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SHORT COMMUNICATION

Delayed and Restricted Expression of UAS-Regulated GFP Gene in Early Transgenic Zebrafish Embryos by Using the GAL4/UAS System

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Abstract A stable *Tg*(*UAS*:*GFP*) zebrafish line was generated and crossed with *Tg*(*hsp70*:*GAL4*) line, in which the *GAL4* gene is under the control of an inducible zebrafish promoter derived from the heat shock 70 protein gene (*hsp70*). The dynamic green fluorescent protein (GFP) expression in early zebrafish embryos in the GAL4/UAS binary system was then investigated. We found that, at early developmental stages, expression of GFP effector gene was restricted and required a long recovery time to reach a detectable level. At later developmental stage (after 2 days postfertilization), GFP could be activated in multiple tissues in a shorter time, apparently due to a higher level of *GAL4* messenger RNA induction. It appears that the type of tissues expressing GFP was dependent on whether they had been developed at the time of heat shock. Therefore, the delayed and restricted transgene expression should be taken into consideration when GAL4/UAS system is used to study transgene expression in early developmental stages.

Keywords GFP · GAL4 · UAS · hsp70 · Zebrafish

Introduction

The GAL4/upstream activation sequence (UAS) transgenic system has been popularly used in *Drosophila* (Duffy 2002) and also tested successfully in mice (Ornitz et al. 1991), zebrafish (Scheer and Campos-Ortega 1999), *Xenopus* (Hartley et al. 2002), and *Arabidopsis* (Guyer et al. 1998). In the GAL4/UAS system, the yeast transcription activator GAL4 binds its target sequence UAS and activates transcription of UAS-linked genes. The main benefit of the GAL4/UAS system is that the GAL4 gene and UAS target gene are separated into two transgenic lines to generate a binary transgenic system. In the GAL4 line (activator line), the *GAL4* gene is placed downstream of a selected promoter; the activator protein GAL4 may be present but has no target gene to activate. In the UAS line, UAS is fused to a target or effector gene which is silent in the absence of the GAL4 activator. When the GAL4 and UAS lines are crossed, the target genes are turned on in the double-transgenic progeny, following the expression pattern of GAL4 in the activator lines (Phelps and Brand 1998). Therefore, GAL4/UAS system has been widely used to regulate gene expression in a cell-specific and temporally restricted manner and provided a powerful tool to test the function of the genes that may be lethal or sterile, trace transgene expression during development, and monitor subcellular structures and target tissues for selective ablation or physiological analyses (Halpern et al. 2008).

In the previous paper on the kinetic expression of GAL4- and UAS-regulated gene in the transgenic zebrafish by Scheer et al. (2002), they investigated the stability of the messenger RNA (mRNA) and protein of GAL4 and UAS-regulated gene. However, the expression of UAS-regulated transgene was examined mainly by reverse transcription polymerase chain reaction (PCR) or Western blot. In this paper, we generated a stable UAS-regulated green fluorescent protein (GFP) transgenic line, *Tg*(*UAS*:*GFP*), in order to continuously monitor the spatial and temporal expression of GFP transgene in the GAL4/UAS transgenic system. The dynamic expression of UAS effector gene therefore could be investigated directly by observation of GFP fluorescence in live developing embryos.

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We chose *Tg*(*hsp70*:*GAL4*) as the activator line, which is the same activator line used by previous study (Scheer et al. 2002). The zebrafish *heat shock 70* (*hsp70*) promoter was commonly used to activate transgene expression ubiquitously in inducible expression systems, and it expresses at low, often undetectable, level at normal temperature (28.5°C) but is robustly induced in all the tissues at 37°C (Lele et al. 1997). Therefore, the *hsp70* promoter *Tg* (*hsp70*:*GAL4*) has been widely used in activating UAS downstream gene in stable transgenic lines (Hans et al. 2004; Scheer et al. 2001, 2002). Since dynamic expression of *GAL4* mRNA and protein has been well studied in previous report, we focused only on the temporal and spatial expression of UAS-GFP in this study. Our major conclusion is that, for the early stage of embryos, longer time is required to activate GFP expression in the *hsp70*-controlled GAL4/UAS system and the expression of GFP is restricted to only certain early developed tissues. In contrast, for later stage of embryos, GFP expression could be activated to a visible level in a relatively short time, and GFP expression could be observed ubiquitously in multiple tissues. The result observed in this paper would be helpful for the study of transgene expression at early developmental stage using GAL4/UAS system. Delayed and restricted expression should be taken into consideration when the transgene was studied at early stages using GAL4/UAS system.

