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Supplementary online material

Transgenesis procedures in *Xenopus*

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X-omics Transgenesis Workshop Report

Material and methods

Harvesting eggs and in vitro *fertilization*

Eggs were obtained from adult female frogs by hormone-induced egg laying and *in vitro* fertilization using standard methods. Briefly, frogs were primed with 10 units of hCG (human chorionic gonadotropin) 24–72 h prior to the injection experiments. The females that had responded to the priming (red cloaca) were preferentially selected and injected with 300–500 units of hCG depending on their mass. For the purpose of the workshop, 30 females coming from three different breeding centres (Rennes, France; Nasco, U.S.A.; Laboratoire Evolution et Développement, Université Paris Sud, France) were used. A combination of females from different sources was made for each experimental procedure and frogs were exchanged among the training platforms to avoid bias in egg quality. For each session, two males were killed and testes were pooled to prepare a sperm suspension used for both the meganuclease and integrase procedures. For the REMI procedure, a frozen sperm nuclei preparation previously tested in transgenic experiments was used.

All experiments were approved by the Direction Départementale de Services Vétérinaires de l'Essonne, Evry, France.

Plasmids used

The CMV-EGFP-DI-*attb* and the pCMV-GFP3-SceI constructs were used for the integrase and meganuclease procedures respectively (Allen and Weeks, 2005; Pan et al., 2006). The pCSGFP3 containing a CMV (cytomegalovirus) promoter driving GFP3 expression was provided by Professor Enrique Amaya (The Healing Foundation Centre, Faculty of Life Sciences, University of Manchester, Manchester, U.K.) and used for the REMI method. Thus all the constructs used for the transgenic procedures contained a CMV–GFP reporter construct that gives a ubiquitous expression of the reporter gene.

Transgenesis Methods

The transgenic procedures were performed as previously described by Pan et al. (2006), Allen and Weeks (2005) and Amaya and Kroll (1999) with the modifications from Sparrow et al. (2000). The meganuclease procedure was conducted as described, except that the injections were performed at room temperature (20°C).

A variety of micromanipulators and injectors were used for both meganuclease and integrase procedures. The injection apparatuses combined MK-1 micromanipulator (Singer) and MM33 or MD4 micromanipulators (Drummond), and either Picospritzer II (Parker Instrument), FemtoJet (Eppendorf) or Nanojects automatic injectors (Drummond). Some participants chose not to use micromanipulators for their injections.

The injection setup for the REMI method was as described in the original publication by Kroll and Amaya (1996).

Injected embryos were cultured according to the original protocols and maintained at 18°C.

Workshop training session organization

Three training platforms were organized, one for each transgenic method, with two instructors and one researcher from the host institution to provide technical

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support. The participants were split into three groups of equal size, and half-day rotations were organized between the various platforms. For each platform, a special area was made available to allow the participants to come back at their will for embryo screening and scoring.

Media preparation, animal care, egg collection and batch *in vitro* fertilization were carried out by a team of eight researchers from the host institution.

Data collection and analysis of GFP expression

Each participant had the opportunity to perform transgenic experiments using the three different procedures. Each injection experiment was considered as an individual lot. A mean of 60 one-cell-stage embryos were injected per lot for the meganuclease and integrase methods and a mean of 125 eggs per lot for the REMI procedure.

Embryos were scored for viability during the initial phase of segmentation, then at gastrula, neurula, tailbud and tadpole stages. GFP fluorescence was assessed using an Olympus SZX12 fluorescent microscope. GFP expression was scored at stage NF46 (Nieuwkoop and Faber, 1994).

Evaluation of data

The percentage of viable individuals was calculated at major stages of development with respect to the number of injected embryos (Figure S1). The total number of embryos injected was: 700 without integrase, 1000 with integrase, 900 without meganuclease, 900 with meganuclease, 3500 for REMI1 (workshop participant), 1000 for REMI2 (beginner, second round) and 3500 for REMI3 (trained user of the method).

The percentage of embryos expressing GFP was calculated at stage NF46 with respect to either the total number of injected embryos (see Figure S3A) or over the number of viable individuals at stage NF46 (see Figure S3B). The percentage of all embryos expressing GFP (Figure S3, open bars) is presented, as well as the percentage of homogenously expressing embryos among these (Figure S3, closed bars).

