Aquaculture 522 (2020) 735134

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

The endogenous EF-1α promoter is highly active in driving gene overexpression in developing embryos of the Pacific oyster *Crassostrea gigas*

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ARTICLE INFO

Keywords:
EF-1α promoter
Enhanced green fluorescence protein
Crassostrea gigas

ABSTRACT

Gain and loss-of-function analyses are powerful genetic approaches to uncover gene functions in basic and applied research. However, the genetic analysis in bivalve molluscs has been challenged by the lack of effective gene promoters for driving gene overexpression. The cosmopolitan Pacific oyster (*Crassostrea gigas*) is an economically important marine bivalve and a representative model species for ecological, evolutionary, and developmental studies. Here, we isolated the elongation factor1-α (EF-1α) gene promoter from the Pacific oyster and compared the promoter activity with the commonly used cytomegalovirus (CMV) and Xenopus EF-1α promoters in oyster embryos. We found that the 3465 bp DNA sequence prior to the ATG start codon in oyster EF-1α was able to drive enhanced green fluorescent protein (EGFP) expression in oyster embryos. In contrast, CMV and Xenopus EF-1α promoters failed to direct EGFP expression in oyster embryos. It appeared that the first intron sequence in oyster EF-1α gene was required for the promoter activity. DNA construct without the first intron located upstream of the ATG start codon failed to drive EGFP expression in oyster embryos. Collectively, these studies indicate that the endogenous EF-1α promoter is preferred promoter for driving gene expression in bivalves.

1. Introduction

Many molluscs are important aquaculture species and selective breeding for genetic enhancement has been conducted over years. Whereas the genetic analysis in commercially important molluscs remains to be relatively weak. The Pacific oyster, *Crassostrea gigas*, is a representative bivalve mollusc that is widely cultured in the world. In the past few decades, significant efforts have been made in genetic breeding to select new oyster strains with desired colours, faster growth and disease resistance (Burge et al., 2007; Ge et al., 2015a; Wang and Li, 2017; Xing et al., 2017). Oyster strains with these beneficial traits have been developed (Burge et al., 2007; Ge et al., 2015b; Wang and Li, 2017; Xing et al., 2017). However, the genetic mechanisms behind these new beneficial traits are not clear. Linkage analyses to map and identify candidate genes responsible for these traits have not been very successful (Ge et al., 2015a, b; Wang and Li, 2017; Song et al., 2018). This is mainly due to the lack of fine markers for genetic mapping and no reliable genetic approaches for gain and loss-of-function analyses have been established in oyster, such as gene transfer and gene knockout, respectively.

Our recent studies showed that CRISPR/Cas9 system is very effective in inducing genetic mutations in the Pacific oyster (Yu et al., 2019). By microinjecting CRISPR/Cas9 ribonucleoprotein complexes into fertilized eggs, we demonstrated that the CRISPR technology could be used as a powerful tool for gene functional studies (Yu et al., 2019). With the complete oyster genome sequence available (Zhang et al., 2012), the reverse genetic approach can be used to perform gene specific knockout. However, this has yet to be accomplished due to the poor survival of the CRISPR/Cas9 injected embryos. An alternative approach to microinjection of sgRNA/Cas9 complex is through the expression of an all-in-one construct that expresses both sgRNA and Cas9 protein *via* DNA transfection (Ran et al., 2013). This alternative approach through DNA transfection could be more appropriate because large number of embryos can be treated in a high throughput fashion.

Abbreviation: EF-1α, elongation factor1-α; CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein

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https://doi.org/10.1016/j.aquaculture.2020.735134
Received 7 January 2020; Received in revised form 17 February 2020; Accepted 18 February 2020
Available online 19 February 2020
0044-8486/

This requires an active promoter to drive Cas9 gene expression in oyster embryos.