Materials and Methods

Fish Maintenance

Zebrafish were maintained essentially according to the method described by Westerfield (1995). The fish embryos were grown in egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.0001% methylene blue) in a 28.5ºC incubator until 4 to 5 days postfertilization (dpf). Juvenile 1-month-old fish were transferred to 10-l containers of the Aquatic Habitats stand-alone System (USA). During the spawning period, the fish were kept with the photoperiod of 14-h of light and 10-h of dark.

Development of *Tg*(*UAS*:*GFP*) Line

The 5× UAS fragment was cut from pBlueScript (KS+)-UAS vector (Scheer and Campos-Ortega 1999) with *Not*I and *Eco*RI and then inserted into vector pEGFP1 (Clontech, USA) to make pUAS-EGFP construct. The linearized pUAS-EGFP DNA at a final concentration of 150–200 ng/μl was injected into cytoplasm of zebrafish embryos at one-cell stage. Each embryo was injected with 2.3 nl of DNA. Injected embryos were raised to sexual maturity and crossed with wild-type fish. Positive transgenic founder fish were identified by PCR screening using DNA from the offspring. Generally, genomic DNA was extracted from pools of 50–100 embryos (2 to 3 dpf) from the crosses and primers used to screen *Tg*(*UAS*:*GFP*) line are: GFP-F: AGCAAGGGCGAGGAGCTG; GFP-R: CTGCTGGTAG TGGTCGGC. For testing the function of *Tg*(*UAS*:*GFP*) stable line, the acidic ribosomal phosphoprotein P_0 (*arp*) promoter (0.8 kb) was cut from pARP-EGFP (Ju et al. 1999) by *Eco*RI/*Bam*H I and was cloned to the upstream of the translational start site of GAL4 (Scheer and Campos-Ortega 1999) to make pARP-GAL4 construct. The circular pARP-GAL4 DNA at a final concentration of 100 ng/μl was injected into cytoplasm of *Tg*(*UAS*:*GFP*) embryos to activate the UAS-GFP expression.

Observation of GFP Expression

Observation of GFP expression and photography were performed under a Zeiss Axiovert 25 fluorescence microscopy. GFP was observed under a blue filter (450–490 nm). Photos were taken by using a charge-coupled device digital camera (Optronic Co.) attached to the microscope (Zeiss, Germany).

Heat Shock Treatment

Tg(*hsp70*:*GAL4*) transgenic line was obtained from the Zebrafish International Resource Center, OR, USA. For heat shock treatment of transgenic embryos, around 50 embryos from the cross of *Tg*(*hsp70*:*GAL4*) and *Tg*(*UAS*:*GFP*) at different stages were transferred into a prewarmed 100-ml 37°C water batch for 45 min. Embryos after heat shock were recovered in a 28.5°C incubator and GFP expression was observed under the fluorescence microscope every hour.

RNA Exaction and Real-Time PCR

After heat shock treatment, around 30 embryos from the cross of *Tg*(*hsp70*:*GAL4*) and *Tg*(*UAS*:*GFP*) at different stages were collected and total RNA was extracted by TRIzol reagent (Invitrogen, USA). Equal amounts of total RNA from each sample were reverse transcribed to complementary DNA (cDNA) using SuperScript™ II RT Kit (Invitrogen, USA) according to the manufacturer's instructions. The cDNA samples were used for quantitative real-time PCR analysis using the Lightcycler 480 system (F. Hoffmann-La Roche, Ltd., Switzerland) with Lightcycler 480 SYBR Green I Master kit (F. Hoffmann-La Roche, Ltd., Switzerland) according to the manufacturer's instructions. Primers used for *GAL4* amplification were 5′-CTTTCACTAACAGTAGCAAC-3′ and 5′-GGTATCTT

CATCATCGAATAG-3′, and primers used for *GFP* amplification were 5′-CTTCAAGATCCGCCACAACATC-3′ and 5′-CTTCTCGTTGGGGTCTTTGCTC-3′.

