SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
EUKARYOTE
TRANSPOSITION
TRANSGENIC ORGANISMS

Received
25 June 2013

Accepted
12 November 2013

Published
3 December 2013

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Enhanced efficiency of P-element mediated transgenesis in *Drosophila*: Microinjection of DNA complexed with nanomaterial

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The efficiency of genetic transformation technology to generate stable transgenics depends upon the successful delivery of plasmid DNA in embryonic cells. The available gene vectors facilitate efficient plasmid DNA delivery to the cellular milieu but are exposed to nuclease degradation. Recent *in vitro* studies suggest encapsulation of plasmid DNA with nanomaterial(s) for better protection against nucleases. Therefore, in this study, we tested if complexing of free plasmid DNA with linear polyethylenimine (LPEI, 25 kDa) based nanoparticle (LPN) enhances the efficiency of transformation (transgenesis) by using *Drosophila* based germ-line transformation technology. Here, we show that the LPN-DNA complex not only enhances the efficiency of this transgenic technology at a DNA concentration of 0.04 μg/μl but also reduces the DNA quantity required to generate transgenics by ten folds. This approach has potential applications for other types of transgenesis and nucleic acid injection methods in *Drosophila* as well as other popular genetic model systems.

Transgenic organisms are the key to various biological questions. Genetic transformation technologies to generate stable transgenics owe their success to various physical and biological methods available for delivering transgenes to cells. Of the various methods, microinjection is a popular option for delivering vector DNA to the cells[1]. Vector DNA, delivered into the cell, translocates to the nuclei for its ultimate integration (either random or site specific) into the genomic DNA. However, the free vector DNA circulating in the cellular milieu is exposed to nuclease degradation[2]. Additionally, the ability of DNA to cross the cell membrane, its timely escape from the endosome, and size as well as charge of DNA are critical determinants of DNA entry into the nucleus[3]. The available gene vectors facilitate efficient vector DNA delivery into the cell but are exposed to nuclease degradation. In this perspective, it is important to note that higher transfection efficiency *in vitro* has been achieved by encapsulation of DNA with organic/inorganic macro, micro and nanomaterial based carriers[4–6]. In addition, nanomaterial based DNA encapsulation and delivery is emerging as a promising prospect for *in vivo* gene delivery. In the last few years, linear polyethylenimine (LPEI) emerged as an attractive cationic polymer for efficient gene delivery *in vitro* and *in vivo* due to its negligible cytotoxicity[7,8]. Recently, Goyal *et al*[9] have shown enhanced DNA delivery *in vitro* when free DNA is complexed with LPEI based nanoparticles (LPN). Taking cues from these technologies, we hypothesized that complexing of the free plasmid DNA with a nanomaterial and subsequent delivery/release to the cellular milieu might lead to increased efficiency of the *in vivo* transgenic technology. To test this hypothesis, we used *Drosophila* based transgenic technology, which has been exploited by the research community for decades to address diverse biological questions.

In *Drosophila*, germ-line transformation[10,11] is a popular genetic technology used to generate transgenics. In addition, numerous gene vectors are available, based on the principle of transposition or site specific recombination to facilitate the delivery and integration of transgene into genomic DNA[10–15]. Among these, P-element-based vector constitutes a highly successful system for routinely creating transgenic strains of *D. melanogaster*[15]. This involves the insertion of gene of interest (transgene) into the P-element based vector and microinjection of this DNA along with a helper plasmid that encodes the transposase into the germ cells of the early *Drosophila* embryo. Within the germ cells, transposase encoded by the helper plasmid facilitates transposition of the transgene from P-element based vector into the genomic DNA of the fly. This technology generally offers 25–40% success rate in

the generation of transgenics[16]. Therefore, in the present study, we tested if complexing of the P-element based vector DNA and helper plasmid with LPN would help to enhance the efficiency of this transgenic technology. We show here that such LPN-DNA nanoplex not only enhances the efficiency of the technology but also reduces the required DNA quantity by ten folds to generate transgenics.