Results

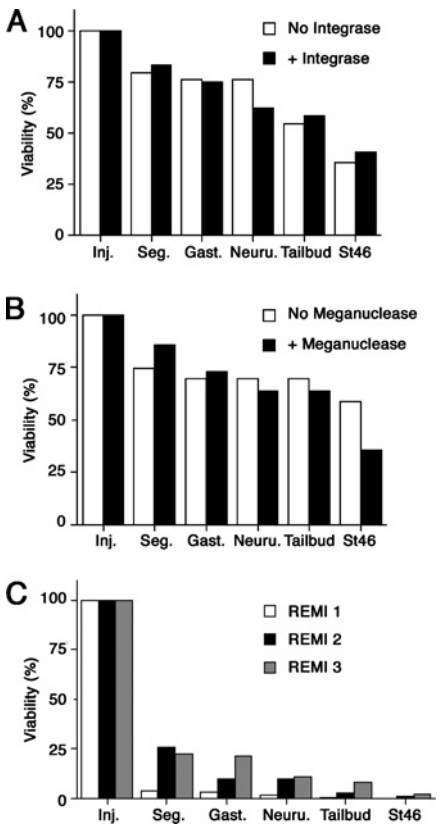
Technical challenges of the injection procedures for transgenesis

When performing transgenic experiments, the injection step appears critical for making the experiment successful. We have tested various microinjection setups with three different types of injectors. Two of them rely on gas pressure for injecting aqueous solutions (Picospritzer and FemtoJet). The third one uses a positive displacement that delivers a fixed quantity of non-compressible fluid, such as mineral oil (Nanoject).

Gas pressure injectors are very user-friendly. Capillaries are filled using long tips and directly placed onto the injectors. The solutions to be injected are therefore never in contact with other solutions that could affect it. This is particularly important when dealing with solutions containing RNA (integrase method). Capillaries are then broken with forceps to

Figure S1 | Survival rates of embryos following the transgenic procedures

Percentage of viable individuals over the number of injected embryos were calculated at various stages of development using the phi-C31 integrase (**A**), the I-SceI meganuclease (**B**) or the REMI procedures (**C**). REMI1, inexperienced researchers, REMI2, inexperienced researchers, second round; REMI3, experienced researchers. Inj., one-cell-stage injected embryos; Seg., segmentation stage; Gast., gastrulation stage; Neuru., neurulation stage; St46, stage NF46.



a diameter that gives the appropriate volume. The estimation of the volume is based on the size of the drop that is being made when applying pressure. This can become highly inaccurate without proper calibration. Many researchers use micrometric reticules to standardize the size of the drop. Usually, this method leads to a fairly good estimation of the volumes initially delivered. However, when the needle gets clogged, it is common to re-clip the end until a drop of solution is being formed. Thus, during the course of the experiment, the delivered volumes tend to vary. Nanoject injectors required more time for the initial set up and this preparation can be tricky. Needles are backfilled with mineral oil and a plunger is inserted into the needle. The needle tips ended up being larger than those used for the gas pressure apparatus. However, the delivered volume was accurate and remained constant during the course of the experiment. Using Nanoject injectors, the holes produced by the injections where much bigger than with a Picospritzer apparatus. However, the survival rates were comparable for either apparatus, with 83 and 79% survivors at segmentation stage and 56 and 53% survivors at stage NF46 when using Nanoject or Picospritzer injectors respectively. We conclude that the type of injector used had no effect on viability and both setups produced glowing embryos.

The REMI procedure requires injecting into unfertilized eggs using an infusion pump. This step has proven to be technically challenging for most workshop participants, including those experienced with microinjections. The combination of soft eggs and big injection needles makes it easy to damage the embryos. There are no alternatives to this step, and thus the REMI method requires some initial training in nuclei transfer.

Survival rates following transgenic experiments

We have monitored the survival rates of injected embryos following the various transgenic procedures (Figure S1). The integrase and meganuclease methods show very similar results, and the data obtained in terms of viability at a given stage of development resemble what is expected from uninjected animals (Figures S1A and S1B). Indeed, depending on the batch of eggs, the survival rate varies greatly and ranges from 50 to 80% at swimming stages (Nishimura et al., 1997; Godfrey and Sanders, 2004). During the course of the workshop, the mean survival rate for injected embryos was close to 50% at stage NF46 for both methods. The addition of RNA encoding the integrase or the meganuclease had no obvious affect on survival rates. Thus, we feel that these two methods are easily accessible, and suitable for the generation of large numbers of healthy embryos.

