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Knockdown of Actin and Caspase Gene Expression by RNA Interference in the Symbiotic Anemone *Aiptasia pallida*

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Abstract. Since the discovery of the ancient eukaryotic process of RNA-mediated gene silencing, the reverse-genetics technique RNA interference (RNAi) has increasingly been used to examine gene function in vertebrate and invertebrate systems. In this study, we report on the use of RNAi, adapted from studies on animal model systems, to manipulate gene expression in a symbiotic marine cnidarian. We describe gene knockdown of actin and of acasp—a cysteine protease, or caspase—in the symbiotic sea anemone *Aiptasia pallida.* Knockdown was assessed qualitatively with *in situ* hybridizations for both genes. Quantitative PCR and caspase activity assays were used as a quantitative measure of knockdown for *acasp.*

Introduction

RNA interference (RNAi) was introduced by Fire *et al.* (1998) after experimental injection of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* resulted in specific silencing or "interference" of genes homologous to the dsRNA. Discovery of this mechanism of gene regulation earned Fire and Mello the Nobel prize in 2006. RNAi has since been shown to exist in plants, fungi, other invertebrates, and vertebrates, indicating that it is a highly conserved process. RNAi can control gene expression and act as a molecular immune system against viral dsRNA, transposons, retrotransposons, and retrovirus infection within eukaryotes (Hamilton and Baulcombe, 1999; Plasterk, 2002; Bagasra and Prilliman, 2004).

Since the discovery of RNAi, its functions and potential applications have been widely explored. The use of reverse genetics is now established in functional investigations in plants (Kusaba, 2004), invertebrates (Lamitina, 2006), and vertebrates (Silva *et al.,* 2004). It is being used in the treatment of diseases such as cancer (Gartel and Kandel, 2006), neurological disorders (Boudreau and Davidson, 2006), and viral infections (Tan and Yin, 2004). We were interested in developing RNAi in the model marine symbiotic cnidarian *Aiptasia pallida* (Verrill 1864) to aid in examinations of gene function. Previous studies have explored the use of RNAi in the freshwater cnidarian *Hydra magnipapillata,* using electroporation transformation of dsRNA (Lohmann *et al.,* 1999; Smith *et al.,* 2000; Cardenas and Salgado, 2003). The authors of those studies acknowledged that the electroporation technique can damage tissues and cells; however, in a more recent study (Amimoto *et al.,* 2006), polyps survived for at least 6 days after transfection. Here we describe an alternative technique of chemical transfection to deliver dsRNA into *A. pallida.*

Caspases are a family of cysteine proteases that act in a highly specific proteolytic cascade as part of the inflammatory response and cell death (Thornberry and Lazebnik, 1998). Programmed cell death and apoptosis are intrinsically mediated pathways leading to deletion of specific cells. These ancient and highly conserved processes, present in all multicellular organisms, play a key role in tissue homeostasis, health, development, growth, and responses to stress and pathogens. Cnidarian apoptosis, initially identified by Cikala *et al.* (1999), is active in different cellular processes in freshwater *Hydra* (David *et al.,* 2005) and in

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Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference.

bleaching of symbiotic *A. pallida* (Dunn *et al.,* 2002, 2004). However, the evolutionary origin of these pathways remains elusive. Recent bioinformatics evidence of two apoptotic genes suggests that cnidarian apoptosis is closely linked to vertebrate apoptosis (Dunn *et al.,* 2006). We were interested in developing reverse-genetics techniques as a part of an effort to understand the apoptotic role of caspases in the symbiotic *A. pallida.* In this study we report on the development of RNAi in *A. pallida* and on successful knockdown of both actin and *acasp,* a caspase. This is the first study to knock down a gene associated with cnidarian apoptosis.