Results

We successfully synthesized LPN [through 11% cross-linking of LPEI (25 kDa) with 1,4-butanediol diglycidyl ether (BDE), see supplementary Fig. S1 online] and characterized these particles as in Goyal *et al*[9]. The average particle size is represented in Table 1. Subsequently, pDNA [a mixture of pUAST-GFP or sympUAST-CG33943[17] with helper plasmid (Δ2–3 -transposase[18])] was complexed with the LPN synthesized. To generate the LPN-pDNA (LPN-pUAST or LPN-sympUAST) complex, it was critical to determine the ratio at which LPN not only retards the mobility of pDNA but also protects the same from nucleases. In the DNA retardation assay, we observed that mobility of pUAST on agarose gels was completely retarded at a pDNA:LPN ratio of 1 : 0.6 and above (Fig. 1). We observed only partial retardation of pUAST mobility below this ratio (Fig. 1, lane 0.3). Similar trend was observed in DNA retardation assays with sympUAST (see Supplementary Fig. S2 online). From the DNase I protection assay, it is clear that the LPN free pDNA was degraded completely by DNase I within 2 h (Fig. 2A, Lane pDNA alone). In contrast, complexing of pDNA with LPN effectively provided protection in a ratio dependent manner with maximum intensity of pDNA at 1 : 3, when these complexes were incubated with DNase I for 2 h at 37°C (Fig. 2A, lanes 0.5–3 & Fig. 2B). Similar trend was observed in DNase I protection assays with sympUAST (see Supplementary Fig. S3 online). Based on these data from retardation and protection assays, we inferred 1 : 3 (pDNA:LPN) as the working ratio for microinjections. Keeping this ratio constant, we prepared the LPN-pDNA complexes with varied quantities (2, 4, 6, 12 μg) of pDNA. The average hydrodynamic diameter of LPN-pDNA complex, as measured by DLS (Dynamic Light Scattering using Zetasizer), is represented in Table 1. However, over a period of time, we observed that the LPN-pDNA complex precipitated out of the solution at concentrations of 4 μg and above. We injected pDNA alone or LPN-pDNA complexes into embryos of w^{1118} flies of *Drosophila melanogaster* and carried out microinjections following the standard protocol[10,11]. The present study included five groups for LPN-pUAST based microinjections: three nanoparticle based and two control groups. Embryos injected with LPN-pUAST complexes at a pDNA concentration of 0.04, 0.08 or 0.4 μg/μl formed the nanoparticle groups. Embryos injected with pUAST (along with helper) alone at a concentration of 0.4 μg/μl (as a positive control) or 0.04 μg/μl (to match the lowest concentration in the nanoparticle based group) served as controls. In the control groups, we observed that the efficiency of transgenesis depended on the concentration of the pDNA injected: 25% and 0.5% of transgenesis in pUAST groups containing 0.4 μg/μl and 0.04 μg/μl, respectively

Table 1 | Average particle size of LPN alone and LPN + pDNA complexes

Sample	Average particle size in nm (Mean ± SE) (in injection buffer)
LPN	239.6 ± 2.04
pUAST:LPN (2 μg : 6 μg)	221.3 ± 1.56
pUAST:LPN (4 μg : 12 μg)	409.7 ± 4.91
pUAST:LPN (6 μg : 18 μg)	310.1 ± 8.62
pUAST:LPN (12 μg : 36 μg)	521.3 ± 2.82
pUAST:LPN (20 μg : 60 μg)	534.4 ± 17.78
sympUAST:LPN(2 μg : 6 μg)	240.7 ± 2.02

