including sequences that facilitate transgene genome integration, a selectable marker and promoter elements driving a coding gene. Linking these four elements in a DNA construction, however, can be a rate-limiting step in the design and creation of transgenic organisms. In order to expedite the construction process and to facilitate cross-species collaborations, we have incorporated the four common elements of transgenesis into a modular, recombination-based cloning system called pTransgenesis. Within this framework, we created a library of useful coding sequences, such as various fluorescent protein, Gal4, Cre-recombinase and dominant-negative receptor constructs, which are designed to be coupled to modular, species-compatible selectable markers, promoters and transgenesis facilitation sequences. Using pTransgenesis in *Xenopus*, we demonstrate Gal4-UAS binary expression, Cre-loxP-mediated fate-mapping and the establishment of novel, tissue-specific transgenic lines. Importantly, we show that the pTransgenesis resource is also compatible with transgenesis in *Drosophila*, zebrafish and mammalian cell models. Thus, the pTransgenesis resource fosters a cross-model standardization of commonly used transgenesis elements, streamlines DNA construct creation and facilitates collaboration between researchers working on different model organisms.

KEY WORDS: Transgenesis, Gateway, *Xenopus*, *Drosophila*, Zebrafish, REMI, I-*Sce*I, Tol2

INTRODUCTION

The ability to engineer genetically modified organisms is essential for establishing the function of genes during development, disease, homeostasis, repair and regeneration (Gama Sosa et al., 2010; Ristevski, 2005). However, a crucial step in engineering genetically modified organisms is the design and generation of the transgene DNA constructions required for a given experiment. For the past thirty years, DNA constructions have been created primarily through restriction enzyme digestion and ligation. However, cloning with restriction enzymes becomes progressively more cumbersome as the complexity of the engineered constructs increases. For this reason, a site-specific recombination-based DNA cloning method was developed that circumvents the use of restriction enzymes (Hartley et al., 2000). The advent of recombination-based cloning brought a series of diverse and pioneering studies showing the utility of this technology in creating DNA constructions for transgenesis (Fisher et al., 2006; Hope et al., 2004; Ikeya et al., 2005; Kappas et al., 2008; Kwan et al., 2007; Nyabi et al., 2009; Semple et al., 2010; Skarnes et al., 2011). However, none had yet been designed specifically for use in *Xenopus*, a widely used model organism (Amaya, 2005), and the ability to use them across multiple models was limited. When we began to develop a transgenesis plasmid resource for *Xenopus*, we sought to design a system that would encapsulate multiple advances demonstrated in previous Multisite Gateway-based cloning projects, but we wished to expand on them to make them more universally useful to the developmental biology community at large. In particular, we wished to decouple the screenable elements from the transgenesis-promoting sequences, thus facilitating the transfer of this plasmid resource across different model systems, such as *Xenopus*, mammals, fish and flies.

By combining these attributes, we created a new modular, cross-species plasmid resource, which we have named pTransgenesis. The pTransgenesis resource is the first modular cloning system that allows the interchange of DNA elements for transgenesis between *Xenopus*, zebrafish, *Drosophila* and mammalian cell culture models. The pTransgenesis design and associated resources will greatly facilitate the efficient generation of transgenic organisms and the transfer of transgenic reagents across various developmental model organisms.

MATERIALS AND METHODS

Plasmid construction

We adapted Invitrogen's Gateway Multisite Cloning Kit (CA, USA) to create the pTransgenesis vectors (note, this is not the Gateway 'Pro'). BP reactions were performed using Invitrogen's BP Recombinase and PCR products were cloned with Invitrogen's pCR8 GW TOPO kit. LR recombinations were performed using Invitrogen's LR Clonase II+ (Ishibashi et al., 2012), bacterial transformations with DH5a competent cells (Invitrogen); typically 50-100% of colonies yield correct recombinations.

Transgenesis

Restriction enzyme-mediated integration (REMI) transgenesis was performed as described (Breckenridge et al., 2001; Kroll and Amaya, 1996). I-*Sce*I transgenesis was performed by injecting 2 nl of a 10 pg/nl reaction mixture as described previously (Ishibashi et al., 2012). The *Cre* mRNA and *Tol2* mRNA was made using the SP6 mMessage Machine