The REMI procedure had the most deleterious effect of all three methods on early embryo survival (Figure S1C). For an experienced researcher, approx. 25% of injected embryos can be selected at cleavage stages (REMI3). For inexperienced researchers (REMI1), only 3.7% of injected embryos will properly initiate development. When an inexperienced researcher practices a second round of REMI transgenesis (REMI2), the survival rate of the injected embryos increases to closely resemble that of an experienced researcher (compare REMI2 and REMI3 on Figure S1C). From the gastrula to swimming stages, the decrease in viability follows a trend similar to that observed for the integrase or meganuclase procedures in all cases (REMI-1, -2 and -3). These data suggest that it is mainly the initial stages of development (segmentation) that are affected by the REMI procedure, especially for beginners. However, a short training in nuclei transplantation is beneficial and results in a rapid improvement in the survival rate following the injection step when adequate expertise and equipment are provided.

Monitoring transgenic efficiency using GFP as a reporter

We have assessed the efficiency of transgenesis by scoring the embryos for GFP fluorescence at stage NF46 (Figures S2 and S3). GFP fluorescence on its own is not a proof of transgene integration; however, previous reports have shown a good correlation between the ability to detect GFP at late stages of development and the incorporation of the transgene into the genome (Etkin and Pearman, 1987).

Traditionally, the efficiency of transgenesis is shown at a given stage by comparing the number of GFP-positive animals with the number of viable tadpoles at stage NF46 (Figure S3B). We found that scoring the embryos this way does not accurately reflect the number of embryos that must be generated to perform a transgenic experiment. We therefore also expressed the percentage of glowing embryos reported relative to the number of injected embryos (Figure S3A).

Figure S2 | Pattern of GFP distribution generated by the various methods of transgenesis

Embryos expressing GFP following either the I-SceI meganuclease transgenic procedure (**A** and **B**) or the REMI method (**C**) using a CMV–GFP reporter construct. Embryos obtained using the phi-C31 integrase procedure exhibited a pattern of GFP distribution similar to that obtained with the meganuclease procedure. Two types of GFP expression were observed. Embryos expressing GFP in a few cells only were called mosaic (**A**). The cluster of GFP-positive cells is delineated with a broken line in the example shown. Embryos expressing GFP ubiquitously (**C**) or on one side (**B**) were called homogeneous. GFP expression on one side of the embryo is indicated with a broken line in the example of hemi-transgenic embryo shown in (**B**).

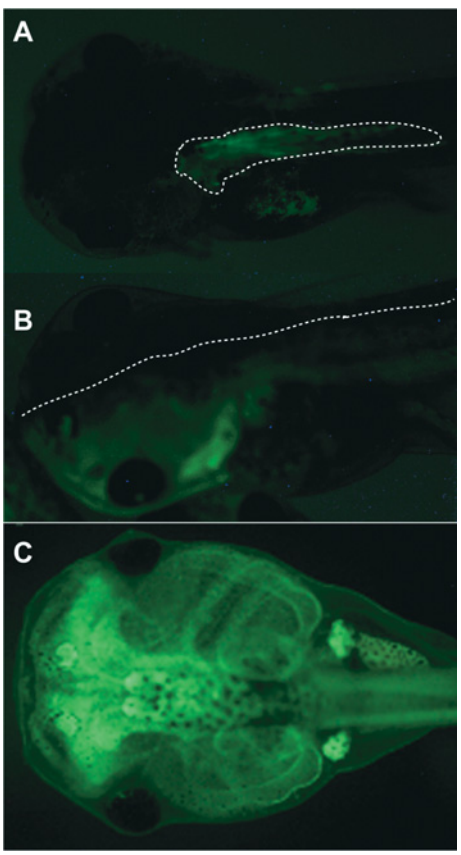
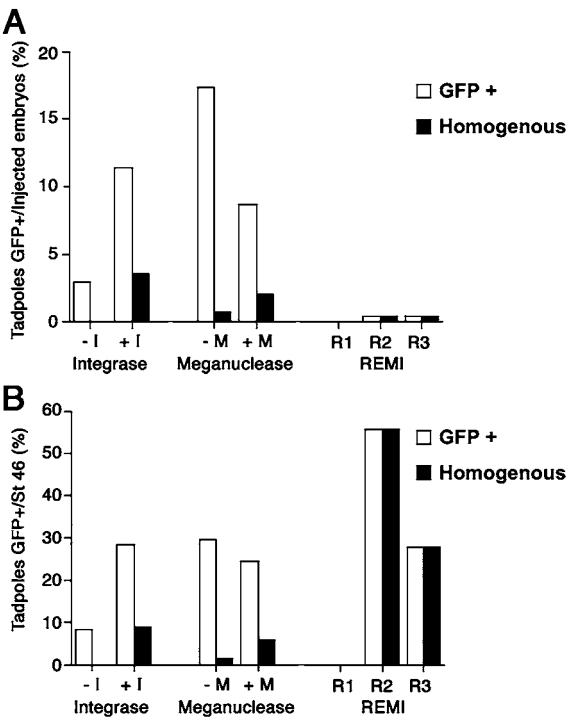