Results and Discussion

Generation of *Tg*(*UAS*:*GFP*) Stable Transgenic Line

Of 105 pUAS-GFP-injected founder fish, three produced offspring carrying UAS-GFP fragment in the genome, but only two lines were successfully maintained: line 11 and line 37. To test the functionality of *Tg*(*UAS*:*GFP*) transgenic lines, F_2 embryos from lines 11 and 37 were injected at one to two cell stages with pARP-GAL4, a GAL4 construct under a strong ubiquitous zebrafish promoter derived from acidic ribosomal phosphoprotein P_0 (*arp*) gene (Ju et al. 1999). GFP expression could be observed at 24 h postfertilization (hpf) in the injected embryos in both transgenic lines (data not shown), suggesting the functionality of *Tg*(*UAS*:*GFP*) line. Between two *Tg*(*UAS*:*GFP*) lines, line 37 showed higher GFP fluorescence expression after pARP-GAL4 injection so that it was chosen for further experiments. So far, line 37 has been maintained for six generations and transgene inheritance always followed the Mendelian ratio as anticipated based on a single insertion.

Activation of GFP in *Tg*(*UAS*:*GFP*) by Transgenic GAL4 Expression

To investigate GFP transgene activation in *Tg*(*UAS*:*GFP*) with the existence of transgenic GAL4, *Tg*(*UAS*:*GFP*) transgenic line was crossed with a previously characterized GAL4 transgenic zebrafish line *Tg*(*hsp70*:*GAL4*), which is capable of activating UAS downstream genes in stable transgenic lines (Scheer et al. 2001). To activate the expression of GAL4 and GFP, 24 hpf progeny embryos from the cross of *Tg*(*hsp70*:*GAL4*) and *Tg*(*UAS*:*GFP*) were heat-shocked for 45 min and the GFP expression was monitored continuously. The earliest GFP expression could be observed in the notochord at 32 hpf (8 h after heat shock), but the GFP expression was quite weak (data not shown). At 36 hpf (12 h after heat shock), strong GFP expression was observed in the notochord, floor plate, hypochord, and skin epithelium (Fig. 1a, b). The GFP expression was increased gradually in the following 12 h. At 48 hpf (24 h after heat shock), strong GFP could be observed in lens, floor plate, notochord, hypochord, and skin (Fig. 1d, e). GFP expression was decreased in the following 24 h. At 60 hpf (36 h after heat shock), GFP expression level was much weaker than that at 48 hpf. Although GFP expression in lens was still strong, the expression in the floor plate, notochord, and hypochord was quite weak (Fig. 1g, h). At 72 hpf (48 h after heat shock), GFP expression in lens became weaker than that at 60 hpf, and even weaker expression of GFP was observed in floor plate, notochord, and hypochord (Fig. 1j, k). In non-heat-shock control embryos, no GFP could be observed in these stages of embryos (Fig. 1c, f, i, l), indicating that the GFP expression could only be induced under the heat shock condition. These results indicated that GFP expression could be activated in *Tg*(*UAS*:*GFP*) by transgenic GAL4. High GFP expression started from 12 h after heat shock and increased in the following 12 h but decreased after 24 h of heat shock. The GFP dynamic expression in this experiment was consistent with the findings from previous reports (Scheer et al. 2001, 2002). However, our result also showed that GFP expression was restricted to certain tissues in stable GAL4/UAS system. In comparison, when *hsp70* promoter was used to control GFP expression directly, strong GFP expression could be observed in nearly all cells immediately after heat shock in *Tg*(*hsp70*:*GFP*) embryos at all the examined stages (Halloran et al. 2000). Therefore, it seems that activation of GFP expression was reduced and restricted in the GAL4/UAS system compared to the direct expression system.