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Accepted 20 October 2011

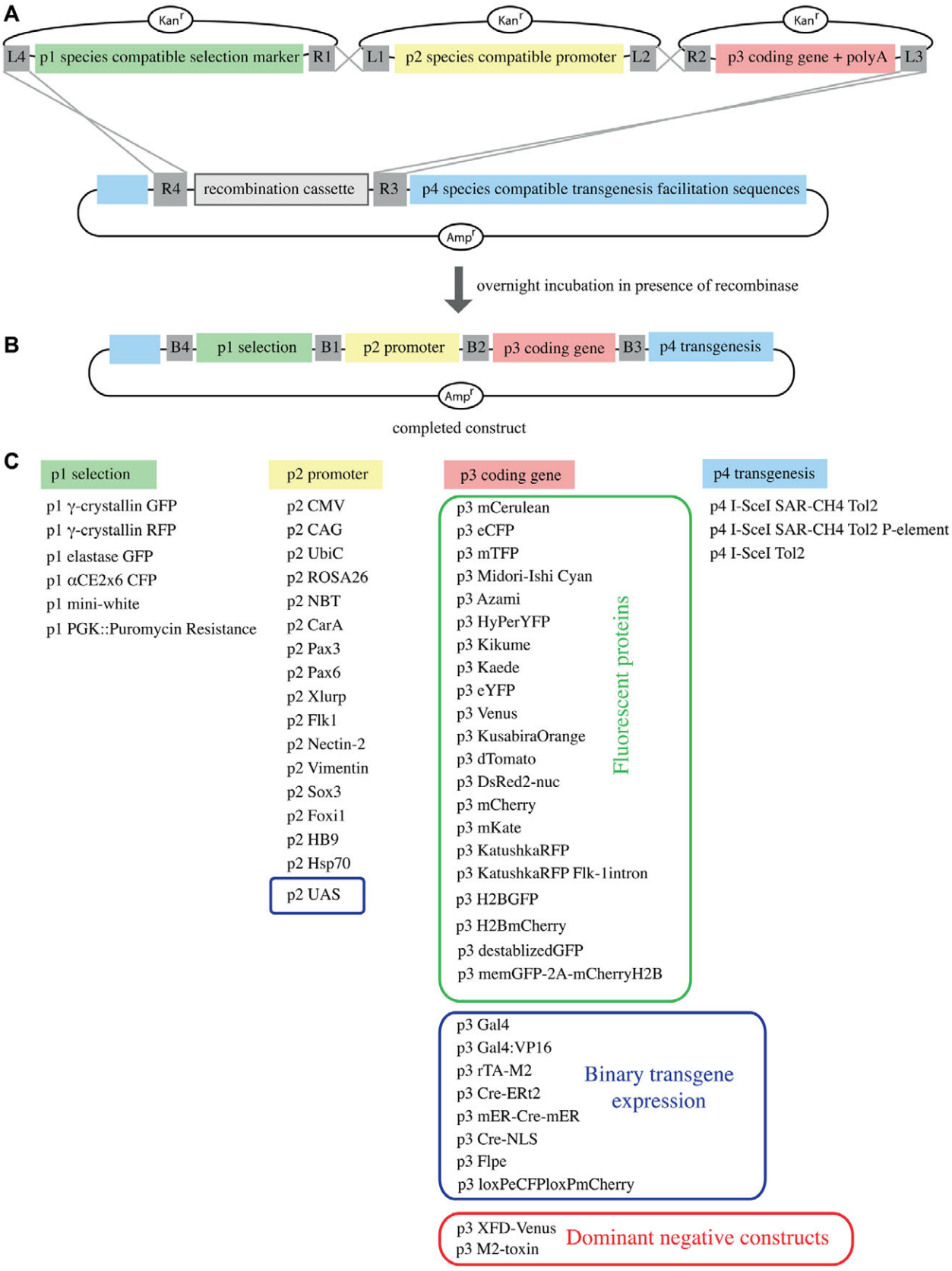

Fig. 1. The pTransgenesis framework. (**A**,**B**) Four separate vectors (p1, p2, p3 and p4) are recombined without restriction enzymes in a sequential and predictable order. Note: The 'L' and 'R' elements contained within the grey boxes represent the sequences that facilitate the in vitro recombination between plasmids. B1-B4, attB sequences produced following 'LR' recombination. (**C**) A selection of p1, p2, p3 and p4 constructs available within the pTransgenesis system.

mRNA Kit (Ambion) from a pCS2-CreNLS or pC2-TP template (Kawakami, 2007). For zebrafish injections, ~1 nl of a 20 pg/nl DNA solution with or without 25 pg *Tol2* mRNA was injected at the 1-cell stage. HeLa cell lines were made by plasmid transfection (Lipofectamine 2000, Invitrogen), with a selection of 3 μg/ml of puromycin in DMEM performed at 48 hours post-transfection. Transgenic *Drosophila* were made using P-element mediated transgenesis (Bestgene, CA, USA), and were crossed with *engrailed*-Gal4 (Bloomington Stock Center) (Brand and Perrimon, 1993; Millard and Martin, 2008), *srp*-Gal4 (Bruckner et al., 2004) and *eval*-Gal4 (Bloomington Stock Center) (Landgraf et al., 1999) driver lines.

Immunohistochemistry and in situ hybridization

Sectioning and immunostaining was performed as described previously (Chalmers et al., 2003) using a primary mouse anti-green fluorescent protein (GFP) antibody (1:500, Roche). For in situ hybridization, digoxygenin (DIG)-labelled probes were generated using the *X. tropicalis* expressed sequence tag (EST) clones from the TTpA043k14 (Vimentin) and TGas140h10 (Nectin2) clones using 10× DIG labelling mix (Roche) (Gilchrist et al., 2004). DIG probe hybridization and staining was performed according to methods described by Harland (Harland, 1991).