Materials and Methods

Synthesis of double-stranded RNA

Double-stranded RNA (dsRNA) was synthesized for both the caspase, *acasp,* and β actin from *Aiptasia pallida.* For this process, PCR products were generated for specific regions of *acasp* and actin, using primers tagged with T7 sequences for dsRNA synthesis. The *acasp* forward primer (5′-TAATACGACTCACTATGGGTCCATCCAAGGTCCGTTGA-3′) and reverse primer (5′-TAATACGACTCACTATGGCTGGAGGTGCATCAGTTGC-3′) generated a 600-bp product. The β actin forward primer (5′-TAATACGACTCACTATAGGGGATGGACAGGTCATCACCA-3′) and reverse primer (5′-TAATACGACTCACTATAGGGTGCTTGGAGATCCACATCTG-3′) generated a 340-bp product. The PCR products were cleaned with Montage filters (Millipore), 1:1 phenol (pH 7.8)/chloroform, followed by chloroform only with Phase Lock Gel tubes (PLG, Eppendorf) and centrifuged at 12,000 $\times$ *g* for 30 min. The supernatants were precipitated using 2.2 volume of 100% EtOH and 0.1 volume of 3 mol l^{-1} NaAc treated with dimethyl pyrocarbonate (DMPC). Samples were centrifuged at 12,000 $\times$ *g* for 10 min; pellets were washed with DMPC-treated 70% EtOH, re-centrifuged, and then air-dried. DNA pellets were resuspended in DMPC-H_2O and quantified using a Beckman DU530 spectrophotometer. The dsRNA synthesis mixture was adapted from the T7 RNA polymerase protocol (MBI Fermentas). A master mix consisted of 3 μg of PCR product, 90 μl of 5$\times$ transcription buffer (MBI Fermentas), 90 μl of 10 mmol l^{-1} nucleotide triphosphates (MBI Fermentas), 12 μl of RNAsin (Promega), 15 μl of T7 RNA polymerase (MBI Fermentas), and distilled water (dH_2O) to a final volume of 450 μl. The mix was subdivided into three aliquots and incubated at 37 °C for 2 h. Afterward, 1 μl of DNAse (Gibco) was added to each aliquot and incubated at 37 °C for 15 min. Aliquots of dsRNA were then placed in PLG tubes and cleaned with 1:1 phenol (pH 4.5)/chloroform, followed by chloroform only. The dsRNA was precipitated, washed with 70% EtOH, resuspended in 10 μl of dH_2O, measured using an ND1000 spectrophotometer (NanoDrop), and adjusted with sterile filtered seawater to the required concentration. In initial trials, several concentrations (0.5 μg/μl, 0.75 μg/μl, 1 μg/μl, and 1.25 μg/μl) of both *acasp* and actin were applied to anemones over 24- and 48-h periods. After these optimization trials, concentrations of 0.8 μg/μl for *acasp* and 1 μg/μl for β actin were used.

Incubation of anemones in double-stranded RNA

Anemones about 1 cm in height were removed from batch culture (26 $\pm$ 0.5 °C, practical salinity 34, photon flux density 50 μmol quanta m^{-2} s^{-1}) and placed in individual wells of a 24-well plate (Falcon Becton Dickinson) with 100 μl of sterile seawater. The liposomal compound 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide linked to cholesterol (DMRIE-C; Invitrogen/Gibco) was used as an RNA transfection agent. Once the anemones had reacclimatized, the sterile seawater was removed and replaced with between 50 and 70 μl of a dsRNA/DMRIE-C (0.15 μl per 50 μl) seawater solution to a level that allowed the anemone to remain submerged. After 24 and 48 h, 100 μl of sterile seawater was added to each well.

After 72 h of incubation, anemones were removed and processed for tissue sectioning and *in situ* hybridization or for the caspase activity assay. Control treatments were (1) no dsRNA; (2) a non-reversible caspase inhibitor Z-Val-Ala-Asp-fluormethylketone (ZVAD-fmk, Enzyme Systems Products, final concentrations: 12.5 μmol l^{-1} and 25 μmol l^{-1} in 0.2% DMSO); and (3) DMRIE-C only, in seawater. For a positive control, dsRNA-treated and non-dsRNA-treated anemones were incubated at a hyperthermic stress of 33–34 °C, which has previously been shown to induce apoptosis (Dunn *et al.,* 2004).

Synthesis of digoxigenin RNA probe

To observe localization and treatment knockdown of acasp and actin, digoxigenin (DIG)-labeled RNA probes were synthesized and used in *in situ* hybridizations of *A. pallida* tissue sections.

Sense and antisense DIG probes were synthesized from PCR products generated from *A. pallida* cDNA. The *acasp* sense PCR product was generated using a T7-tagged forward primer (5′-TAATACGACTCACTATGGGTCCATCCAAGGTCCGTTGA-3′) and a reverse primer (5′-CTGGAGGTGCATCAGTTGC-3′). For the *acasp* antisense product, the forward primer (5′-GTCCATCCAAGGTCCGTTGA-3′) and a T7-tagged reverse primer (5′-TAATACGACTCACTATGGCTGGAGGTGCATCAGTTGC-3′) were used. The actin sense PCR product was generated using a T7-tagged forward primer (5′-TAATACGACTCACTATAGGGACCAACTGGGATGACATGGA-3′) and a reverse primer (5′-CGCTCTGTCAGGATCTTCAT-3′) and for the actin antisense, a forward primer (5′-ACCAACTGGGATGACATGGA-3′) and a T7-tagged reverse primer (5′-TAATACGACTCACTATAGGGCGCTCTGT-