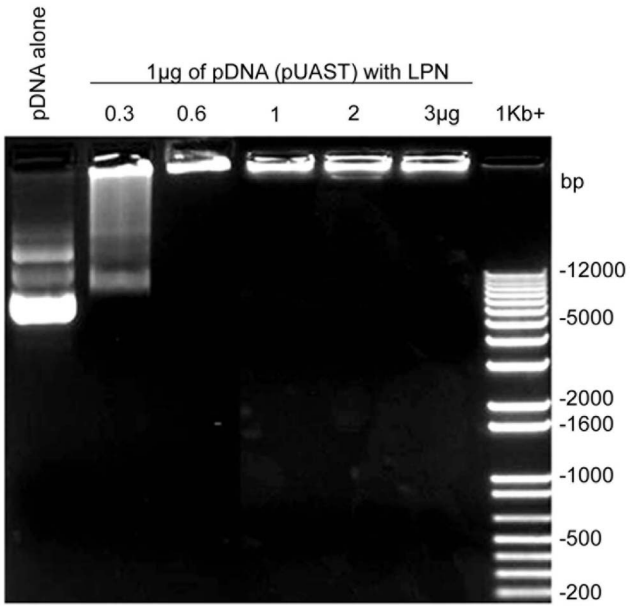

Figure 1 | Retardation of mobility of pDNA complexed with LPN. Lane pDNA alone represents free plasmid DNA without LPN. Lanes 0.3 to 3 μg represent the corresponding amounts of LPN with 1 μg of pDNA (pUAST in this case). Lane 1 Kb^+ represents 1 Kb plus DNA ladder (Life Technologies, USA; range 100–12000 bp).

(Table 2). The efficiency of transgenesis observed in the positive control group in the present study is comparable to that generally observed in P-element mediated transgenesis[16]. In contrast, we observed that LPN-pUAST complexes with pDNA concentration of 0.08 or 0.4 μg/μl did not yield transgenics (Table 2). However, this is not surprising given our observation above regarding precipitation of LPN-pUAST complexes at these concentrations. Interestingly, we found that approximately 30% of adults emerging from embryos injected with LPN-pUAST complexes with a pDNA concentration of 0.04 μg/μl yielded transgenics (Table 2). To determine if similar efficiency can be observed with other genes, we injected embryos with LPN-sympUAST complex or sympUAST alone (at a pDNA concentration of 0.04 μg/μl.) We observed that 27% of adults emerging from embryos injected with LPN-sympUAST complexes (at a concentration of 0.04 μg/μl) yielded transgenics (Table 2). The efficiency of transgenesis observed in LPN-sympUAST or sympUAST alone is comparable to those in equivalent pUAST groups (Table 2). This observed efficiency is significantly higher than that observed with similar concentration of pDNA without LPN ($p < 0.001$ with pUAST groups and $p < 0.003$ with sympUAST groups, according to Student's T-test; Table 2). Moreover, the observed efficiency in these groups is statistically at par with that of the positive control group ($p > 0.05$). Please refer to supplementary Table S1 online, for details on the number of embryos injected, larvae hatched, adults emerged, fertile/infertile G_0 crosses and the percentage of transgenesis for various replicates under each group.

Discussion

In this study, we tested if complexing of plasmid DNA with LPEI based nanoparticle (LPN) enhances the efficiency of the *in vivo* transformation (transgenesis) by using *Drosophila* based germ-line transformation transgenic technology. For in vivo studies, it is essential to use reagents that are non-toxic. We selected LPN for this study based on two observations. First, LPN synthesized through cross-linking of LPEI with BDE (which involves conversion of secondary amino-groups in LPEI into tertiary amino-groups) retained the