Temporal GFP Expression in Double-Transgenic Zebrafish *Tg*(*hsp70*:*GAL4*;*UAS*:*GFP)*

To further investigate the dynamic expression of GFP effector gene in stable GAL4/UAS transgenic system, the temporal expression of GFP was examined at different developmental stages. The progeny embryos were heat-shocked at various developmental stages between 12 and 72 hpf, and the initiation of GFP expression after heat shock and GFP intensity was monitored. As shown in Table 1, the interval between heat shock and the initiation of GFP expression in *Tg*(*hsp70*:*GAL4*,*UAS*:*GFP*) embryos was different at different stages. For example, when the embryos were heat-shocked at 12 hpf, no GFP could be observed until 9 h after heat shock. At more advanced stages, the interval between heat shock and initiation of GFP expression became shorter. When the embryos were heat-shocked at 24, 48, and 72 hpf, the time required for the initial GFP expression was shortened to 8, 5, and 3 h, respectively. This indicated that embryos at younger stages need longer recovery time to activate GAL4-mediated GFP expression. However, despite the different intervals between heat shock and initiation of GFP expression at different stages, the dynamic GFP expression, i.e., strong GFP expression starting between 12 and 24 h and decreasing after 48 h after heat shock, was consistent for all these tests at different developmental stages.