Currently, the CMV promoter from cytomegalovirus and the elongation factor 1α (EF-1α) gene promoter are two commonly used promoters to drive ubiquitous gene expression in various *in vitro* and *in vivo* systems. It has been shown that CMV promoter is useful for directing high-levels of transient expression in human cell lines and Xenopus and zebrafish embryos (Turner and Weintraub, 1994). The strength of its promoter activity, however, varied considerably depending on cell type (Qin et al., 2010). Moreover, silencing of the viral CMV immediate early enhancer promoter can be a problematic in certain cell types (Teschendorf et al., 2002). By comparison, the EF-1α promoter is often useful in conditions where other promoters (such as CMV) have diminished activity. EF-1α gene promoters of many species have been identified and used in variety of expression vectors (Kim et al., 1990). The EF-1α promoter could drive gene expression across taxa, allowing the effective use of Xenopus EF-1a promoter in zebrafish (Kawakami et al., 2004). However, the activity of these promoters in oyster is unknown. Successful transgene expression has been reported in the Pacific oyster using the PiggyBac transposon system (Chen et al., 2018), and in eastern oyster (*Crassotrea virginica*) embryos using the CMV promoter (Buchanan et al., 2001). The expression activity was relatively low, likely due to the use of exogenous promoters because no endogenous ubiquitous gene promoter has been isolated and characterized in the Pacific oyster.

In this study, we isolated the EF-1α gene promoter from *C. gigas* and constructed reporter plasmids using the *C. gigas* EF-1α promoter sequence. Moreover, we compared the *C. gigas* EF-1α promoter activity with CMV and Xenopus EF-1α promoters in oyster embryos. The development of a ubiquitous expression system using an endogenous promoter from *C. gigas* could facilitate future studies in bivalve gene functional analysis.

2. Materials and methods

2.1. Synthesis of EGFP mRNA in vitro

The EGFP mRNA was synthesized using T3 RNA polymerase by *in vitro* transcription. DNA template for the *in vitro* transcription was generated by PCR using the DNA plasmid pT2AL200R150G that contains the EGFP coding sequence (Kawakami et al., 2004). The PCR was carried out using the EGFP-F1 forward primer containing the T3 promoter sequence and an EGFP-R1 reverse primer (Table 1). *In vitro* transcription of capped mRNA was carried out using the T3 RNA Polymerase Kit (Ambion). The EGFP mRNA transcripts were purified using the Megaclear Kit (Ambion).

2.2. Plasmid construction

Two plasmids were constructed using the *C. gigas* EF-1α promoter sequence. The p(−3.4 kb EF1α:EGFP) construct contains the 2310 bp promoter and the 1059 bp first intron sequence, whereas the p(−2.3 kb EF1α:EGFP) construct contains the promoter without the first intron. Briefly, the DNA fragments containing the 5′ flanking sequences of *C. gigas* EF-1α were amplified using the High-Fidelity Phusion DNA Polymerase (Thermo Fisher Scientific). The PCR primers including one forward sequence primer and two reverse primers were listed in Table 1. The PCR fragments were cloned into the BamHI linearized pEGFP-1 vector (www.miaolingbio.com) using the In-Fusion HD Cloning Kits (Clontech). These two DNA constructs, designated as p(−3.4 kb EF1α:EGFP) and p(−2.3 kb EF1α:EGFP), were confirmed by sequencing. In addition, several other constructs were obtained from other labs, including the pCS2:EGFP plasmid (Li et al., 2005) and the pT2AL200R150G plasmid (Kawakami et al., 2004). The pCS2:EGFP construct contains a CMV promoter upstream the EGFP coding sequence, and a SV40 poly A signal downstream the EGFP coding region (Li et al., 2005). The pT2AL200R150G plasmid is a Tol2 transposon-based construct expressing the EGFP directed by the Xenopus EF-1α promoter (Kawakami et al., 2004).

2.3. Microinjection of oyster embryos

Pacific oysters at mature stage were collected from a local oyster farm in Rushan, Shandong province, China. The oysters were sexed and gametes were then obtained by stripping the gonad. *In vitro* fertilization was performed as described previously (Li et al., 2011). The capped EGFP mRNA and DNA constructs were microinjected into fertilized oyster embryos at one cell stage. EGFP mRNA was directly microinjected into fertilized oyster eggs at a concentration of 500 or 1000 ng/μl. The DNA construct pCS2:EGFP and pT2AL200R150G were injected into fertilized eggs at concentration of 750 or 1000 ng/μL. The DNA construct p(−3.4 kb EF1α:EGFP) and p(−2.3 kb EF1α:EGFP) were injected into oyster embryos at various concentration of 750, 500, 200, 100, 50 and 10 ng/μl. A final concentration of 0.5% phenol red was added to the injection solution for easy visualization during microinjection. Approximately 0.1 nl of the mixture solution was microinjected into oyster embryos before the first cleavage. The 0.5% phenol red was used as injection control. Various final concentrations of mRNA and plasmids were tested and microinjection was carried out as described previously (Yu et al., 2019). The injected embryos were incubated in filtered seawater at 22 °C.