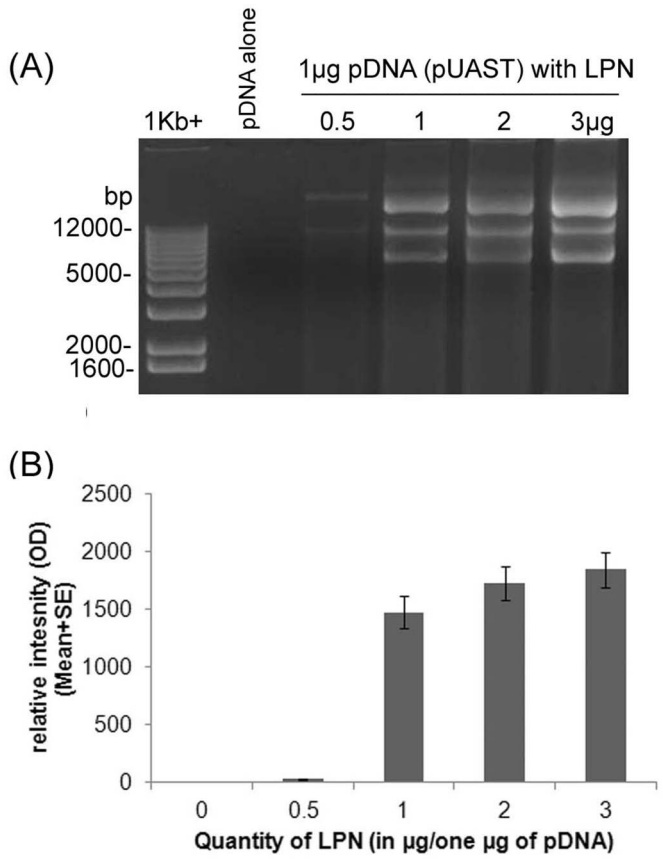

Figure 2 | Protection for pDNA when complexed with LPN against DNase I. Protection was assayed through qualitative (A) and semi-quantitative (B) measures. Samples were run on 0.8% agarose gels and panel A represents the DNA levels without LPN (lane pDNA alone) and with LPN (lanes 0.5–3 µg) at different ratios (as represented on top of lanes) when subjected to DNase I digestion for 2 h at 37°C. Lane 1 Kb^+ represents 1 Kb plus DNA ladder (Life Technologies, USA; range 100–12000 bp). Panel B represents the semi-quantitative analysis of cumulative relative intensities of DNA profiles observed without LPN (0 µg) and with LPN (0.5–3 µg) through densitometry using quantity one software (Bio-Rad, USA). Data provided is the average of three replicates along with standard error (Mean ± SE) for each ratio.

non-toxic nature of the base polymer[9]. Second, exposure of *Drosophila* to LPN during development neither caused mortality nor induced any cellular stress/damage in the exposed organisms[9]. In addition, the rate of development and morphology of flies exposed to LPN were similar those in controls[9].

We analyzed the efficiency of transgenesis in terms of percentage by scoring the number of transformants (in terms of red/orange/yellow eyed flies) in the adults from the injected w^{1118} embryos. The number of transformants obtained in the present study represents the number of crosses that yielded transformants, reflecting independent integration events. In the present study, we observed the efficiency of transgenesis in LPN-pUAST group (with effective pDNA concentration of 0.04 µg/µl) is at par with that of the positive control group (pDNA alone with effective concentration of 0.4 µg/µl). To rule out the possibility of enhanced transgenesis due to gene/sequence specific interactions of LPN, we have carried out additional injections with a modified pUAST plasmid (sympUAST) consisting of a different gene (CG33943[17]). Similar enhancement of efficiency even in this case rules out any such LPN-gene specific interactions. Thus, complexing of pDNA with a nanoparticle (LPN in this case) indeed reduces the required effective pDNA concentration generally used for germ-line transformation by 10 folds. This indeed is helpful not only to reduce the requirement of the molecular reagent (pDNA in this case) but also relieves the researcher from generating large quantities of highly concentrated DNA required for the regular transformation protocol.