Figure S3 | GFP expression in embryos following the transgenic procedures

The percentage of glowing embryos (GFP+) was calculated compared with either the number of injected embryos (**A**) or the number of surviving embryos at stage NF46 (**B**). R1, REMI1, inexperienced researchers; R2, REMI2, inexperienced researchers, second round; R3, REMI3, experienced researchers.



Two types of GFP expression patterns were observed (see the Material and methods section and Figure S2). First, some embryos were scored GFP-positive, but exhibit GFP expression in only few cells (Figure S2A). These embryos were called mosaic. A simple explanation for this GFP expression pattern is that mosaic embryos probably result from late transgene integrations. Alternatively, the observed mosaic GFP distribution may reflect a variegated expression of the transgene that depends on its chromosomal environment. For transgenic procedures that generate a majority of single-copy insertion events, such as the integrase method, this can be a real concern. In our experiments, we have used a construct containing insulator elements flanking the CMV–GFP reporter in order to minimize the role of chromosomal environment on the transgene. Finally, it is worth noting that the high level of expression observed in the few cells of the so-called mosaic embryos is likely to mask other weaker signals. Thus some early integration events cannot easily be assessed in such a background by our screening procedure. In summary, we cannot rule out that some embryos that we scored mosaic on the basis of GFP expression have, in fact, non-mosaic integrations.

The second type of glowing embryos exhibited homogenous GFP expression (Figures S2B and S2C). Among these, we have observed embryos that expressed GFP on a single side with a left–right asymmetry, suggesting that the transgene likely inserted in one of the two blastomeres at the two-cell stage. Together, these embryos are likely to be due to early integration events and were called homogeneous. For the reasons stated above, this category of GFP-positive embryos is likely to be underestimated by our screening procedure.

In control experiments, when plasmid DNA was injected alone into one-cell-stage embryos, GFP expression was detected in a mosaic fashion in 3 and 17% of injected embryos for the integrase and meganuclease procedures respectively (Figure S3A). The amounts of plasmid DNA injected can explain the differences observed between the two protocols. Indeed, 5 pg of DNA are injected for the integrase procedure compared with 50 pg for the meganuclease method. When RNA encoding the integrase is added, the percentage of glowing embryos increases from 3 to more than 11%, among which, 3.6% are homogenous. We conclude that the phi-C31 integrase efficiently promotes integration of the transgene. When the I-SceI meganuclease is added to the injection mixture, we observed a drop in the percentage of glowing embryos. However, the ratio of homogeneous compared with mosaic embryos increases dramatically when the endonuclease is provided: from 0.045 without meganuclease to 0.24 with meganuclease. These data indicate that, although the amount of plasmid DNA needed in the meganuclease protocol leads to a high percentage of mosaic embryos, the addition of I-SceI meganuclease promotes early transgene integration.

We have to mention that for both the integrase and meganuclease methods, although a certain percentage of embryos were scored homogenous, none expressed GFP ubiquitously, which is a pattern expected from a CMV–GFP construct (Figure S2C). Thus, although these embryos represent early integration events, the insertions have likely occurred after the first division during these experiments.