CAGGATCTTCA-3′) were used. The PCR products were cleaned as above. DIG RNA was synthesized using a mix of 300 ng of DNA template, T7 reaction buffer (Fermentas), T7 RNA polymerase (Fermentas), DIG RNA label mix (Enzo, Roche), RNAse out (Promega), and DMPC H_2O to a final volume of 20 μl. Reactions were incubated at 37 °C for 2 h, then 1000 units of DNAse I (Gibco) was added to each preparation and incubated at 37 °C for 15 min. DIG RNA preparation quality was visualized by resolving a subsample on an agarose gel. The remainder of each DIG RNA preparation was precipitated using 0.2 volume of 0.5 mol l^{-1} EDTA, 0.1 volume of 4 mol l^{-1} LiCl, and 2.2 volume of 100% EtOH and stored at −20 °C for 2 hours. Samples were thawed and centrifuged at 12,000 × *g* for 30 min. DIG RNA pellets were resuspended and washed with DMPC-70% EtOH and re-centrifuged prior to air drying and re-suspension in DMPC H_2O. DIG RNA probe concentration was determined using dot blots of a serial dilution of stock DIG-labeled RNA (Enzo-Roche) according to the manufacturer's instructions.

Sectioning and in situ *hybridization of tissue*

Whole anemones were embedded in TBS tissue freezing medium (Triangle Biomedical Sciences) and stored at −80 °C. Tissue sections (20–25 μm) were produced using a 2800 Frigocut N cryostat microtome (Reichert-Jung), mounted within a PAP pen (Daido Sangyo Co, Ltd) restricted area of poly-L-lysine coated slides (LabScientific, Inc), and returned to −80 °C. Slides were later removed from the −80 °C, allowed to briefly thaw, and then placed into a Coplin jar containing 4% paraformaldehyde in phosphate buffered saline (PBS, 2 mmol l^{-1} NaH_2PO_4, 7.7 mmol l^{-1} Na_2HPO_4, 0.14 mol l^{-1} NaCl in DMPC-H_2O, pH 7.0) for 45 min at room temperature. Slides were washed twice in PBST (1% Triton X-100 in PBS) for 5 min before a MeOH dehydration series (25%, 50%, 75%, 100%, 75%, 50%, 25%) in PBST for 2 min. Slides were rinsed in PBST twice for 5 min and once in 0.1 mol l^{-1} TEA (1.33% triethanolamine, 0.3% HCl) for 5 min, incubated in acetylation buffer (0.25% acetic anhydride in 0.1 mol l^{-1} TEA) for 10 min, and then given two 5-min PBST rinses.

For pre-hybridization, sections were incubated in hybridization buffer (50% formamide, 10% 2× SSC [0.3 mol l^{-1} NaCl, 30 mmol l^{-1} sodium citrate, pH 7.0], 2.5% tRNA 10 mg ml^{-1}, 2% 50× Denhardt's solution, 10% dextran sulfate, 0.25% 10 mg ml^{-1} denatured salmon sperm) in a humid chamber at 37 °C for 45 min. During the pre-hybridization, antisense and sense probes in hybridization buffer (final concentration 0.5 μg ml^{-1}) were heated at 80 °C for 10 min and then placed briefly on ice prior to application. The pre-hybridization solution was removed and replaced with the probe in hybridization buffer and incubated within a humid chamber at 55 °C overnight. The following morning, the probe was removed and the slides were placed in a Coplin jar containing 2× SSC and 50% formamide for 5 min at 52 °C. This was followed by two 15-min rinses of 2× SSC at 37 °C, followed by two 15-min rinses of 1× SSC at 37 °C. Slides were then placed in a separate Coplin jar containing a 37 °C pre-warmed RNAse solution (2% RNAse A 10 μg ml^{-1} [Promega], 0.2% RNAse T [Sigma] in NTE [0.5 mol l^{-1} NaCl, 10 mmol l^{-1} Tris pH 8]) for 30 min. Slides were rinsed twice in 0.1× SSC at 37 °C for 5 min, followed by two 10-min rinses in Buffer 1 (100 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, pH 7.5). Slides were then incubated in blocking solution (0.1% Triton X-100, 2% sheep serum in Buffer 1) for 45 min at room temperature. The blocking solution was replaced with anti-DIG antibody solution (0.1% Triton X-100, 1% sheep serum, 0.001× Anti-Dig Digoxigenin-AP FAB conjugate, Enzo-Roche, in Buffer 1) and incubated for 1.25 h. Slides were removed from the chamber and rinsed twice in Buffer 1 for 10 min, followed by a rinse in NTMT buffer (0.58 mmol l^{-1} NaCl, 0.05 mmol l^{-1} $MgCl_2$, 1.21 mmol l^{-1} Tris, 0.1% Triton X-100, pH 9.5) for 10 min. Slides were incubated in detection buffer (1:2 BCIP:NBT, Calbiochem, 1 mmol l^{-1} levamisole in NTMT) until color precipitation was observed. Staining was stopped by rinsing in dH_2O prior to mounting with glycerol mountant. Sections were viewed using an Olympus Vanox-T AH2 microscope and Sony DKL 5000 digital camera.