Table 1
Primers used in this study.

Primer name	Sequence
EGFP-F	AATTAACCCTCACTAAAGGGAGAAACGTGCTGGTTGTTGTGCTGT
EGFP-R	CTGCATTCTAGTTGTGGTTTGTCC
CgEF-1αF	CGCGGGCCCGGGATCCACTATATGACGGACTGGACAAC
CgEF-1αR1	GGCGACCGGTGGATCCCGTTGCTCCTTGTTTTTATTACTC
CgEF-1αR2	GGCGACCGGTGGATCCCTCAGAGTTCGCCACAGCAA

The blue sequence in EGFP-F is the T3 promoter sequence. The underline sequences in CgEF-1αF, CgEF-1αR1 and CgEF-1αR2 are homologous to each end of BamH I-digested pEGFP-1 (BamH I restriction endonuclease site marked red).

2.4. Image collection

EGFP expression in the injected embryos was observed under an Olympus BX53 fluorescence microscope (Olympus, Japan). EGFP expression was monitored every hour after fertilization and fluorescence images were captured using a DP80 camera and the cellSens Entry software (Olympus, Japan).

3. Results

3.1. Expression of pCS2:EGFP and pT2AL200R150G in the injected embryos

To test whether CMV promoter and Xenopus EF-1α promoter could drive foreign gene expression in oyster embryos, microinjection was performed with pCS2:EGFP or pT2AL200R150G plasmid DNA in oyster embryos. After microinjection, all embryos died in the 1000 ng/μl injected group. Approximately 15% of the injected embryos survived in the 750 ng/μl injected group. The live embryos were observed for EGFP expression every hour after microinjection. No EGFP expression could be detected in the pCS2:EGFP or pT2AL200R150G injected embryos from 1 to 24 hours post-fertilization (hpf), suggesting that the CMV viral promoter and the exogenous Xenopus EF-1α promoter might not be able to drive strong gene expression in *C. gigas* embryos.

To ensure the lack of EGFP expression was not due to poor microinjection technique, capped EGFP mRNA was directly microinjected into fertilized oyster eggs. EGFP expression was clearly detected in the injected embryos at 24 hpf (Fig. 1). Interestingly, EGFP expression was only detected in the 1000 ng/μl injected group, suggesting that lack of EGFP gene expression could be due to the low activity of exogenous CMV and Xenopus EF-1α promoters in oyster embryo during early development.

3.2. Characterization of EF-1α gene structure and promoter region in C. gigas

To identify an endogenous promoter that can drive EGFP expression in *C. gigas,* we decided to isolate the EF-1α gene from *C. gigas* and characterize its promoter activity *in vivo*. Comparison of transcriptome and genome data revealed that the EF-1α gene of *C. gigas* contains 6 introns (Fig. 2) (Hedgecock et al., 2007; Roberts et al., 2009). The first exon, consists of 75 nucleotides which encodes part of the 5′-UTR sequence upstream of the ATG start codon located in exon 2. Consequently, the first intron sequence was prior to the ATG start codon.

The EF-1α gene structure appeared to be conserved during evolution. The first exon and part of the second exon encode the 5′-UTR sequence. The ATG start codon is located on exon 2 after intron 1. This type of gene structural organization is also found in EF-1α homologs in other bivalve, such as *Mizuhopecten yessoensis*, and in some vertebrate species, such as *Homo sapiens* and *Danio rerio*. The intron 1 is longer than other introns in EF-1α genes of these animal species and lengths of intron 1 sequence vary considerably. The intron 1 sequence of *C. gigas* EF-1α is 1059 bp long which is similar with the intron 1 sequence in EF-1α genes of human and mouse but longer than that of zebrafish. Interestingly, the EF-1α gene of *Caenorhabditis elegans* does not contain an intron 1 that splits the 5′UTR sequence in two exons (Fig. 2).