Microscopy

Whole-mount imaging was performed using a Leica MZ FLIII fluorescent stereomicroscope and Northern Eclipse software, HeLa cells were imaged with a Olympus IX70 inverted fluorescent microscope with Northern Eclipse software and confocal imaging was performed with an Olympus Fluoview FV1000 imaging system and accompanying software.

RESULTS AND DISCUSSION

The pTransgenesis framework

The pTransgenesis plasmid resource utilizes four separate, modular plasmid libraries (also referred to as reservoirs or positions, Fig. 1A). We named these plasmid positions with the following terminology: p1, the selection marker, designed to contain all necessary DNA sequences to allow selection of transgenic organisms; p2, the promoter DNA sequence, which is sufficient to drive expression of a coding gene; p3, the coding gene with a poly-adenlyation signal; and p4, containing sequences that facilitate genome integration and/or chromosomal attachment sites. Incubation of a ‘p1 selection marker’, ‘p2 promoter’, ‘p3 coding

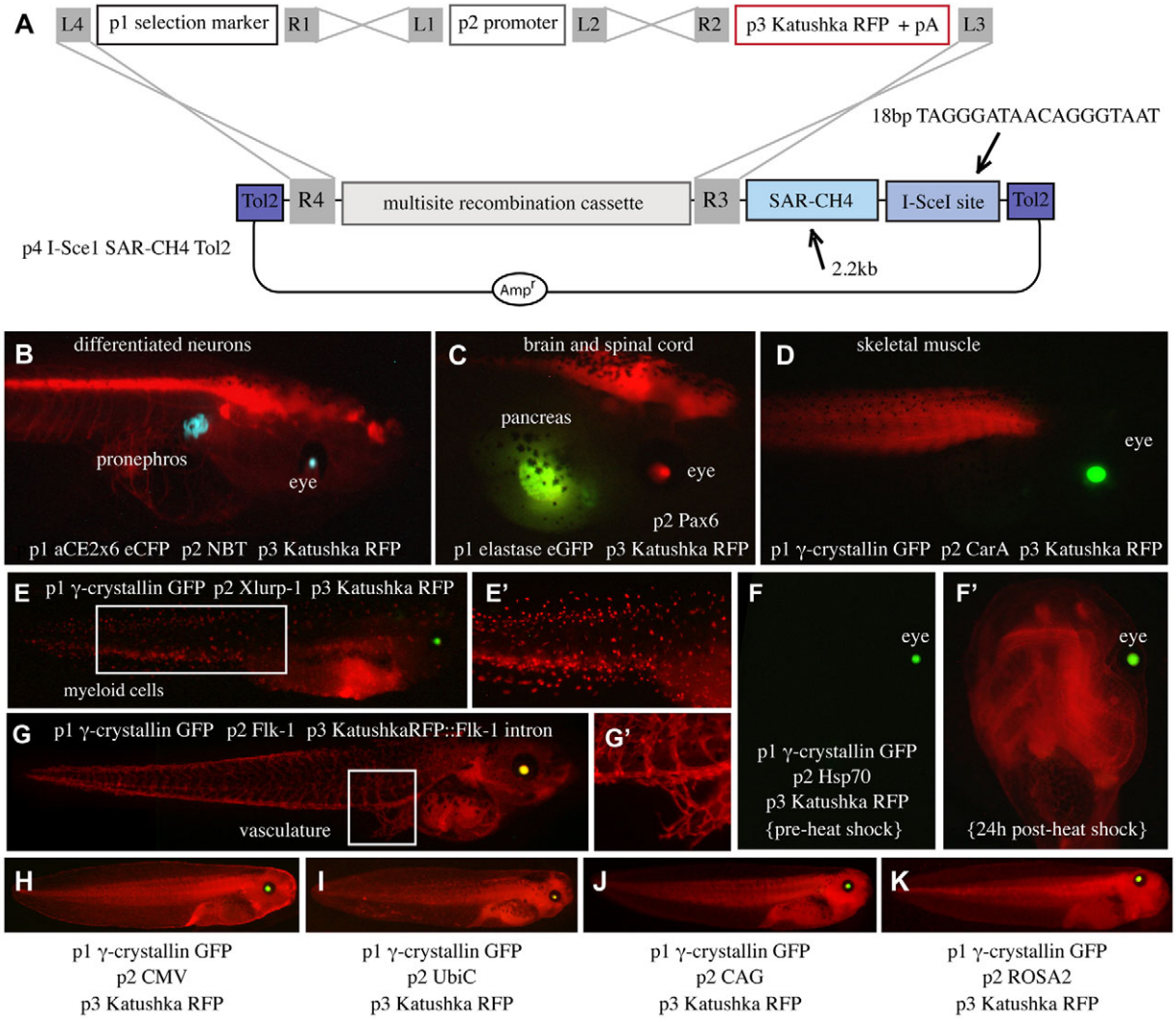

Fig. 2. Incorporation of *Xenopus*-compatible elements into pTransgenesis. (**A**) Schematic of recombination with a *Xenopus*-compatible p4 vector containing a single I-*Sce*I site, Tol2 elements, and SAR-CH4 sequences, p3 Katushka RFP and *Xenopus*-compatible p1 and p2 constructions. (**B-K**) Transgenic tadpoles from the resulting recombinations are shown. Individual p1, p2, p3 constructions and expression domains are written on each panel in white. Images in E′ and G′ are magnified images of the boxed regions in E and G, respectively. Image in F′ shows the induction of RFP from the heat-shocked tadpole in F.

gene' and a 'p4 transgenesis' vector in the presence of recombinase enzyme in vitro produces a final DNA construction that contains these individual elements in a predictable order (Fig. 1B).