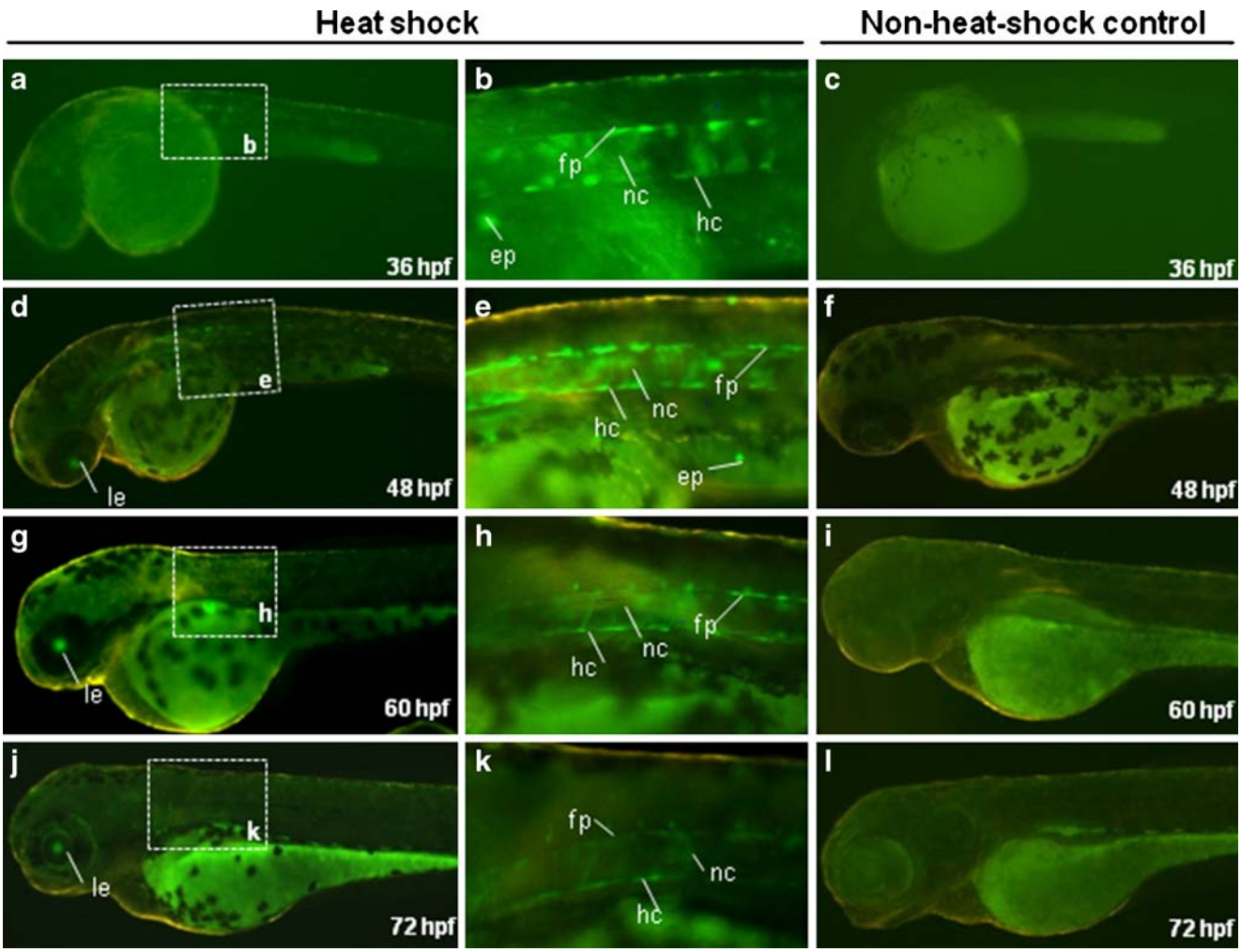

Fig. 1 Dynamic expression of GFP in *Tg*(*hsp70*:*GAL4*; *Tg*(*UAS*:*GFP*)) embryos. The embryos were heat-shocked at 24 hpf and recovered for 12 h (36 hpf, **a** and **b**), 24 h (48 hpf, **d** and **e**), and 36 h (60 hpf, **g** and **h**), 48 h (72 hpf, **j** and **k**), respectively. **b**, **e**, **h**, **k** Enlarged view of GFP expression at 36-, 48-, 60-, and 72-hpf embryos shown as boxed region in (**a**, **d**, **g**, **j**), respectively. **c**, **f**, **i**, **l** Control non-heat-shock embryos at 36, 48, 60, and 72 hpf, respectively, as indicated. Abbreviations: *le*, lens; *ep*, skin epithelium; *nc*, notochord; *fp*, floor plate; and *hc*, hypochord

Table 1 Dynamic expression of GFP after heat shock at different stages

Recovery time (h)	Developmental stage at heat shock			
	12 hpf	24 hpf	48 hpf	72 hpf
3	−	−	−	+
5	−	−	+	+
9	+	+ (8 h)	+	++
12	++	++	++	+++
24	++	++	+++	+++
48	+	+	+	++

+++ strong GFP expression in most of tissues, ++ strong GFP expression in restricted tissues, + weak GFP expression, − invisible GFP expression

GFP Expression in Multiple Tissues in Double-Transgenic Zebrafish *Tg*(*hsp70*:*GAL4*;*UAS*:*GFP*)

Since different recovery time for initiation of GFP expression was required when heat shock was performed at different stages, we next investigated whether the GFP expression pattern would be different when UAS-GFP was activated at different stages. As shown in Fig. 1 and Table 1, when heat shock was performed at certain developmental stage, the expression pattern of GFP had no obvious difference between 12 and 24 h after heat shock. At 24 hpf (12 h after heat shock), GFP could be observed mainly in restricted tissues including notochord, skin, lens, and neural tube (Fig. 2a–c). GFP expression in the notochord showed the strongest expression among all the