Quantitative real-time-PCR

To quantify changes in transcript level as a function of incubation in *acasp* dsRNA, quantitative real-time-PCR (QPCR) was performed on control and *acasp* dsRNA-incubated animals. Three anemones were incubated per treatment for 48 h. Specific primers for *acasp* and β actin were designed for real-time PCR amplification. The *acasp* forward primer (5′-GAATGGGCATGGTTATCCA-3′) and reverse primer (5′-TCGTTAGTCCTTGACTGGTG-3′) and the β actin forward (5′-CTGATGGACAGGTCATCAC-CAT-3′) and reverse (5′-CTCGTGGATACCAGCAGAT-TCC-3′) primers resulted in products of approximately 100 bp in length. Total RNA was extracted and 500 ng per sample was used for cDNA synthesis. One microliter of cDNA was used with 5 μmol l^{-1} of the above primers, dH_2O, and iQ SYBR Green Supermix (BioRad) and ROX (Invitrogen) in a 20-μl reaction amplified using an ABI Prism 7000 sequence detection system (Applied Biosystems). Reactions were performed in triplicate. PCR efficiency was determined using LinReg PCR (Ramakers *et al.,* 2003) and normalized using delta QT of *acasp* and β actin, a housekeeping gene, as previously described by Rodriguez-Lanetty *et al.* (2006). Log10-transformed data were expressed as a ratio of *acasp* to actin quantity. Statistical

analysis using a paired *t*-test was undertaken with Minitab (ver. 12) statistical software (Minitab Inc).

Assay of caspase activity

The caspase *acasp* from *A. pallida* is homologous in deduced amino acid sequence and secondary structure to vertebrate executioner caspase 3 (Dunn *et al.,* 2006). Therefore, to investigate knockdown in *acasp,* a caspase 3 activity assay (ApoAlert, BD Bioscience Clontech) was used on anemone extracts. After experimental incubation, individual anemones were homogenized in 150 μl of cell lysis buffer according to the manufacturer's instructions. For the remainder of the protocol, the sample and working volumes were reduced by half. The remaining homogenate was frozen for protein measurement. Caspase activity was measured in units of fluorescence (FU), using a SpectraMAX Gemini XS fluorometer and SOFTmax Pro (ver. 4) software (Molecular Devices). The total protein concentration of each sample was measured using a Micro BCA protein assay kit (Pierce), a BSA protein standard (Pierce), and a Vmax Kinetic spectrophotometer microplate reader and SOFTMax software (ver. 2.34, Molecular Devices). Caspase activity therefore was expressed as FU · mg anemone protein^{-1}. Data from the caspase activity assay were tested for normality and heteroscedasticity using Minitab (ver. 12) statistical software (Minitab Inc.). Log10-transformed data were analyzed using one-way ANOVA and Tukey *post hoc* pairwise comparison.

Results

Optimization of acasp *and* β *actin double-stranded RNA concentration and phenotypic effects*

In initial trials, anemones treated with either dsRNA at concentrations of 1.25 μg/μl died within 24 h. Anemones incubated in 1.25 μg/μl β actin dsRNA displayed a shrunken morphology followed by fatal widespread tissue disassociation during the incubation period. Anemones incubated in 1.0 or 1.25 μg/μl of *acasp* dsRNA displayed the same shrunken morphology followed by widespread loss of algae and mortality. Anemones incubated in 0.5 and 0.75 μg/μl of β actin dsRNA and 0.5 μg/μl *acasp* dsRNA showed no phenotypic change, and any effect of RNAi was undetectable. At maximum concentrations of 0.8 and 1.0 μg/μl for *acasp* and β actin, respectively, anemones displayed the shrunken "stress" morphology but then recovered after 48 h. These concentrations of dsRNA were used in future assays.

Acasp *and actin* in situ *hybridization, localization, and knockdown*

Knockdown of *acasp* transcript in *Aiptasia pallida* (n = 3) tissues was observed by *in situ* hybridization. Hybridizations with *acasp* antisense probes of all control anemone sections strongly labeled endoderm in both column and tentacles (Fig. 1A, B). No labeling was seen in sections hybridized with negative control *acasp* sense probes (Fig. 1C). In contrast to the controls, sections from all *acasp* dsRNA-incubated animals showed greatly reduced hybridization with the antisense *acasp* (Fig. 1D). Knockdown of actin transcript was also demonstrated. Whereas control animals that were hybridized with actin antisense probes labeled strongly in the mesenteries, which are rich in epitheliomuscular cells (Fig. 1E), actin dsRNA-incubated animals showed almost no hybridization with actin antisense probes (Fig. 1F).