The low survival rates obtained for the REMI procedure makes it difficult to analyse the efficiency, as described above (Figure S3C). None of the embryos generated during the workshop survived to stage NF46 and only 0.5% of injected embryos made by an experienced researcher express GFP at this stage (REMI3). If we now express the percentage of glowing embryos at stage NF46, the data indicate that over 30% of the embryos generated during the REMI procedure performed by experienced researchers (REMI2 and REMI3) are glowing. In addition, all the GFP-expressing embryos made by REMI are homogeneous and express the reporter ubiquitously (Figure S2C). We conclude that, although the REMI method impinges on the survival rate, it is a method that results in a majority of non-mosaic transgene insertions.

Discussion

The availability of three methods of transgenesis makes it possible to choose the procedure of choice for the experiment to be performed. Importantly, it seems that the methods described here are compatible with the generation of transgenic lines, although germline transmission remains to be demonstrated for the integrase procedure. An interesting perspective is the transfer of these technologies to *Xenopus tropicalis*. Indeed, the REMI method is extremely difficult to perform in *X. tropicalis*. The diploid state of this species makes a highly mutagenic method, such as REMI, unsuitable. The integration of multiple transgene copies at different sites along with recombination events are probably too damaging to the integrity of the genome, a process perhaps better tolerated in tetraploid species, such as *X. laevis*. To generate lines of transgenic *X. tropicalis*, a less invasive method would be better suited. The high level of survival rates obtained using the integrase or meganuclease procedures makes them attractive to begin raising tadpoles. Indeed, the density of the tadpoles is critical for long-term survival. Thus we view the integrase and meganuclease method as promising methods to generate transgenic *Xenopus* lines.

When analysing these results, one has to keep in mind that in the REMI procedure the embryos are generated by nuclei transplantation into unfertilized eggs using an infusion pump. Therefore, when the injection flow rate is set appropriately, at best, a third of the injected embryos are expected to receive a single male pronucleus and to develop properly (Figures 1 and 5, and Kroll and Amaya, 1996). The results presented here are in agreement with these considerations, as nearly 25% of the injected embryos

generated by an experienced researcher cleaved properly (REMI2 and REMI3). Furthermore, during the REMI procedure, activation of the egg is triggered by the injection itself. The stab of the needle is supposed to mimic sperm entry, the exact location of which remains to be shown for *X. laevis*. In practice, the injection will be performed randomly according to the position of the egg in the injection dish. This leaves open the question of whether the location of the injection is crucial or not. We can only suspect that the male pronucleus will be in an hostile environment when injected in the vegetative pole and likely to get trapped by the vitellus. The injection leaves a hole of the diameter of the needle tip that remains open for a few seconds. The recovery of the embryo from the injection depends on the size of the hole. In practice, many investigators have difficulties getting the needle inside, usually squashing the soft unfertilized eggs. When the eggs are pierced, big holes are made, and as the needle is withdrawn, large amounts of cytoplasm are dragged outside the cell. We have observed that this type of injected egg, which represented the majority of eggs injected by inexperienced researchers (REMI1), is not viable. Finally, the location of the delivered pronucleus within the egg remains unknown and is probably extremely variable. Thus the events initiated by and just after the sperm entry, such as activation and cortical rotation, must be greatly influenced by the REMI procedure. Finally, the REMI method has been shown to be highly mutagenic and the generation of embryos carrying important chromosomal aberrations, as well as haploid and triploid individuals, could influence the overall survival rates (Figure 5). This is not an issue for the integrase and meganuclease methods that use *in vitro* fertilized eggs.

Together, it appears that the survival rate obtained using the REMI procedure mostly relies on the quality of the injection itself and the relative integrity of the genomic material delivered. In fact, we have noticed that a very high percentage of transgenic individuals correlated with a higher degree of mortality and abnormal development among the injected embryos. This suggests that a fine balance must be reached to allow both recovery of healthy embryos and generation of transgenic individuals using the REMI method. Unlike the other transgenic methods, we conclude that the REMI procedure requires a training period for the injection step. Indeed, we show that a few practices are beneficial to inexperienced researchers. After two rounds of transgenic experiments, the results obtained by a beginner compare favourably with an experienced researcher in REMI transgenesis (Figure S2, compare REMI2 and REMI3). Thus, when the technical expertise is provided, the REMI method can become amenable to all. Transgenic platforms that could provide these expertise and technical support would be particularly beneficial. In addition, the establishment of a standardized 'transgenic kit' containing both egg extracts and sperm nuclei would contribute to improve the reproducibility of experiments among laboratories and to make this method more user friendly.