The important outcome of this design is that p3 coding sequences, like GFP, which are used repeatedly in experiments across a wide variety of species, are easily coupled to modular p1, p2 and p4 constructions. Hence, we created a p3 coding sequence library that can be shared and utilized in any compatible species. For this, we cloned into the p3 reservoir 16 different fluorescent proteins, sequences for Gal4-UAS binary transgenic approaches, variations of the Cre-recombinase, and coding sequences that allow genetic manipulation in vivo, such as the dominant-negative Fgf receptor construct (Fig. 1C).

Incorporation of *Xenopus* compatible elements into pTransgenesis

To demonstrate the utility of the pTransgenesis cloning framework, we used *Xenopus*, a model which did not possess a recombination-based cloning system. Notably, the *Xenopus* model allows the generation of non-mosaic, fully transgenic organisms in the F0 generation via REMI transgenesis, thus allowing us to rapidly validate pTransgenesis elements using this method (Kroll and Amaya, 1996).

We incorporated three *Xenopus*-compatible selection markers into the p1 selection marker position: the γ-crystallin promoter driving GFP in the lens (Offield et al., 2000), the elastase promoter driving GFP in the pancreas (Beck and Slack, 1999), and the αCE2x6 promoter driving eCFP (enhanced cyan fluorescent protein) in the lens and pronephros (Matsuo and Yasuda, 1992). Into the p2 position, we inserted five previously characterized tissue-specific promoters, one heat shock-inducible promoter, and four ubiquitous promoters (Fig. 2, Table 1). In addition, we created p4 vectors amenable to I-*Sce*I- and Tol2-mediated transgenesis, some containing SAR-CH4 sequences (human interferon-β scaffold attachment region-chicken β-globin DNase I hypersensitive site 4), reported to protect integrated transgenes from positional effects (Fig. 2A, Fig. 1C) (Allen and Weeks, 2005; Ramezani et al., 2003; Sekkali et al., 2008).

Following recombination with selectable markers in p1, Katushka red fluorescent protein (RFP) in p3 and transgenesis elements in p4, as shown in Fig. 2A, we tested the functionality of several p2 promoter constructs by REMI transgenesis in F0 *X. laevis* tadpoles (Fig. 2B-K; Fig. 3D,E, middle panels). In all cases, RFP expression was appropriate to the promoter sequences driving it. We next confirmed the functionality of the p4 I-*Sce*I site by establishing transgenic lines via I-*Sce*I-mediated transgenesis (supplementary material Fig. S1).

The pTransgenesis vector framework allows the rapid testing of uncharacterized DNA sequences for promoter activity owing to a commercially available PCR product cloning kit. This method allows cloning of PCR products directly into to the p2 position of the pTransgenesis framework, allowing immediate recombination upstream of a p3 coding gene and downstream of a desired p1 selection marker (Fig. 3A-C). Using this strategy, we tested functionally the promoters of two genes; *vimentin*, which is highly expressed in glia during *Xenopus* neural development (Fig. 3D, upper panels) (Yoshida, 2001) and *nectin-2*, which is expressed in the superficial layer of the neuroepithelium (Fig. 3E, upper panel) (Morita et al., 2010). The new promoters were found to be capable of driving VenusGFP expression in a pattern similar to the endogenous expression patterns of *vimentin* and *nectin-2* in both the F0 and F1 generation, validating the use of these promoters for the study of neural development (Fig. 3D,E, middle and lower panels).

Also, we generated a large repertoire of fluorescent proteins in the p3 coding sequence position, including 15 fluorescent proteins with peak emissions ranging from 475 nm to 630 nm (supplementary material Fig. S2A) (Belousov et al., 2006; Mizuno et al., 2003). Using the p2 CMV promoter, we validated these 15 fluorescent proteins in p3 by linking these sequences with a p1 γ-crystallin RFP or p1 γ-crystallin GFP construction and created F0 transgenic tadpoles using REMI (supplementary material Fig. S2B,C). Taken together, these experiments validated the use of pTransgenesis framework to create a wide variety of transgenic lines in *Xenopus*.

Conditional transgene expression using pTransgenesis

We adapted the pTransgenesis system to be compatible with two powerful binary transgenic strategies, the Gal4-UAS system, utilized in a variety of model species, including *Drosophila*, zebrafish and *Xenopus* (Brand and Perrimon, 1993; Fischer et al., 1988; Hartley et al., 2002; Scheer and Campos-Ortega, 1999) and the Cre-loxP recombination system.