It has been reported that the EF-1α promoter in human genome contains a typical TATA box for transcription initiation (Uetsuki et al., 1989). The TATA box has also been identified in the promoter of Xenopus EF-1α gene. Interestingly, Xenopus genome contains two EF-1α genes. In addition to the ubiquitously expressed EF-1α, Xenopus expressed a germline specific EF-1α in the oocytes, and thus named EF-1αO (Frydenberg et al., 1991). In contrast to the ubiquitously expressed EF-1α, TATA box sequence is missing in the Xenopus EF-lαO promoter (Frydenberg et al., 1991). To characterize the promoter region of *C. gigas* EF-1α, we performed a sequence analysis on the 5′ flanking sequence. No typical TATA box could be identified at upstream flanking region of oyster EF-1α gene. However, a potential downstream promoter element (DPE) that includes a G-A-C-G consensus sequence was found at 16 nucleotides downstream of the putative transcription start site. This alternative downstream promoter element has been found in Drosophila jockey, Drosophila Antennapedia P2, Drosophila Abdominal-B, and human IRF-1 genes (Burke and Kadonaga, 1996, 1997).

3.3. EGFP expression in oyster embryos driven by C. gigas EF-1α promoter

The EF-1α promoter activity was analyzed in oyster embryos by driving EGFP expression. A 3465 bp 5′-flanking sequence including the first intron (Fig. 3) was cloned into the pEGFP-1 vector. The DNA construct p(−3.4 kb EF1α:EGFP) was injected into oyster embryos at various concentration. EGFP expression could be detected in embryos at 4 h after microinjection of DNA at 750, 500 and 200 ng/μL (Fig. 4). Injection of DNA plasmid at the reduced concentration of 100, 50 and 10 ng/μl delayed the appearance of EGFP detection by 1, 4 and 6 h, respectively (Fig. 4). Injection of DNA at 200 ng/μl resulted in 47.3% survival rate, while the lower concentration did not improve the survival rate (48–54.5%). Injection of DNA at higher concentrations showed an adverse effect to embryonic development in pacific oyster. Only 16.0% and 20.5% embryos could survive when injected with DNA at 750 and 500 ng/μl, respectively (Fig. 4). Injection with 0.5% phenol red resulted in 64.0% embryos survival.

To follow the EGFP expression during development, the p(−3.4 kb EF1α:EGFP) construct was injected at 200 ng/μl into oyster embryos, and followed by scoring the EGFP expression and embryo survival. Out of 44 embryos injected, 63% showed the EGFP expression. The EGFP expression appeared to be ubiquitous, and the ubiquitous expression could be detected as early as at morulae stage (Fig. 5B). EGFP expression was subsequently detected in various stages from morulae to

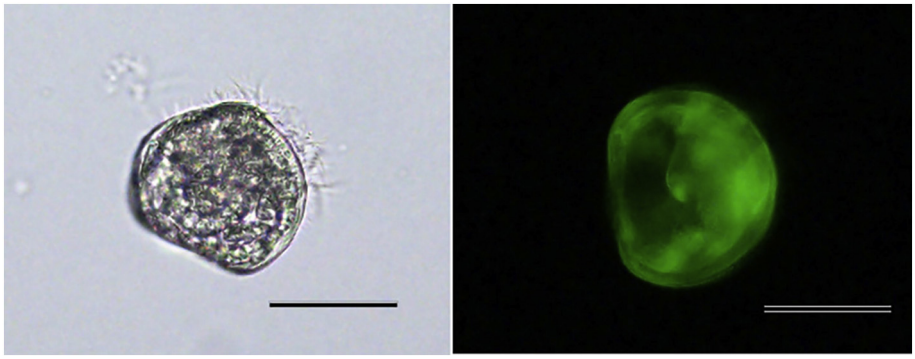

Fig. 1. Fluorescence images of oyster embryos microinjected with EGFP mRNA at the concentration of 1000 ng/μl. D-larvae under white light (left) and fluorescence images of D-larvae (right). Bar = 50 μm.