Nanotized linear polyethylenimine is known to deliver significant amounts of DNA into cells through endocytic routes and the associated pDNA escapes within 4 h from the endosomes[19,20]. Further, complexing of DNA with nanoparticles appears to provide superior protection to the DNA from circulating nuclease activity[21]. Therefore, microinjection of pDNA along with nanomaterial is likely to provide better transgenesis efficiency when compared to that of pDNA alone. Consistent with this, the efficiency of transgenesis in LPN-pDNA group (with effective pDNA concentration of 0.04 µg/µl) was 15–60 times (depending upon the plasmid in use) more than that observed with similar concentration of pDNA alone. These results indicate that delivery of pDNA by complexing with a nanoparticle such as LPN enhances the efficiency of the P-element mediated transgenic technology. The enhanced efficiency might be a consequence of the better protection of pDNA by LPN against circulating nucleases. Alternatively, it is also possible that the interaction between positively charged LPN and the negatively charged pDNA[22,23] might have facilitated spatial/temporal delivery/availability in the cellular milieu, thereby increasing the efficiency of transgenesis. Nevertheless, the increased efficiency of P-element based transgenic technology by 10-folds through microinjection of pDNA complexed with LPN in the present study indeed supports our hypothesis. However, given our observation regarding the precipitation of LPN-pDNA at higher concentrations, the required nanoparticle concentration should be tailored according to the pDNA used. Further, as with other P-element mediated germ-line transformations, transformants should be tested to ascertain the number of insertions of transgene in a given transgenic line.

The nanoparticle based microinjection concept reported in this study has potential application to not only for *Drosophila* but also for other model organisms ranging from lower invertebrates (such as *C. elegans*) to higher vertebrates, including mammals. The efficiency of

Table 2 | Efficiency of transgenesis through microinjections involving pDNA alone or LPN + pDNA complexes in germline-tranformation of *Drosophila melanogaster*

Group	Effective pDNA concentration (µg/µl)	% transgenesis (Mean ± SE)	% of DNA compared to standard (STD)
Positive control (20 µg pUAST) (STD)	0.4	25.34 ± 1.91[a]	100%
pUAST:LPN (20 µg : 60 µg)	0.4	0 ± 0.00[b]	100%
pUAST:LPN (4 µg : 12 µg)	0.08	0 ± 0.00[b]	25%
pUAST:LPN (2 µg : 6 µg)	0.04	30.20 ± 3.73[a]	10%
pUAST alone (2 µg; control)	0.04	0.59 ± 0.59[c]	10%
sympUAST alone (2 µg)	0.04	2.09 ± 1.10[d]	10%
sympUAST:LPN (2 µg : 6 µg)	0.04	27.33 ± 3.65[a]	10%

The proportion data were arcsine transformed and the pair wise comparisons were done through Student's T-test. Values with same alphabet are statistically non-significant ($p > 0.05$) and values with different alphabet are differ significantly ($p < 0.003$), according to Student's T-test.

transgenesis and mRNA, dsRNA injection methods reported for these models may be enhanced by a simple modification to the standard protocols through complexing of pDNA with the LPN reported here. Further, the time taken for the whole procedure, from injection to established transgenic stocks, is similar to that of traditional microinjection based transgenesis methods. Finally, our study highlights the potential of *Drosophila* as an alternate model for testing the *in vivo* efficiency of DNA delivery reagents, thereby adding another feature to the repertoire of the versatile *Drosophila* model system.

Methods

Preparation and characterization of Linear Polyethylenimine nanoparticles (LPN). Linear polyethylenimine (LPEI, 25 kDa) based nanoparticles (LPN) were synthesized by cross linking with 1,4-butanediol diglycidyl ether (BDE) and subsequently characterized as in Goyal et al[9]. Briefly, to an aqueous solution of pre heated LPEI at 45°C (100 ml, 1 mg/ml), a solution of (BDE; 66.48 µl, 1 µl/ml in water, for the attempted 30% crosslinking) was added drop wise over a period of 30 min with continuous stirring. The stirring was continued overnight at the same temperature and then the volume of the reaction mixture was reduced to one third of the total volume on a rotary evaporator. The remaining solution was subjected to dialysis against water for 72 h with changes at every 24 h. Thereafter, the solution was concentrated in a speed vac to obtain a white residue of nanoparticles (~85% yield). The nanoparticles were then characterized through DLS by using a zetasizer (Malvern, UK).