Compatibility of Gal4-UAS binary transgene expression and pTransgenesis was achieved by generating one construct to express p3 Gal4 under the control of a p2 promoter (selectable by RFP in the lens RFP; supplementary material Fig. S3A) and

Table 1. Partial list of p2 promoters

Construct name	Species of origin	Expression domain	Reference
p2 NBT	*Xenopus laevis*	Differentiated neurons	Huang et al., 2007
p2 Pax6	*Xenopus laevis*	Brain, eye, spinal cord	Hartley et al., 2001
p2 CarA	*Xenopus laevis*	Skeletal and cardiac muscle	Kroll and Amaya, 1996
p2 Xlurp-1	*Xenopus laevis*	Myeloid cells	Smith et al., 2002
p2 Hsp70	*Xenopus laevis*	Heat shock inducible	Beck et al., 2003
p2 Flk-1	*Xenopus laevis*	Vasculature	Doherty et al., 2007
p2 Pax3	*Xenopus laevis*	Neural ectoderm	Our unpublished results
p2 Nectin-2	*Xenopus tropicalis*	Superficial neuroepithelium	This paper
p2 Vimentin	*Xenopus tropicalis*	Neural progenitors/glia	This paper
p2 Sox3	*Xenopus tropicalis*	Ectoderm/neural progenitors	Our unpublished results
p2 Foxi1	*Xenopus tropicalis*	Ionocytes	Our unpublished results
p2 HB9	*Danio rerio*	Motor neurons	Flanagan-Steet et al., 2005
p2 CMV	*Cytomegalovirus* (CMV)	Widespread	Werdien et al., 2001
p2 UbiC	*Homo sapiens*	Widespread	Lois et al., 2002
p2 CAG	CMV and *Gallus gallus*	Widespread	Sakamaki et al., 2005
p2 ROSA26	*Mus musculus*	Widespread	Gross et al., 2006
p2 UAS	*Saccharomyces cerevisiae*	GAL4 inducible	Brand and Perrimon, 1993