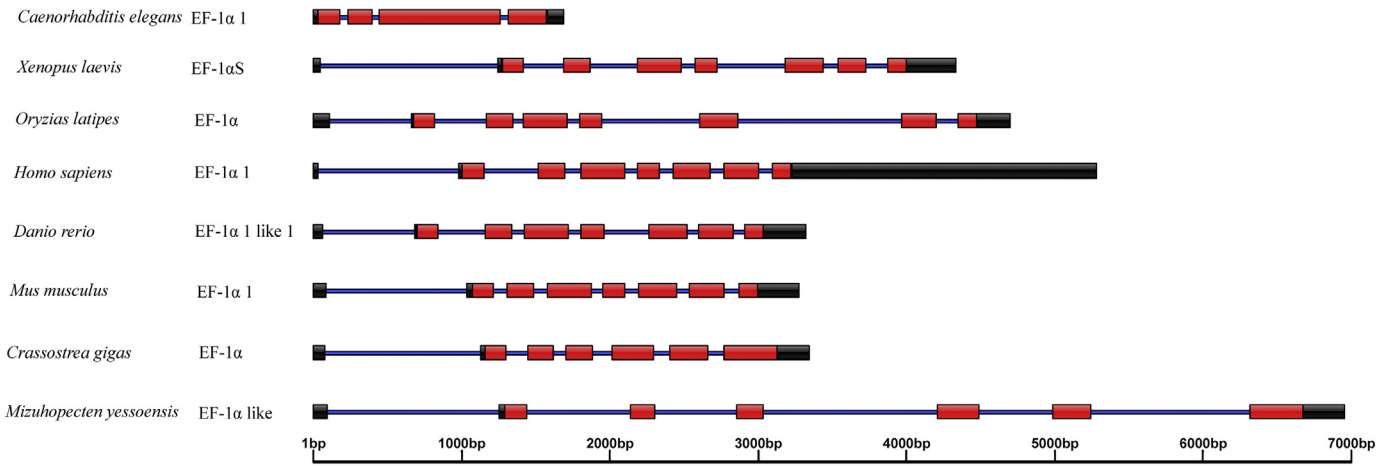

Fig. 2. The organization of *C. gigas* EF-1α gene and representative EF-1α genes from other species. Black boxes, red boxes and the blue lines represent the non-coding regions of exons, protein-coding regions and introns, respectively. The size is indicated by a scale plate at the bottom of this figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

blastulae, gastrulae, trochophore and D-larvae (Fig. 5). EGFP signal gradually increased at later stages and D-larvae showed the strong EGFP expression at 24 hpf.

To test whether the first intron sequence located upstream of the ATG start codon is critical for *C. gigas* EF-1α promoter activity, we generated a second DNA construct p(−2.3 kb EF1α:EGFP) by cloning the 2384 bp 5′-flanking without the first intron into the pEGFP-1 vector (Fig. 3). In contrast to p(−3.4 kb EF1α:EGFP), injection of the p (−2.3 kb EF1α:EGFP) construct into oyster embryos showed no detectable levels of EGFP expression. This demonstrated that that the 3465 bp 5′-flanking sequence including the first intron is critical for EF-1α gene expression.

4. Discussion

In this study, we tested the promoter activity of two commonly used promoters, the CMV promoter and the Xenopus EF-1α promoter in oyster embryos. The data showed that both exogenous promoters failed to direct EGFP expression in Pacific oyster embryos. To identify a promoter that is active in oysters, we isolated the *C. gigas* EF-1α gene and characterized its promoter activity in oyster embryos. Our data revealed that the endogenous EF-1α promoter could induce efficient EGFP expression in oyster embryos. The identification of this ubiquitous promoter provides a useful tool for gene overexpression studies to uncover gene function in bivalves.

4.1. Low activity of CMV promoter and Xenopus EF-1α promoter in oyster embryos

The viral CMV and EF-1α promoters are commonly used promoters to drive gene expression in various *in vitro* and *in vivo* systems in vertebrates. It has been reported that the CMV promoter could be silenced in rapidly proliferating cells (such as embryonic stem cells), thus causing problems of no expression in certain cell types (Teschendorf et al., 2002). Our data revealed that the CMV promoter in construct pCS2:EGFP was not effective in driving EGFP expression in oyster embryos. The poor activity of CMV promoter in oyster is consistent with previous studies in DNA transfection in oyster cell culture and early embryos using liposomes and high velocity particle bombardment, respectively (Boulo et al., 1996; Cadoret et al., 1997). The reason is not known at present. We speculate that the silencing of CMV promoter in oyster might be associated with epigenetic modification *via* DNA methylation and/or histone modification because CMV promoter silencing has been observed in other species, such as mice (Brooks et al., 2004; Mehta et al., 2009).