Quantitative PCR

The average ratio (n = 8) of *acasp* to β actin transcript quantity was 0.17 in untreated controls compared to 0.51 in DMRIE-C controls. These ratios were not significantly different (P = 0.068). However, the ratio of transcripts in *acasp* dsRNA-treated anemones was 0.05, significantly lower than untreated controls (P = 0.02; Fig. 2) and DMRIE-C controls (P = 0.012) (data not shown). Three of the 8 anemones incubated in dsRNA were within $\pm$ 1 standard deviation of the control mean.

Assay of acasp *caspase activity*

The fluorometric assay of caspase 3 activity, which detects activated caspase 3, showed that there was a significant difference in activity between controls and treatments (one-way ANOVA $_{(6)}$ $P < 0.0001$). Caspase activity in control animals averaged 44.4 $\pm$ 1.7 FU · mg protein^{-1} (Table 1, Fig. 3). In contrast, anemones subjected to a hyperthermic stress had a significantly higher caspase activity of 64.4 $\pm$ 4.7 FU · mg protein^{-1}, a 45% increase over controls. Caspase activity was reduced in all anemones incubated in dsRNA when compared to individuals from the control. Anemones treated with the caspase inhibitor ZVAD-fmk exhibited a significant dose-dependent decrease in activity. Activities of those treated with 12.5 μmol l^{-1} ZVAD-fmk decreased by 35%, to 28.7 $\pm$ 3.1 FU · mg protein^{-1}, and those treated with 25 μmol l^{-1} ZVAD-fmk decreased by 87%, to 5.5 $\pm$ 0.0 FU · mg protein^{-1}. RNAi using *acasp* dsRNA was effective at knocking down caspase activity, as indicated by a significant 30% decrease in activity, to 31.1 $\pm$ 1.1 FU · mg protein^{-1}, in *acasp* dsRNA-incubated animals. *Acasp* RNAi was even effective in animals subjected to a hyperthermic stress. The mean activity in these animals of 30.1 $\pm$ 1.7 was 31% lower than in controls and 53% lower than in hyperthermic-stressed animals. Caspase activities in animals incubated in 12.5 μmol l^{-1} ZVAD-fmk and those incubated with *acasp* dsRNA were not significantly different, suggesting that