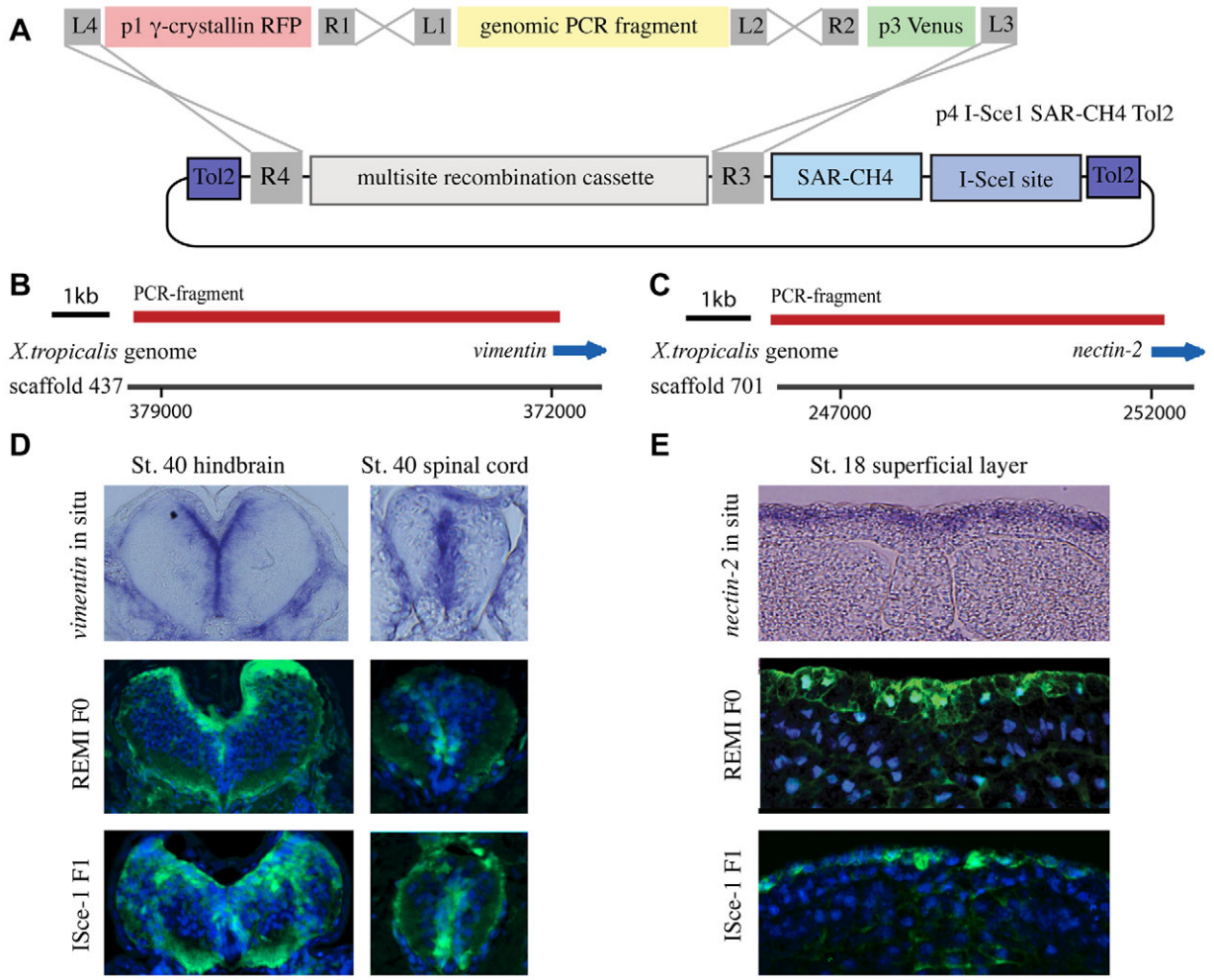

Fig. 3. A highly efficient method of testing promoters and creating transgenic lines using pTransgenesis. (**A**) Genomic PCR fragments are cloned directly into the p2 position, thus allowing the recombination shown. (**B**,**C**) PCR products encoding regions 5′ to *nectin-2* and *vimentin* were generated and tested for transcriptional activity in F0 *X. laevis* and in F1 *X. tropicalis*. (**D**) Transverse sections through the hindbrain and spinal cord of embryos (dorsal side up) stained for endogenous *vimentin* expression (upper panels), or Venus transgene expression (green) in F0 transgenic *X. laevis* (middle panels) and F1 transgenic *X. tropicalis* (lower panels). (**E**) Transverse sections through the neural plate of stage 18 embryos (dorsal side up) stained for endogenous *nectin-2* expression (upper panel), or Venus transgene expression (green) in F0 transgenic *X. laevis* (middle panel) and F1 transgenic *X. tropicalis* (lower panel). Nuclei are stained with DAPI (blue) in middle and lower panels in D and E.

another expressing a p3 transgene of interest downstream of p2 UAS repeats (selectable by GFP in the lens; supplementary material Fig. S3B). Double transgenic lines are easily identified owing to the co-expression of GFP and RFP. We generated a construction expressing Gal4 under the control of the NBT promoter (i.e. expressed in the nervous system; supplementary material Fig. S3C), and VenusGFP or a dominant-negative form of the FGFR1 (XFD) tagged with VenusGFP downstream of p2 UAS (supplementary material Fig. S3D,E). VenusGFP expression was only observed in the nervous system in the double transgenics showing that the binary Gal4-UAS system works (supplementary material Fig. S3F). However, double-transgenic embryos expressing XFD-VenusGFP showed a consistent tail elongation phenotype, suggesting a role for FGF signalling in the nervous system during tail elongation (supplementary material Fig. S3G,H). Importantly, the tail and body length phenotype was also observed in the F1 generation by crossing F0 NBT:Gal4 and UAS:XFD-VenusGFP founder frogs (supplementary material Fig. S3I-K). These data showed the use of pTransgenesis to investigate the role of signalling pathways under strict spatial or temporal control. A more detailed examination of this phenotype is beyond the scope of this report and will be addressed in a separate study.

Another widely used binary transgene approach is via Cre-loxP recombination, in which a floxed transgene is excised by Cre, resulting in the conditional gene activation of another transgene, lying downstream of the distal loxP site (Lakso et al., 1992; Mosimann et al., 2011; Werdien et al., 2001). Using the pTransgenesis resource, we have successfully established via I-*Sce*I transgenesis the first transgenic *X. tropicalis* line amenable to Cre-loxP recombination. The line expresses cyan fluorescent protein (CFP) ubiquitously in the absence of Cre recombinase (supplementary material Fig. S4A-E), but in the presence of Cre, recombination leads to the replacement of CFP with RFP. We demonstrated the functionality of this transgenic line, by injecting synthetic Cre-recombinase mRNA into one of the two blastomeres

at the two-cell stage in the F2 generation of this line, causing the expected hemispheric RFP expression by the neurula stage (supplementary material Fig. S4F-N). This transgenic line will be valuable in long-term fate-mapping studies in *X. tropicalis*.

Expanding the scope of pTransgenesis

The pTransgenesis resource is designed such that the p3 coding sequence library can be easily adapted to other model species, namely, by including species-compatible selection p1 markers and p2 promoters and, if necessary, species-compatible p4 transgenesis constructs (Fig. 4A). In this section, we outline experiments showing the compatibility of pTransgenesis in *Drosophila*, zebrafish, and HeLa cells.