In comparison with the CMV promoter, the activity of EF-1α promoter tends to be more stable. The EF-1α promoter derived from *Xenopus laevis* EF-1α gene has been routinely used in driving gene expression in embryos of many vertebrate species. However, the application of EF-1α promoter has not been extensively reported in invertebrate species. Our data demonstrated that the Xenopus EF-1α promoter was silenced as CMV promoter in oyster embryos. The EF-1α promoter in construct piggBac system has also been used to drive

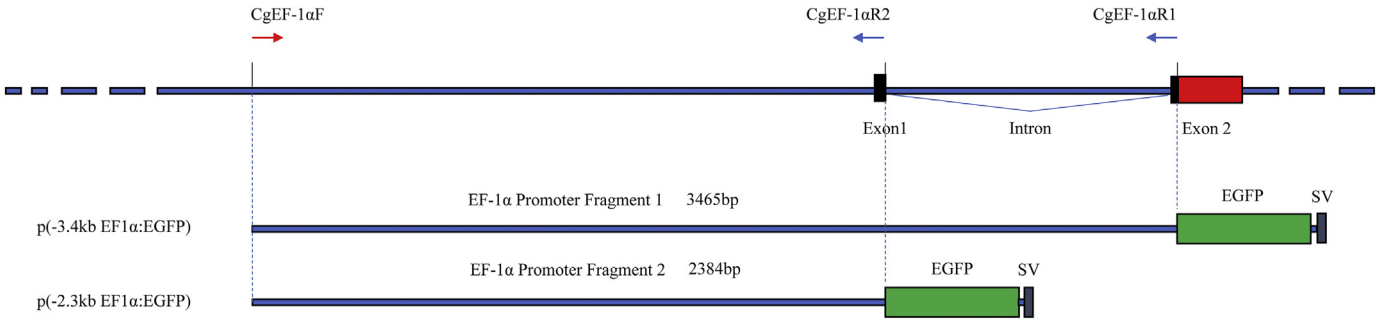

Fig. 3. Diagram of plasmid DNA construction. The EF-1α Promoter Fragment 1 prior to the first ATG codon was amplified using CgEF-1αF forward and CgEF-1αR1 reverse primers. The EF-1α Promoter Fragment 2 containing Exon 1 was amplified using CgEF-1αF forward and CgEF-1αR2 reverse primers. Red and blue arrows show the position of forward primer and reverse primers. Black boxes show the non-coding region of exons and the red one represents the protein-coding region in Exon 2. The DNA construct p(−3.4 kb EF1α:EGFP) and p(−2.3 kb EF1α:EGFP) showed the constructed plasmids that inserted with EF-1α Promoter Fragment 1 and EF-1α Promoter Fragment 2, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