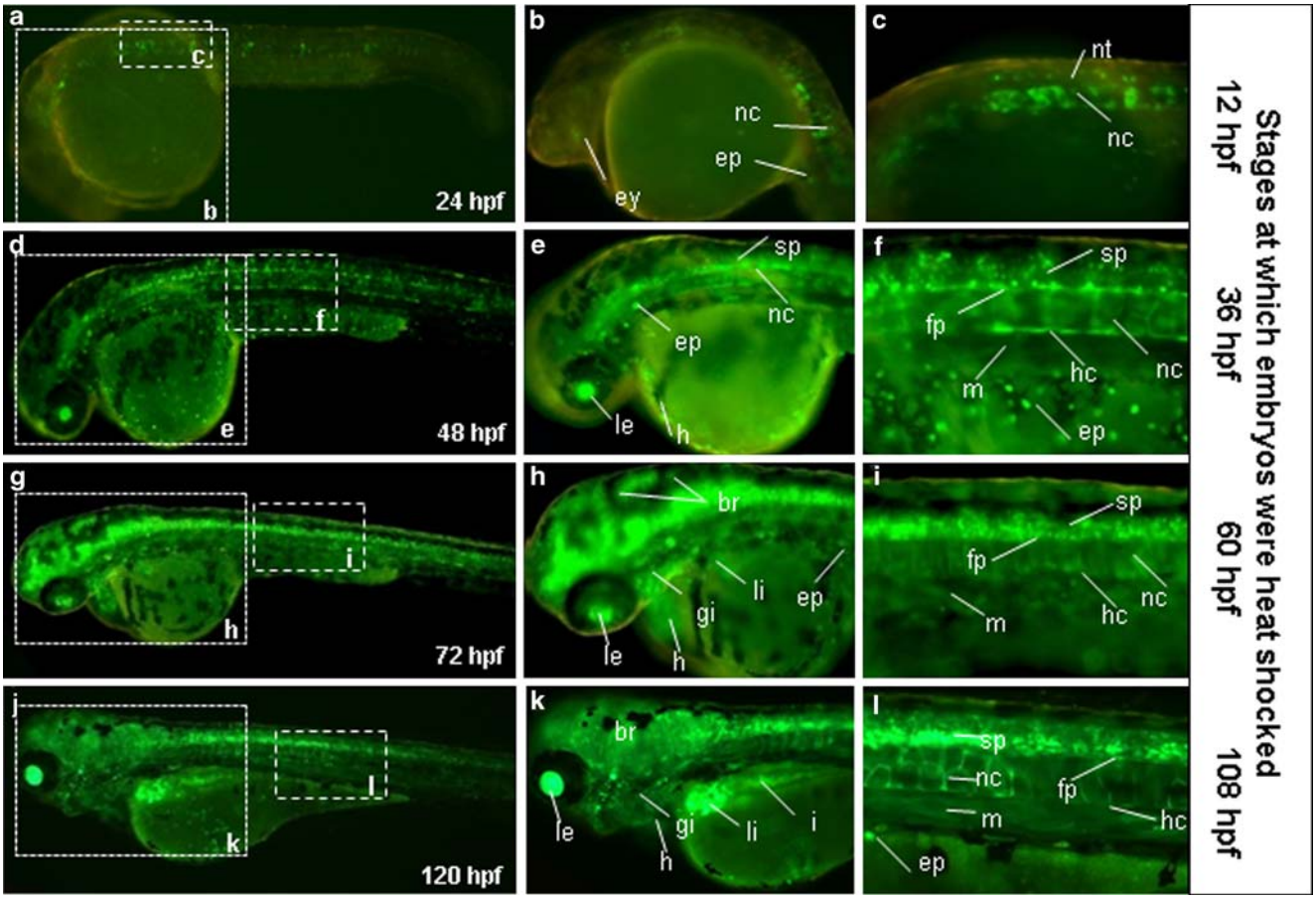

Fig. 2 GFP expression pattern in *Tg*(*hsp70:GAL4*;*Tg*(*UAS:GFP*)) embryos at different stages after heat shock. All the embryos were heat-shocked for 45 min at different stages as indicated on the right and GFP expression was pictured at 12 h after heat shock. **a**–**l** Side view of transgenic embryos showing GFP expression at 24 hpf (**a**–**c**), 48 hpf (**d**–**f**), 72 hpf (**g**–**i**), and 120 hpf (Panels **j**-**l**), respectively, as indicated. **b**, **c** Enlarged view of GFP expression at 24-hpf embryos shown as *boxed* region in **a**; **e**, **f** enlarged view of GFP expression at 48-hpf embryos shown as *boxed* region in (**d**); **h**, **i** enlarged view of GFP expression at 72-hpf embryos shown as *boxed* region in **g**; **k**, **l** enlarged view of GFP expression at 120-hpf embryos shown as *boxed* region in **j**. Abbreviations: *le*, lens; *ep*, skin epithelium; *nc*, notochord; *br*, brain; *sp*, spinal cord; *fp*, floor plate; *hc*, hypochord; *nt*, neural tube; *h*, heart; *gi*, gill; *li*, liver; *i*, intestine; *m*, muscle; and *ey*, eye