For zebrafish, we first confirmed that the p1 γ-crystallin RFP, p2 CMV construction, and a p3 fluorescent protein (Midori-Ishi Cyan) properly express in this model organism. We injected a vector with recombined p1 γ-crystallin RFP, p2 CMV and p3 Midori-Ishi Cyan constructs with or without *Tol2* mRNA. We found, like others, that the p1 γ-crystallin RFP screenable marker was functional in the zebrafish eye lens (Fig. 4B, arrow), and the CMV promoter was able to drive widespread Midori-Ishi expression in the fish body (Fig. 4B) (Davidson et al., 2003). Furthermore, we cloned the zebrafish HB9 promoter (Flanagan-Steet et al., 2005) into the p2 position, recombined this with the p1 γ-crystallin RFP screenable marker and p3 VenusGFP (supplementary material Fig. S5A,B) and confirmed its activity in zebrafish using Tol2-mediated transgenesis

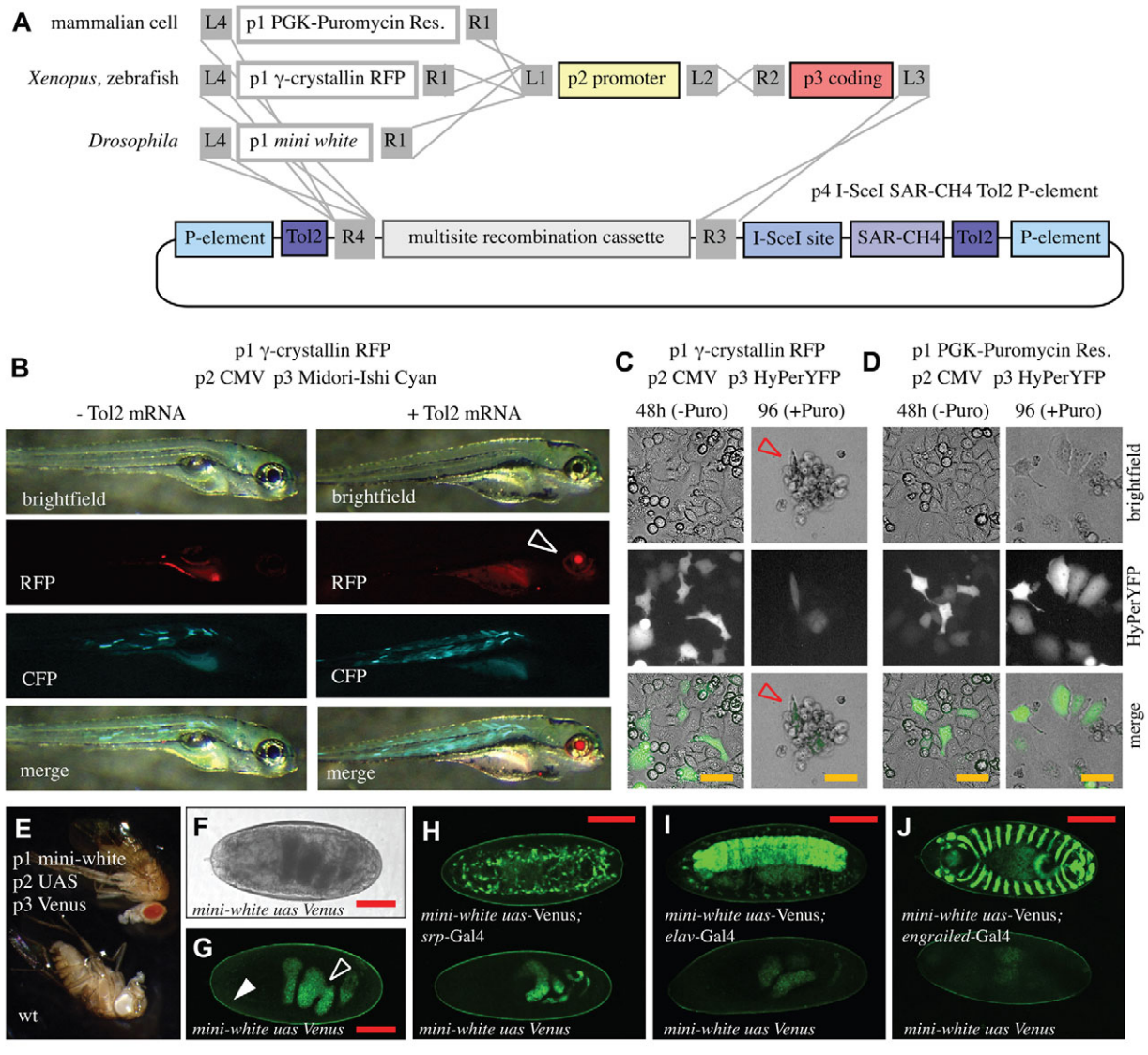

Fig. 4. pTransgenesis in various models. (**A**) Schematic showing plasmid recombinations yielding plasmids compatible with transgenesis in HeLa cells, *Xenopus*, zebrafish and *Drosophila*. (**B**) Images of zebrafish injected with the indicated p1, p2, p3 and p4 elements with or without *Tol2* mRNA. Open arrowhead points to activity of the p1 γ-crystallin RFP marker. (**C**,**D**) Results from HeLa cell transfections and puromycin (Puro) selection using pTransgenesis-engineered constructions. Red open arrow indicates dying cells. (**E**) *Drosophila melanogaster* engineered with the indicated pTransgenesis constructs versus wild type. (**F**,**G**) Phase contrast and fluorescence image of pTransgenesis-engineered *Drosophila* embryo. Open and closed arrowheads indicate gut and cuticle autofluorescence, respectively. (**H-J**) Confocal images from the indicated Gal4 crosses. (H) *srp*-Gal4, stage 16 embryo, GFP-labelled hemocytes. (I) *eval*-Gal4, stage 16 embryo, GFP-labelled CNS. (J) *engrailed*-Gal4, ventral view of stage15 embryo during dorsal closure process, GFP-labelled *engrailed* domain segments. Scale bars: 25 μm in C,D; 100 μm in F-J.