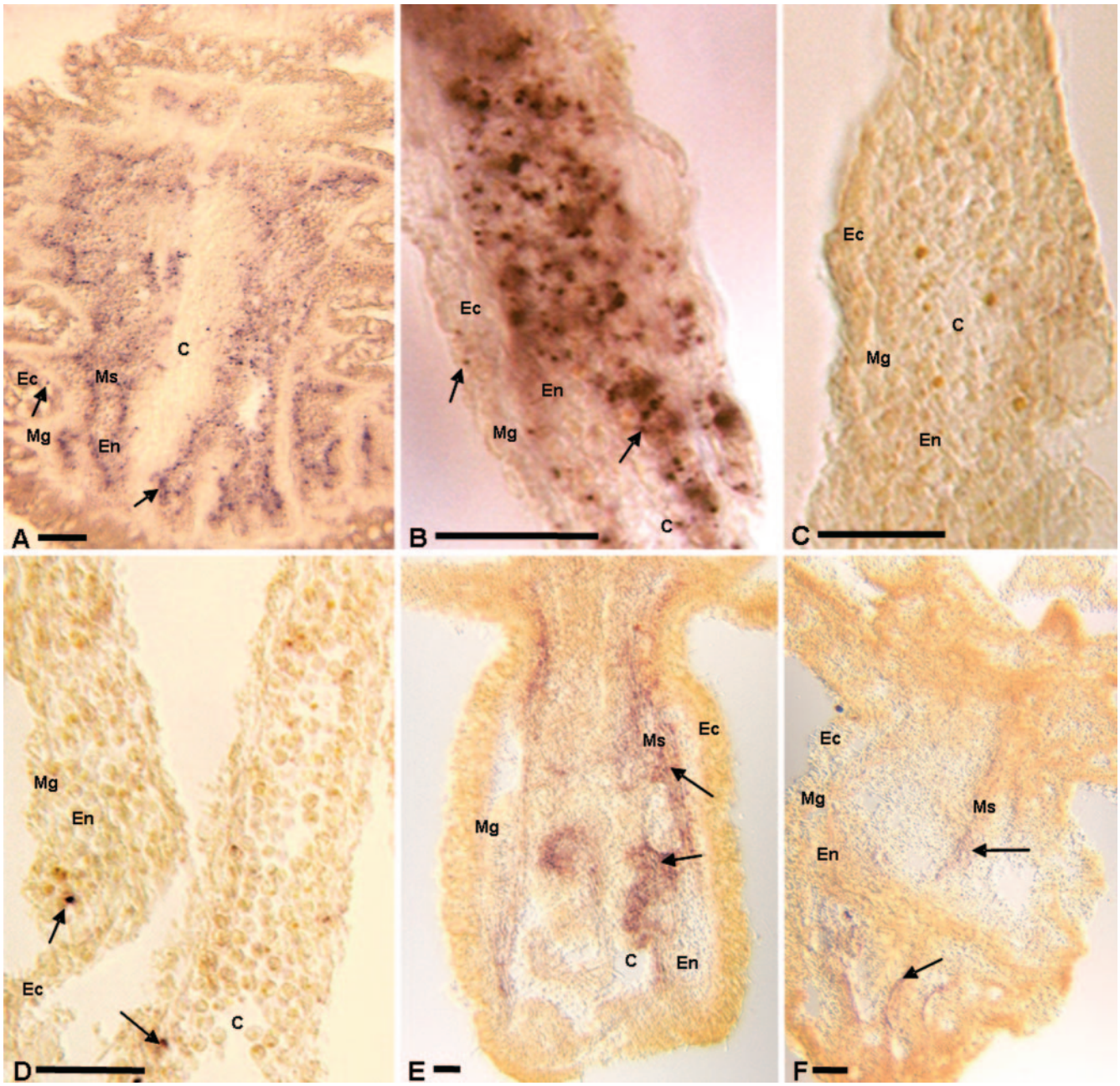

Figure 1. *In situ* hybridizations of *Aiptasia pallida* tissue sections with *acasp* and actin probes. Localization of probes is shown by arrows in *acasp* antisense-treated column (A) and tentacle (B), and in *acasp* sense-treated tentacle tissue (C) and *acasp* dsRNA-treated tentacle tissues (D). Actin antisense localization is shown by arrows in control (E) and actin dsRNA-treated (F) tissue sections. C = coelenteron, Ec = ectoderm, En = endoderm, Mg = mesoglea, and Ms = mesenteries. Scale bar = 80 μm.

reduced caspase activity from RNAi knockdown was comparable to that at the low concentration of inhibitor. Finally, the mean caspase activity of anemones incubated with actin dsRNA (46.5 $\pm$ 3.5 FU $\cdot$ mg protein^{-1}) was not significantly different from activity in control anemones, indicating that β actin dsRNA did not have a nonspecific effect of silencing caspase activity.

Discussion

In this study we report the successful use of an RNAi technique for gene knockdown in the symbiotic sea anemone *Aiptasia pallida.* Specifically, dsRNA synthesized from *acasp,* a caspase with homology to vertebrate apoptotic executioner caspase 3 (Dunn *et al.,* 2006), achieved RNAi

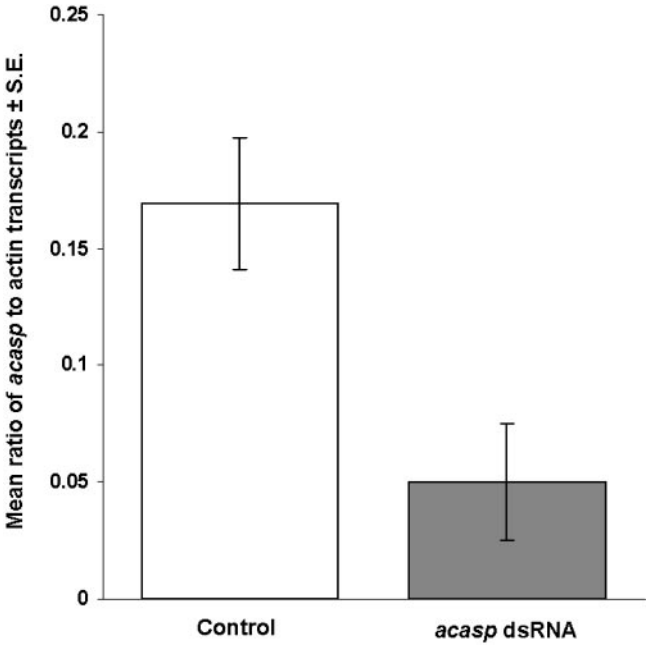

Figure 2. The mean ratio of *acasp* to actin transcripts in untreated controls and dsRNA treated anemones, measured by QPCR. $n = 8$ anemones.

of caspase 3 activity, using an optimized chemical transfection delivery. The advantage of this method is that it overcomes the problems associated with electroporation in high-saline media. This success enables future in-depth studies of gene function in cellular and physiological processes in *A. pallida* and other marine cnidarians.