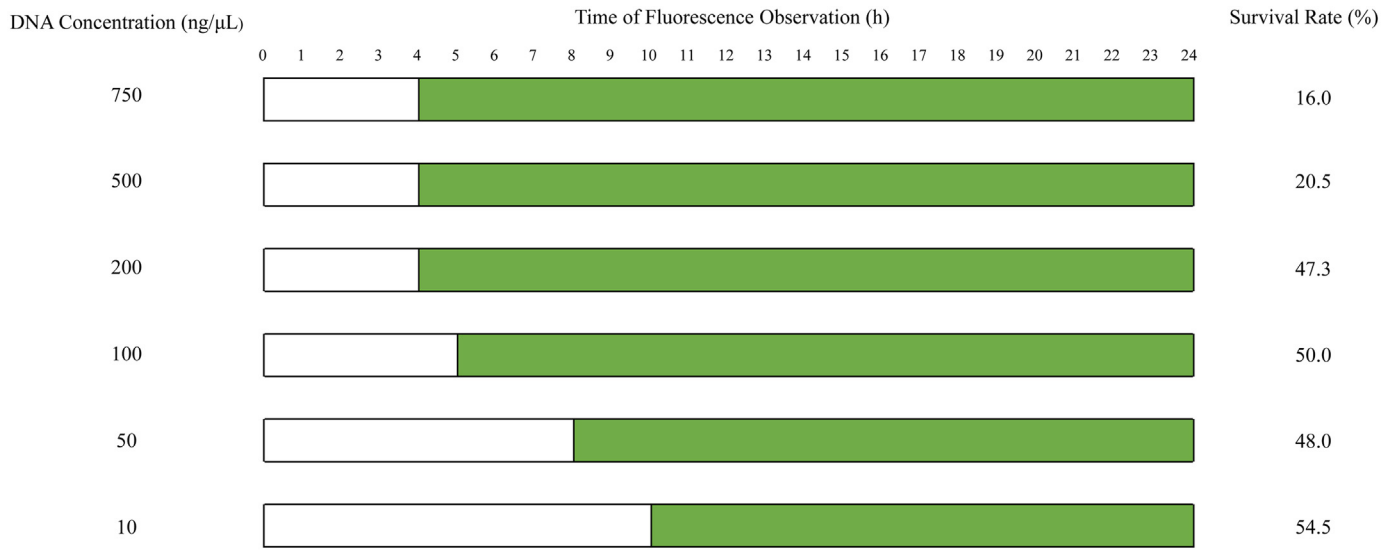

Fig. 4. Observation of EGFP expression in oyster embryos microinjected with p(−3.4 kb EF1α:EGFP). From left to right, the columns represent DNA concentration used for microinjection, fluorescence detection time after microinjection and survival rate of oyster embryos, respectively. Green boxes mean that the green fluorescence could be observed and blank boxes show no fluorescence detected in embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reporter gene expression in oyster embryos through DNA electroporation. The resulting GFP expression efficiency was extremely low, less than 1% (Chen et al., 2018). Together, these data indicate that these exogenous promoters might not be able to direct high levels of gene expression in oyster embryos.

4.2. Strong activity of C. gigas EF-1α promoter and the necessity of the first intron

Gain of function analysis by gene overexpression is a powerful approach to study gene function. We showed in this study that the EF-1α gene promoter from *C. gigas* is a suitable active promoter for driving gene expression in oysters. We found that initial EGFP expression was detected around the morulae stage in the injected oyster embryos. It has been reported that the zygotic gene transcription starts at the mid-blastula stage in zebrafish and medaka embryos, and EF-1α promoter could direct foreign gene expression from mid-blastula stage (Kinoshita et al., 2000). By comparison, the exogenous gene expression driven by EF-1α promoter seemed a bit earlier in oyster embryos compared with that in zebrafish and medaka.

Data in this study revealed that the first intron is essential for EF-1α promoter activity of *C. gigas*. It has been shown in cultured mammalian cells that the human EF-1α gene promoter with the first intron was more active than the promoter without the first intron in driving reporter gene expression (Kim et al., 1990). Subsequent analysis suggested that the 5′-flanking region and the first intron was essential for human EF-1α promoter activity (Wakabayashi-Ito and Nagata, 1994). Moreover, the human EF-1α first intron could enhance expression of foreign genes from the murine cytomegalovirus promoter (Seon-Young et al., 2002). Previous studies in medaka embryos also discovered that the medaka EF-1α promoter containing the first intron showed a stronger activity and the deletion of intron 1 decreased the promoter activity (Kinoshita et al., 2000). Together, these data indicate that it is probably better to include the first intron sequence for efficient EF-1α promoter activity.

4.3. The DPE core promoter element of C. gigas EF-1α

The regulatory elements in the *C. gigas* EF-1α promoter were analyzed. No typical TATA box core element was identified. Interestingly, a DPE was found at downstream of the potential transcription start site. The TATA box is one of the most extensively characterized eukaryotic core promoter elements that is responsible for basal transcription activity. Without the typical TATA box, the DPE serves as a functional analogue to TATA box (Xu et al., 2016). DPE is often located about 30 bp downstream of the transcription start site, which is characterized by a consensus G-A/T-C-G sequence (Burke and Kadonaga, 1996, 1997). The DPE has been suggested to function cooperatively with the initiator for the binding of transcription factor II D (TFIID) and to direct accurate transcription initiation (Burke and Kadonaga, 1996, 1997). It has been suggested that the DPE sequence might be as common as the TATA box in Drosophila core promoters (Kutach and Kadonaga, 2000). The identification of potential DPE sequence motif in the oyster EF-1α promoter indicated that DPE, rather than a TATA box, might play a role as core promoter element.

5. Conclusion

In this study, we compared the promoter activity of CMV, Xenopus EF-1α and oyster EF-1α in the Pacific oyster embryos. The most significant outcome of this study is the demonstration of *C. gigas* EF-1α promoter in driving reporter gene expression in oyster embryos and the requirement of first intron for its strong promoter activity. Identification of this active ubiquitous EF-1α promoter is expected to contribute to gene overexpression studies for function analysis in *C. gigas* and other bivalve species.

Author contributions

Conceived and designed the experiments: QL. Performed the experiments: CY, HY and HL. Analyzed the data: CY, HY, HL and SD. Wrote and revised the paper: CY, QL and SD.