(supplementary material Fig. S5C-E) and in *Xenopus* using REMI transgenesis (supplementary material Fig. S5F). Notably, we generated a transgenic zebrafish line with this construct using Tol2-mediated transgenesis, showing the functionality of the p4 Tol2 elements in the pTransgenesis system (supplementary material Fig. S5D,E).

To test the pTransgenesis system in mammalian cells, we created a p1 PGK:Puromycin Resistance cassette, which allows selection following application of puromycin to culture media. We linked this selection marker, or the p1 γ-crystallin RFP construction (negative control), to the p2 CMV and p3 HyPerYFP constructs (Fig. 4C,D). Expression of HyPerYFP was observed in ~50% of cells 48 hours after transfection using either construct (Fig. 4C,D, middle left panels). However, following addition of puromycin in the culture media, only the cells bearing the p1 PGK:PuroR cassette survived and continued to express HyPerYFP (Fig. 4D, middle right panel). Cells were successfully cultured for over a month under selection conditions.

We also tested the pTransgenesis resource in *Drosophila*. For this purpose, we created a p1 *mini-white* plasmid, which allows selection for red eyes (Tang and Sun, 2002) and a p4 vector containing sequences enabling P-element mediated transgenesis (Rubin and Spradling, 1982). By linking the p1 *mini-white*, p2 UAS, p3 VenusGFP and p4 P-element constructions, we created transgenic flies via P-element mediated transgenesis selectable by their red eyes (Fig. 4F). We crossed this pTransgenesis-engineered *Drosophila* line with previously established transgenic *engrailed*-Gal4, *srp*-Gal4 and *elav*-Gal4 driver lines (Bruckner et al., 2004; Landgraf et al., 1999; Millard and Martin, 2008) (Fig. 4G-K). Control stage 15-16 embryos lacking *Gal4* expression showed no fluorescence besides the expected autofluorescence of the gut and cuticle (Fig. 4H, arrowheads) (Bainbridge and Bownes, 1981). By contrast, embryos expressing *Gal4* showed the expected Gal4-UAS-driven VenusGFP expression in hemocytes (*srp*-Gal4, Fig. 4I), motor neurons (*elav*-Gal4, Fig. 4J) or *engrailed* domain segments (*engrailed*-Gal4, Fig. 4K).

Together, these data show that pTransgenesis vectors are compatible with other vertebrate, invertebrate and mammalian cell culture models. Compatibility between pTransgenesis vectors and previously established resources using Multisite Gateway cloning depends on the plasmid 'RL'-site design of each resource. For example, the previously reported R4-R3 ROSA26 locus targeting (Kappas et al., 2008) and Tol2-transposon-containing vectors (Kwan et al., 2007; Villefranc et al., 2007) function as p4 vectors in the pTransgenesis system. Moreover, the promoters generated in other zebrafish studies are compatible in the p2 promoter position (Fisher et al., 2006). However, unlike earlier projects, pTransgenesis is not designed for the rapid creation of fusion proteins (Akbari et al., 2009; Villefranc et al., 2007) and, thus, these applications are better served by their original resources.

In conclusion, the pTransgenesis resource markedly streamlines the process of creating DNA constructions for engineering transgenic organisms and provides a straightforward framework for the distribution of constructs across various model organisms. An additional salient feature of the pTransgenesis resource is the substantial reservoir of constructs, which readily allows a wide variety of experimental approaches. Finally, the cross-species utility of pTransgenesis will allow researchers to evaluate more easily whether biological processes, which are increasingly being studied with a view to application, are evolutionarily conserved.

Acknowledgements
We thank Sarah Woolner, Tim Mohun, Shane Woods, Matthew Ronshaugen, Jonathan Slack, Caroline Beck, Robert Grainger, Scott Fraser, Igor Samokhvalov, Koichi Kawakami, Makoto Ikeya, Mototsugu Eiraku, Richard Hawley, Ali Ramezani, Paul Krieg, Lyle Zimmerman, Roberto Mayor and John Gurdon for their kind donations of reagents.

Funding
We thank the Wellcome Trust for their support of the European *Xenopus* Research Centre [M.J.G., E.A.]. This work was funded by the Healing Foundation [N.R.L., R.P., Y.C., E.A.]; the National Science Foundation [N.R.L.]; the Henry Luce Foundation [N.R.L.]; the Winston Churchill Scholarship Foundation [N.R.L.]; and the Wellcome Trust [N.P., E.A.]. K.D. is a Research Councils UK Research Fellow and N.P. is a Wellcome Trust Senior Research Fellow. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066498/-/DC1

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