During optimization of dsRNA, initial trials of both *acasp* and β actin dsRNA showed no significant change in phenotype or successful knockdown at the lowest concentrations, but deleterious phenotypic effects—including cell death, tissue degradation, and animal mortality—were seen at the highest concentrations. Low dsRNA concentrations likely failed to achieve a successful transformation or a strong enough gene-targeting response. At high dsRNA concentrations, although the target gene may have been silenced, additional and possibly deleterious antiviral responses and pathways may also have been initiated (Wang and Carmichael, 2004). Optimal dsRNA concentrations were ultimately determined by observing animal phenotype. Optimal dsRNA concentration resulted in initial phenotypic change to a shrunken "stress" morphology that would disappear after 48 h. Animals incubated in these optimal concentrations for 72 h and afterward removed to sterile seawater were still alive after 2 weeks. The working concentrations of dsRNA in this study are in the same range as those used in invertebrate cell line transfection (Gesellchen *et al.,* 2005) but higher than in vertebrate microinjection (Wargelius *et al.,* 1999) or freshwater cnidarian electroporation (Lohmann *et al.,* 1999). Finally, the fact that the optimal concentrations were different for the two genes examined suggests that these optima are gene-specific and must be determined empirically for each dsRNA, as indicated by Steele (2002).

The template lengths for *acasp* and β actin dsRNAs were designed in accordance with other invertebrate studies. The length of dsRNA has previously been shown to be an important factor in obtaining efficient gene-specific RNAi. For example, in *Drosophila melanogaster* S2 cells transfected with *cyclin* E, both 400-bp and 540-bp dsRNAs were effective. However, 200-bp and 300-bp dsRNAs were less effective, and 50–100-bp dsRNAs were ineffective (Hammond *et al.,* 2000). Likewise, Elbashir *et al.* (2001) reported efficient RNAi with 39–50-bp dsRNA but not with dsRNA of 29–36-bp in size.

Acasp was more abundant in endodermal than ectodermal cells. The observed distribution of *acasp* may indicate a tissue-specific *acasp* function in processes associated with the endoderm such as digestion, symbiosis, immunity, and recognition. This is a topic for future studies. The observed reduction of *acasp* DIG RNA staining within tissues of *acasp* dsRNA-treated anemones indicated a significant reduction of available transcripts and therefore successful *acasp* RNAi. The successful *in situ* labeling of actin and the reduced labeling after delivery of actin dsRNA indicate that this RNAi technique is effective for different genes. These results were corroborated by QPCR data that indicated a

Table 1

Summary of probabilities determined by one-way ANOVA and post hoc *Tukey pairwise comparison of caspase activity assay*

Treatment	Temperature stress	12.5 μmol l^{-1} ZVAD-fmk	25 μmol l^{-1} ZVAD-fmk	*acasp* dsRNA	*acasp* dsRNA temperature stress	Actin dsRNA
Control	<0.05	<0.01	<0.001	<0.05	<0.05	>0.05
Temperature stress		<0.001	<0.001	<0.001	<0.001	>0.05
12.5 μmol l^{-1} ZVAD-fmk			<0.001	>0.05	>0.05	<0.01
25 μmol l^{-1} ZVAD-fmk				<0.001	<0.001	<0.001
acasp dsRNA					>0.05	<0.05
acasp dsRNA temperature stress						<0.05