tissues (Fig. 2a). At 48 hpf (12 h after heat shock), GFP expression was stronger in the notochord, spinal cord, and skin than that at 24 hpf and more tissues, including lens, floor plate, and hypochord, began to express strong GFP (Fig. 2d–f). Weak GFP was also visible in the heart (Fig. 2e) and several muscle fibers at this stage (Fig. 2f). Among all of the tissues expressing GFP, the lens showed the strongest GFP expression at this stage. At later stage from 72 hpf (12 h after heat shock), GFP expression in the central nervous system was much stronger than that in earlier stages and also strong compared with most of other tissues expressing GFP (Fig. 2g–i). The notochord and lens still showed strong GFP expression (Fig. 2h, i). From this stage, strong GFP could be observed in the brain, heart, and gills (Fig. 2h). The liver cells also began to show weak GFP expression at this stage (Fig. 2h). By 120 hpf (12 h after heat shock), GFP could be observed in most of tissues including spinal cord, lens, notochord, brain, heart, muscles, and skin (Fig. 2j–l). GFP expression in lens and central nervous systems was still the strongest among all of tissues. GFP expression in the liver and intestine was increased dramatically at this stage (Fig. 2k) and more muscle cells expressed GFP. Therefore, it seems that GFP could only be induced in restricted tissues at early developmental stages (24–48 hpf) in *hsp70* promoter-controlled GAL4/UAS stable transgenic system. A more ubiquitous GFP expression could be observed at later developmental stage from 72 hpf.

While strong GFP expression could be induced in almost all the cells by heat shock in *Tg*(*hsp70*:*GFP*) line (Halloran et al. 2000), we observed that GFP effector gene activation in GAL4/UAS system required a long recovery time and was restricted to certain tissues at early developmental stages. Our data indicated that effector transgene expression in GAL4/UAS system could only mimic its expression in direct expression system at later developmental stage. The

possible interpretation of the delayed and restricted GFP expression before 2 dpf might be due to the long time needed for GFP synthesis and rapid morphogenesis in the first 2 days after fertilization. According to Scheer et al. (2002), the *GAL4* mRNA was transcribed almost immediately after heat shock and decreased dramatically after 1 hour of heat shock. However, in our GAL4/UAS system, strong GFP expression could only be observed 12 h after heat shock. When we observed the GFP expression in 24-hpf embryos, heat shock was performed at 12 hpf, at which stage only notochord and skin were fully developed. Thus, we observed GFP expression mainly in these two tissues at 24 hpf. The other tissues, however, did not show visible GFP expression at 24 hpf as they had not fully developed at the time of heat shock (12 hpf). This suggests that only those fully developed tissues at the stage of heat shock could express high level of GFP in *hsp70*-induced GAL4/UAS stable transgenic system. Since effector transgene expression level was dependent on the GAL4 protein concentration (Osterwalder et al. 2001), the weak experience/nonexpression of GFP in undeveloped tissues might be due to the low concentration of GAL4 protein, which results from the dilution during rapid morphogenesis in the first 2 days postfertilization (Akimenko and Ekker 1995). After 2 dpf, most of organs and tissues have been fully developed so that enough amount of GAL4 protein could be induced and maintained after heat shock in most tissues/organs to activate GFP effector gene expression. That could also explain that more and more tissues express GFP at later stage and at higher level.

To understand the difference of GFP expression between early and later developmental stages after heat shock in our system, real-time PCR was performed to quantify the *GAL4* and *GFP* expression level. The *Tg*(*hsp70*:*GAL4*;*UAS*:*GFP*) embryos were heat-shocked at early stage (12 hpf) or at later stage (72 hpf), and *GAL4* and *GFP* expression were examined at 1, 3, 6, and 9 h after heat shock. As shown in Fig. 3a, in both 12- and 72-hpf embryos, *GAL4* mRNA expression was dramatically increased immediately after heat shock and then decreased rapidly (Fig. 3a). It is interesting to note that 72-hpf embryos had a higher and more persistent induction of *GAL4* mRNA than 12-hpf embryos (Fig. 3a). This difference was also clearly reflected in GFP expression (Fig. 3b). Thus, the different levels of *GAL4* mRNA induction between early and late stages of embryos may partially explain the longer time required for detectable GFP fluorescence in early embryos.

