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In silico identification of the genes for sperm-egg interaction in the internal fertilization of the newt *Cynops pyrrhogaster*

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ABSTRACT A specific sperm-egg interaction in the oviductal matrix is crucial for internal fertilization of the red-bellied newt, *Cynops pyrrhogaster*. An understanding of the molecular basis of this interaction is expected to elucidate the evolutionary history of internal fertilization in amphibians. Recently, deep sequencing technology has provided global gene information even in nonmodel animals, allowing us to understand specific features of the molecular mechanisms underlying fertilization in *C. pyrrhogaster*. In the present study, we screened *de novo* assembled RNAseq from ovary, testis, and oviduct samples in *C. pyrrhogaster* and identified the base sequences encoding zona pellucida (ZP) proteins, voltage-dependent Ca^{2+} channels, and cysteine-rich secretory proteins (CRISPs), which respectively are sperm receptors for egg envelopes, major mediators of sperm intracellular signaling, and expected extracellular modulators for sperm function in the female reproductive tract. In the ovary, ZP homologues of all six subgroups were found, including a ZP1 homologue that was newly found in amphibians, a ZP4 homologue, and six ZPC homologues. The unique combination of ZP proteins suggests a new mechanism for sperm binding to egg envelopes in the internal fertilization of *C. pyrrhogaster*. In the testis, CaV1.1, 1.2, and 3.2, which are L- and T-type voltage-dependent Ca^{2+} channels, were found as potential mediators for the internal fertilization-specific sperm-egg interaction. We also found CRISP 2 in the oviduct, which is speculated to participate in the sperm-egg interaction. These results indicate that the *de novo* assembled RNAseq is a powerful tool allowing analysis of the specific sperm-egg interactions in *C. pyrrhogaster*.

KEY WORDS: *ZP proteins, Ca^{2+} channel, CRISP, RNAseq, internal fertilization, urodele*

Introduction

Animals reproduce in various environments in water and on land. Some environmental conditions involve unstable factors that influence the success of fertilization and cause the optimization of the sperm-egg interaction (Inoda and Morisawa, 1987; Takai and Morisawa, 1995). Rapid evolution of the genes that are significant for fertilization may involve the modification of the sperm-egg interaction that contributes to the diversification of reproductive modes during adaptation to fertilization environments.

CatSper is a sperm-specific cation channel in mammals (Ren *et al.*, 2001). Its primary role is Ca^{2+} influx into spermatozoa to induce hyperactivated motility, which ensures that spermatozoa are propelled through the oviductal matrix and penetrate the egg coat known as the zona pellucida for fertilization (Yanagimachi, 1994). In primates, the *CatSper* gene has been positively selected

with an accompanying modification of the putative channel function (Podlaha and Zhang, 2003), whereas it has been lost in the genomes of the model animals belonging to Aves, Anura, Teleosts, and Agnatha (Cai and Clapham, 2008). Other molecules, such as the Na$^+$-Ca^{2+} exchanger and voltage-dependent Ca^{2+} channels (VDCCs), appear to be substituted for CatSper to increase the intracellular Ca^{2+} for the initiation or activation of sperm motility in those animals (Morisawa *et al.*, 1999; Vines *et al.*, 2002; Froman and Feltmann, 2005). Zona pellucida (ZP) proteins are also known to be rapidly evolving molecules (Goudet *et al.*, 2008). They are divided into six subgroups, ZP1, ZPA/ZP2, ZPB/ZP4, ZPC/ZP3, ZPAX, and ZPD, and contain a ZP domain that most likely