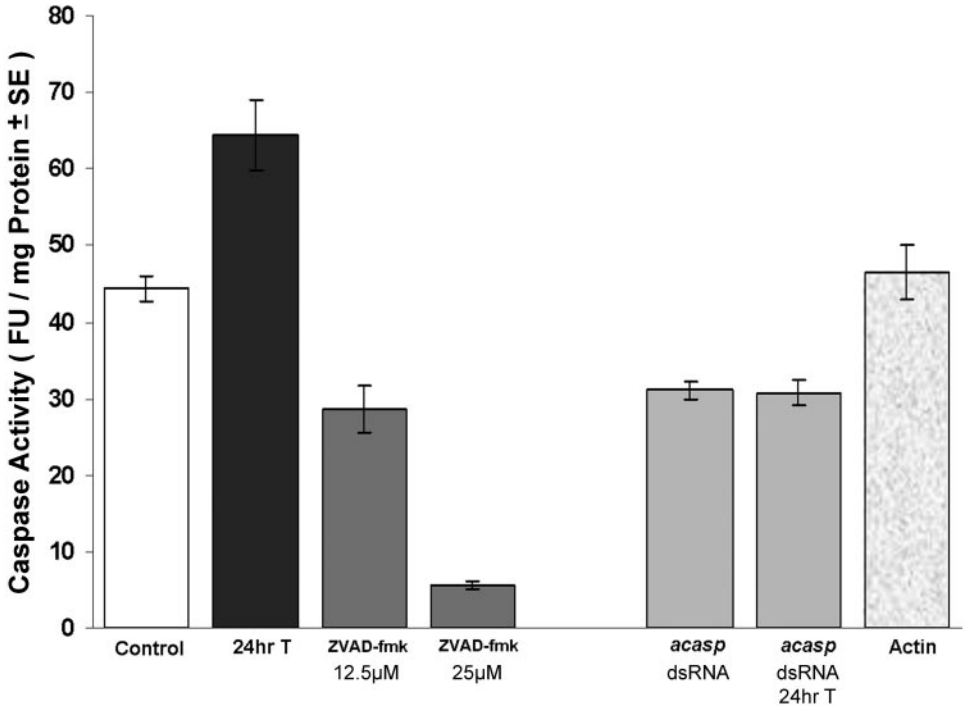

Figure 3. Fluorometric caspase 3 activity in anemones subjected to different treatments and incubated in *acasp* dsRNA to test for RNAi. From left to right, bars represent mean activities (FU · mg protein^{-1}) for animals under control conditions; at elevated temperature stress (T, 33–34 °C for 24 h); in 12.5 and 25 μmol l^{-1} ZVAD-fmk, a caspase inhibitor; in *acasp* dsRNA and *acasp* dsRNA at elevated temperature; and in actin dsRNA. $n = 5$ anemones.

significant reduction of *acasp* transcripts with dsRNA incubation (Fig. 2).

High caspase 3 activity was observed in control animals (Fig. 3). Similarly high activities have been observed in the larvae of *Hydractinia echinata* (Seipp *et al.,* 2006) and in the anemone *Anemonia viridis* (Richier *et al.,* 2006). Such high activity could indicate a high cell turnover during development, metamorphosis, and tissue homeostasis in cnidarians. Successful gene knockdown with RNAi was also demonstrated with the caspase 3 activity assay, which showed the gene-specific phenotypic knockdown of *acasp.* Anemones treated with *acasp* dsRNA had significantly less caspase 3 activity than controls. This confirms that the function of *acasp* is associated with the sequence and structure homology with vertebrate caspase 3 that was previously shown by Dunn *et al.* (2006). Hyperthermic stress, previously shown to increase apoptosis in *Aiptasia* sp. (Dunn *et al.,* 2004), induced increased caspase 3 activity in *A. pallida.* In contrast, pretreatment with *acasp* dsRNA prevented the increase in caspase 3 activity in anemones treated with the same hyperthermic stress. In future studies, prevention of caspase-mediated apoptosis will be a valuable tool in the examination of physiological responses to stress induced by environmental stimuli.

Delivery of *acasp* dsRNA did not achieve 100% knockdown; such failures are common in initial transfection. Large-scale RNAi of caspase activity may require repeated inoculations, as shown with other systems (Zender *et al.,* 2003). However, the aim of this study was to develop a method for knockdown of specific genes in a marine cnidarian for the purpose of gene-manipulation experiments. Further studies may determine the longevity of gene knockdown and whether the effect could be incorporated into progeny or enhanced by multiple applications. Evidence from the inhibitor treatments indicates that caspase activity could be inhibited beyond the knockdown achieved by a single *acasp* RNAi treatment. There was no significant difference in caspase activity between anemones treated with 12.5 μmol l^{-1} ZVAD-fmk and *acasp* dsRNA, but anemones treated with 25 μmol l^{-1} ZVAD-fmk did exhibit significantly lower activities than RNAi-treated animals. However, the further decrease in activity with the higher inhibitor concentration may not reflect inefficient *acasp* RNAi but instead may indicate the inhibition of different caspase isoforms that are not targeted by the *acasp* dsRNA. The successful delivery of actin dsRNA and resulting RNAi of actin, shown by *in situ* hybridization, had no significant effect on caspase 3 activity. This illustrates the specificity of *acasp* dsRNA delivery and RNAi.

There is increasing use of RNAi in familiar higher inver-

tebrate model systems to identify key molecular pathways (Boutros *et al.,* 2004; Agaisse *et al.,* 2005; Hamilton *et al.,* 2005; Philips *et al.,* 2005). The development and use of RNAi techniques in basal metazoan model systems such as cnidarians will help to determine the evolutionary lineage and complexity of homologous pathways such as apoptosis in higher metazoans (Golstein *et al.,* 2003; Zender and Kubicka, 2004; Gesellchen *et al.,* 2005). The use of *acasp* RNAi will also enable us to answer key questions about the roles of cnidarian apoptosis in the onset and breakdown of symbiosis. This technique has been used in recently completed large-scale experiments in which manipulation of apoptosis resulted in a marked effect on symbiosis stability (Dunn *et al.,* unpubl.). Further, using RNAi in cnidarians could help answer key questions about characteristic attributes of indeterminate growth and longevity.

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