It is also interesting to note from Fig. 3 that *GAL4* mRNA was induced by a few thousand fold while *GFP* mRNA was induced in less than tenfold. This observation implied that the GFP effector gene expression may be compromised in the GAL4/UAS system. Consistent with this, we also observed from transient transgenic assays that

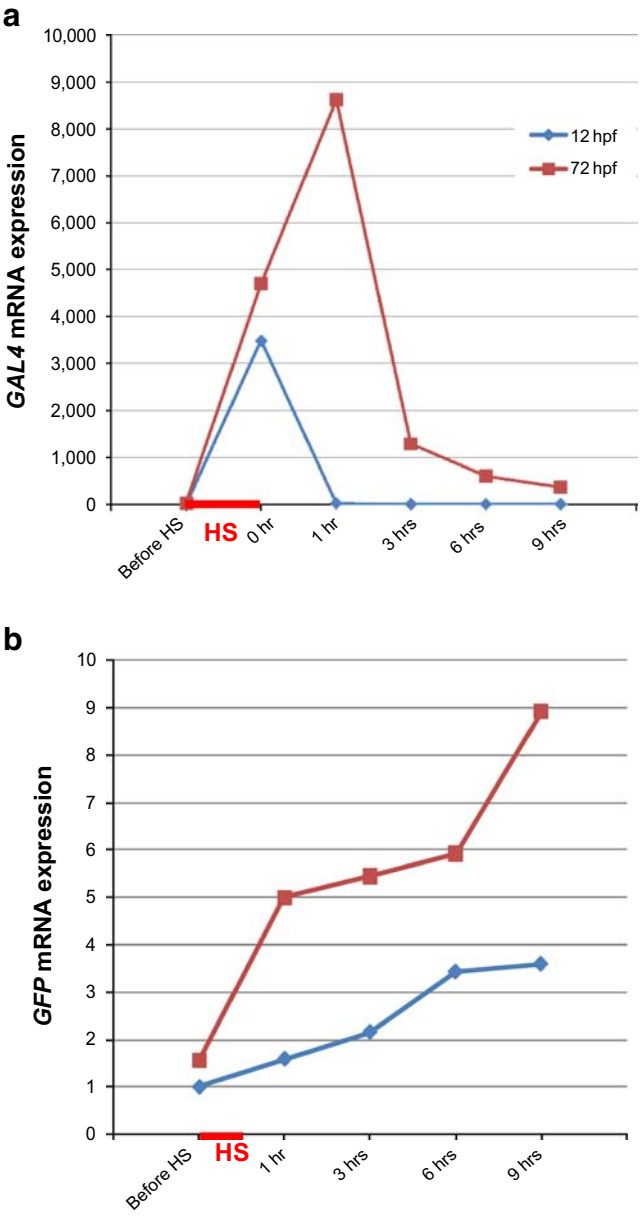

Fig. 3 Time course of *GAL4* and *GFP* mRNA induction in *Tg*(*hsp70*:*GAL4*; *UAS*:*GFP*) embryos after heat shock; 12- and 72-hpf embryos were heat-shocked for 45 min and *GAL4* and *GFP* mRNAs were quantified at 1, 3, 6, and 9 h after heat shock. **a** Comparison of the dynamic change of *GAL4* expression after heat shock when embryos were heat-shocked at 12 and 72 hpf, respectively. **b** Comparison of the dynamic change of *GFP* expression after heat shock when embryos were heat-shocked at 12 and 72 hpf, respectively; 45 min of heat shock period was indicated by red lines before 0 h on *X-axis*. The levels of *GAL4* or *GFP* expression are relative to the levels of *GAL4* or *GFP* mRNAs in 12-hpf embryos before heat shock

the direct expression of GFP reporter gene by injection of a simple promoter-GFP construct was always stronger than that by coinjection of the two GAL4/UAS constructs under the same promoters (data not shown). Thus, the GAL4/

UAS system may compromise the level of effector gene expression.

Overall, our observation demonstrated the feasibility to use GAL4 to activate UAS-linked transgene expression in most, if not all, tissues in zebrafish embryos under the control of *hsp70* promoter. Our newly developed *Tg*(*UAS*:*GFP*) would become a useful line to test the functionality of GAL4 activator lines. However, the ubiquitous expression could only be activated at later developmental stage and long recovery time was required for transgene activation. Therefore, consideration should be taken into account when the GAL4/UAS system is used to study transgene expression at early developmental stage. In addition, the generation of the stable transgenic zebrafish line, *Tg*(*hsp70*:*GFP*), and confirmation of its functionality in the present study should be useful for future tests of GAL4 activator lines in zebrafish.

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