Preparation of plasmid DNA. pUAST-GFP plasmid DNA (referred to as pUAST), Δ2–3 (transposase[18]) plasmid DNA and symPUAST-CG33943 plasmid DNA (Ravi Ram and Wolfner[17]; referred to as sympUAST) were independently transformed into DH5α strain of *E. coli* and transformants were selected using ampicillin marker. From these colonies, endotoxin free DNA was prepared using the Endofree Maxiprep kit (Qiagen, USA) following manufacturer's protocol. Subsequently, 15 µg of pUAST or sympUAST DNA were mixed, independently, with 5 µg of Δ2–3 DNA and the contents were precipitated. The precipitated DNA (pDNA) was resuspended in 50 µl of 1× injection buffer containing KCl (0.5 mM KCl; 0.01 mM, $NaPO_4$ buffer pH7.5) for use in microinjections involving pDNA alone or containing only 0.01 mM $NaPO_4$ pH7.5 for generating LPN-pDNA complexes and subsequent microinjections. We omitted KCl from the injection buffer as the LPN-pDNA complexes were unstable and precipitated in the presence of KCl.

Determination of Nanoparticle to plasmid ratio (N/P ratio) based on DNA retardation assay and DNase I protection assay. We determined the ratio at which LPN and pDNA to be complexed based on DNA retardation assay and DNase I protection assay (as in Goyal et al[9]). For DNA retardation assay, pDNA was mixed with LPN at ratios of 1 : 0.3, 1 : 0.6, 1 : 1, 1 : 2, or 1 µg : 3 µg (pDNA:LPN) and the final volumes were made up to 20 µl using distilled water. The resulting samples were gently vortexed and incubated at 25 ± 1°C for 30 min. Subsequently, samples were electrophoretically separated on 0.8% agarose gels and the DNA mobility patterns were documented using UV transilluminator based gel documentation system (Bio-Rad, USA). For DNase I protection assay, pDNA was mixed with LPN at ratios of 1 : 0.5, 1 : 1, 1 : 2, or 1 µg : 3 µg (pDNA:LPN) and complexes were made as above. Subsequently, these LPN-pDNA complexes (10 µl) were mixed with RNase free DNase I (Qiagen, USA) (10 µl, equivalent to one kunitz unit of DNase I in RDD buffer from Qiagen RNAse free DNase I set) and incubated for 2 h at 37°C. Subsequently, DNase I was inactivated by adding 5 µl of 100 mM EDTA and incubating the mixture at 75°C for 10 min. DNA bound to the LPN was released by incubating the mixture with 10 µl of heparin (5 mg/ml) for 2 h at 25 ± 1°C. Subsequently, samples were electrophoresed on 0.8% agarose gels and the relative intensity of pDNA released from complexes was estimated by densitometry using quantity one software (Bio-Rad, USA). Assays were repeated for at least three times and values given are Mean ± Standard Error (SE).

Formation of nanoparticle/pDNA complexes. To prepare the LPN-pDNA complex, pDNA was added to LPN at ratio of 1 : 3 as described above and the final volume was made up to 50 µl with 1× injection buffer. Keeping this ratio constant, complexes were formed with varied quantities (2, 4, 6, 12 µg) of DNA and the resulting samples were gently vortexed and incubated at room temperature for 30 min. Subsequently, the hydrodynamic diameter of LPN-plasmid DNA complexes were measured by DLS in triplicates using Zetasizer as described in Goyal et al[9].

Preparation of nanoparticle/DNA complexes for microinjection. For microinjection, 20 µl of LPN-pDNA complex was mixed with 2 µl of food color [Carmoisine E122, (Sigma, USA, Cat. No. 52245), 2 mg/ml]. Five microlitres of this mixture was loaded onto the microinjection needle (Eppendorf, USA) and fitted onto the micromanipulator (Eppendorf, USA).