The data presented here are consistent with previously published results with respect to the percentage of embryos expressing the transgene at swimming stages (Table 1). The main difference lies in the production of non-mosaic embryos using the meganuclease and integrase methods.

We have presented the data obtained during a workshop on transgenesis organized by the X-omics consortium. As an introduction to the techniques, there was an attempt made to allow each of the participants an opportunity to inject using each apparatus and to test all three methods. The limited time of the workshop imposed some conditions that are not optimal for each method and the data presented here certainly underestimate the efficiency of the tested procedures. For example, the optimal timing of the injection of one cell embryos using the integrase method may not extend beyond the gathering of pigment commonly seen when the embryo starts to cleave. In the interest of allowing participants to develop familiarity with each apparatus, embryos were often injected well into first cleavage. In order to enhance integration at the one-cell stage, embryos should have their jelly coats removed shortly after fertilization is confirmed (i.e. by seeing rotation that places the animal pole up) and should be injected only until pigment begins to gather. A second variable that was difficult to control in the workshop was injection volume. The accurate delivery of prescribed amounts of plasmid and RNA is critical for reproducibility, and was compromised as needle tips clogged or were broken. A third variable concerns the integrity of the mRNA. Although all participants were careful with the integrase mRNA, any inadvertent contamination of the samples with

RNase would be deleterious to the approach. In addition, injection of large amounts of unintegrated plasmid increases the background expression of the reporter and too little integrase mRNA has been shown to rapidly decrease integration of plasmids. The variables affecting the efficiency of the integrase method, such as timing of injection and volume delivered, also holds true for the meganuclease procedure. An additional variable concerns the temperature at which the injection was being performed. During the course of the workshop, most participants injected at room temperature (20°C). This temperature was shown to be above the optimal range (12.5°C). Thus, in the context of a workshop, the conditions were certainly not optimal and the data presented here underestimate the efficiency of the methods.

The generation of *X. tropicalis* transgenic lines constitutes an important resource to promote transgenesis in this species. For this purpose, we have shown that the REMI method results in a majority of non-mosaic founders. However, the increased mosaicism observed when using the integrase or meganuclease compared to REMI is not necessarily to be considered as a major drawback as long as the frequency of transgene transmission allows the production of a sufficient number of F1 transgenic offspring. Using the meganuclease procedure, these F1 individuals have been shown to transmit the transgene in a mendelian fashion and can be used to generate homozygous F2 embryos (Ogino et al., 2006).

The data obtained during the workshop allowed us to highlight some of the principal features of the three methods. Depending on the type of study, some transgenic methods might indeed be more appropriate than others (Table 1). If one wants to overexpress a gene of interest in a non-mosaic fashion, the REMI method is by far the most efficient. However, using REMI, the embryos generated will mostly contain a high-copy number of the transgene. To get embryos with less than 10 copies of the transgene per genome, thus approaching physiological conditions, the integrase and meganuclease methods may be better suited. The size of the transgene may also influence the choice of transgenic procedure. BACs have been successfully used in REMI procedures (Kelly et al., 2005). So far, the upper size limit of the transgene has not been established for the integrase and meganuclease procedures. The phi-C31 phage DNA is over 41 kb, and it is expected that exogenous DNA of this size will be efficiently integrated using the integrase procedure. However, this remains to be demonstrated. Finally, the equipment and the technical implications required are determining factors for most laboratories. The REMI method is clearly more demanding in terms of special equipment and technical training than the integrase and meganuclease procedures. For some experiments though, the REMI method cannot be substituted with other transgenic procedures. The establishment of transgenic resource centres will clearly benefit researchers who would like to perform transgenic experiments using REMI, particularly for those who are not willing to routinely practice REMI in their laboratories.

Finally, it is important to mention that to date, although the integrase and meganuclease procedures have features compatible with transgenic studies performed in *X. tropicalis*, the community is still not equipped with methods adapted for the generation of high numbers of non-mosaic F0 transgenic animals in this species. This lack of methodologies dedicated to *X. tropicalis* clearly impinges on the elaboration of high-throughput functional and gene expression studies or the initiation of large-scale mutagenesis screens. An important perspective lies in the development of such methodology that is likely to condition the dynamic continuity of transgenesis in *Xenopus*.

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