Declaration of Competing Interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

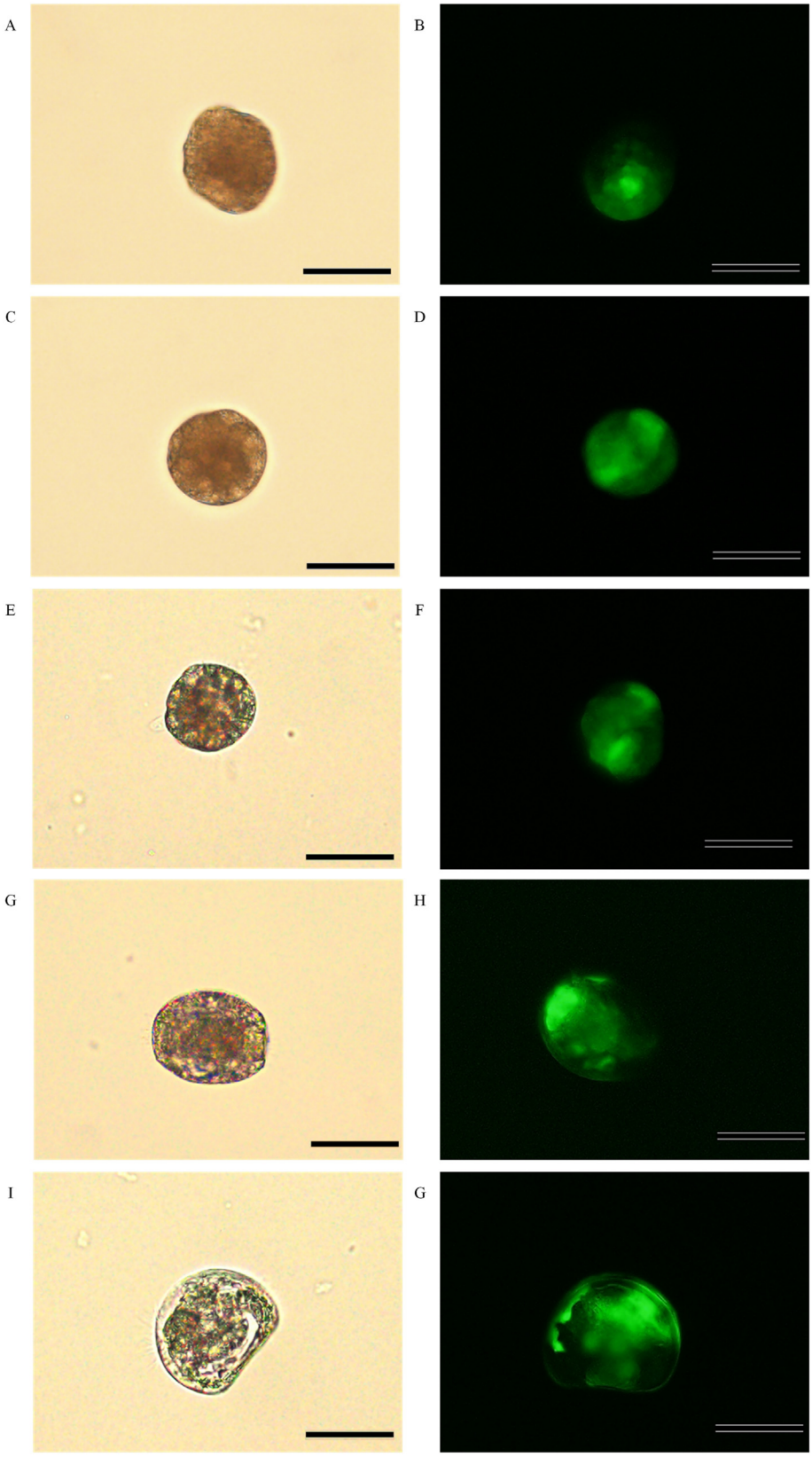

Fig. 5. Fluorescence images of oyster embryos microinjected with p(−3.4 kb EF1α:EGFP) at 200 ng/μl. (A, C, E, G, I) Morulae, blastulae, gastrulae, trochophores and D-larvae under white light. (B, D, F, H and G) Fluorescence images of morulae, blastulae, gastrulae, trochophores and D-larvae. Bar = 50 μm.

Acknowledgments

This research was supported by grants from National Natural Science Foundation of China (31972789 and 31672649), Shandong Province (2017LZGC009), Weihai City (2018NS01), and the Ocean University of China-Auburn University Joint Research Center for Aquaculture and Environmental Science.

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