Generation of Drosophila transgenics. To generate *Drosophila* transgenics, we injected LPN-pDNA complex or pDNA alone into embryos of w^{1118} flies of *Drosophila melanogaster*. We followed the steps described in the traditional microinjection protocol[10,11]. Abundant batches of eggs synchronized in age were collected from w^{1118} flies as in Ravi Ram and Wolfner[17]. Briefly, 300 adults from fresh w^{1118} cultures were transferred into bottles containing small petri plates filled with grape juice-agar food. The first hour's collection was discarded, to avoid those eggs retained by females in anticipation of fresh food. Thereafter, egg collection plates were replaced with new ones at 30 min intervals. To prepare the eggs for microinjection, the outer covering of egg, namely chorion, was manually removed using a sticky ball and these dechorinated eggs were arranged in a vertically linear fashion on a slide with double stick tape (Scotch 665). After optimal dehydration, embryos were covered with a layer of halocarbon oil 700 (Sigma, USA) and the LPN-pDNA complexes or pDNA alone were injected into these eggs under the inverted phase contrast microscope (Nikon, Japan) with the help of micromanipulator (Eppendorf, USA). Approximately, one picolitre was injected into each embryo and about 150–250 embryos were injected for each concentration group. The present study included five groups: three nanoparticle based and two control groups. Embryos injected with LPN + pDNA complexes at a pDNA concentration of 0.04, 0.08 or 0.4 µg/µl formed the nanoparticle groups. Embryos injected with pDNA alone at a concentration of 0.4 µg/µl (as a positive control) or 0.04 µg/µl (to match the lowest concentration in the nanoparticle based group) served as controls. Embryos injected with LPN-sympUAST or sympUAST alone at a pDNA concentration of 0.04 µg/µl served as additional controls for gene specific interactions of LPN. Subsequently, slides containing injected embryos were placed in a petridish containing the moist tissue paper at 22 ± 1°C. After 24–28 h, larvae were collected from these slides using a fine needle and transferred to a vial containing fresh food. Each eclosed adult was crossed to the opposite sex partner from w^{1118} stock. After 10–12 days, next generation was screened for the red/orange/yellow eyed flies, which are transformants. All the experiments were replicated three times per group and each group contained 150–250 embryos per replicate. The efficiency of transgenesis in terms of percentage was determined by scoring the number of transformants yielding crosses involving adults from the injected embryos. The percentage data were transformed into arcsine values through arcsine square root transformation and the differences, if any, between the control and nanogroups in the efficiency of transgenesis was statistically analyzed through Student's T-test.

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Acknowledgments

The authors are thankful to Prof. LS Shashidhara, IISER, Pune, India, for kindly providing the pUAST-GFP and helper plasmids (P{Δ2–3}), to MF. Wolfner, Cornell University, USA for providing sympUAST-CG33943 and to Dr. BN Singh, CSIR-CDRI, Lucknow, India, for extending Microbiology facility to perform bacterial transformation. The assistance rendered by Ms. Vandana Sharma during certain microinjections is highly appreciated. Financial support from Council for Scientific and Industrial Research (CSIR), New Delhi, to M.S., D.K.C., K.R.R., K.C.G. and from University Grants Commission (UGC), New Delhi, to R.G. is gratefully acknowledged. This is CSIR-IITR communication 3166.

Author contributions

K.R.R., K.C.G. and D.K.C. designed the experiments. K.R.R., M.S. and R.G. carried out experiments. M.S., R.G., K.R.R., K.C.G. and D.K.C. analyzed the results. All authors wrote and reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sonane, M., Goyal, R., Chowdhuri, D.K., Ram, K.R. & Gupta, K.C. Enhanced efficiency of P-element mediated transgenesis in *Drosophila*: Microinjection of DNA complexed with nanomaterial. *Sci. Rep.* **3**, 3408; DOI:10.1038/srep03408 (2013).