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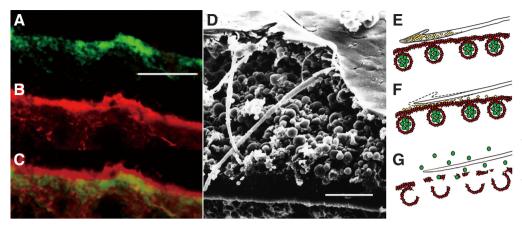
Fig. 1. Acrosome reaction-associated initiation of sperm motility based on the fine structure of the surface of jelly layer in *C. pyrrhogaster*. (A-C) *Immunohisto-chemical localizations of sperm motility-initiating substance (SMIS) (A), acrosome reaction-inducting substance (ARIS) (B), and both merged (C). ARIS and SMIS are localized in the outer surface of egg jelly and just beneath the ARIS-localizing area, respectively. Bar: 75 μm.* **(D)** *Scanning microscopic image of the surface of jelly layer showing sheet structure in the outer surface and granules beneath it. Bar: 10 μm.* **(E-G)** *A possible mechanism for acrosome reaction-associated initiation of sperm motility. Quiescently stored spermatozoa are inseminated on to the surface of jelly layer (E) and undergo acrosome reaction in response to the ARIS (red). Acrosomal enzymes were exposed to lyse the ARIS-localizing sheet structure covering the surface of the jelly layer (F), which results in exposure of the SMIS to affect spermatozoa for the motility initiation (G). Photographs and illustrations are reproduced with permission from Int. J. Dev. Biol. (2010) 54: 591–597.*

mediates the polymerization of those molecules into filamentous components of the vitelline envelope (Killick *et al.*, 1995). A few genes for ZP proteins are disrupted, depending on the species, which results in the expression of different combinations of ZP proteins. The rapid evolution of ZP proteins might influence their function in sperm binding to the vitelline envelope (Hedrick, 2008) which is mediated by ZPA/ZP2 in mouse and human (Bleil and Wassarman, 1988; Avella *et al.*, 2014) and by ZPC/ZP3 in addition to ZPA/ZP2 in *X. laevis* (Tian *et al.*, 1999; Vo and Hedrick, 2000; Hedrick, 2008).

The reproductive mode of amphibians is highly diversified to finally evolve internal fertilization (Duellman and Trueb, 1994). In the newt *Cynops pyrrhogaster*, a specific state of sperm motility to penetrate the jelly layer is induced in association with an acrosome reaction on the basis of fine structures on the jelly surface (Watanabe *et al.*, 2010; Takayama-Watanabe *et al.*, 2015) (Fig. 1). This mechanism is crucial for the internal fertilization of *C. pyrrhogaster* (Takahashi *et al.*, 2006) and contributes to the initiation of sperm motility inside the female body. The motility is pharmacologically suggested to be mediated by a unique molecular mechanism in which L-type and T-type VDCCs act in distinct sites of the sperm midpiece (Takahashi *et al.*, 2013). In addition, *C. pyrrhogaster* spermatozoa bind to the vitelline envelope in passing through the jelly layer, though *Cynops* ZPC, unlike *X. laevis* ZPC, is suggested not to act as a sperm receptor. Elucidation of the substantial changes in the genes involved in the achievement of the internal fertilization of *C. pyrrhogaster* will be significant to understand the mechanism underlying the diversification of reproductive modes in amphibians and to understand the evolutional history leading to internal fertilization in tetrapods. However, these substancial changes are largely unknown because of poor gene information. Recently, global gene information expressing in a specific organ was constructed in newts (Looso *et al.*, 2013; Yokoe *et al.*, 2014; Nakamura *et al.*, 2014). In the present study, we focused on genes for ZP proteins, VDCCs, and cysteine-rich secretory proteins (CRISP) that were prospectively critical for sperm-egg interaction in the internal fertilization of *C. pyrrhogaster*, and features of their expression were examined using the RNAseq data constructed via *de novo* assembly of cDNA from testis, ovary, and oviduct samples.

Results and Discussion

In the *de novo* assembled data on RNAseq from ovary and testis samples, 12 and 15 base sequences were annotated as ZP proteins and $\alpha 1$ subunits of VDCC, respectively. The base sequences for ZP proteins possessed putative coding regions encoding 328-789 amino acids, including two encoding partial amino acid sequences of the ZP domain (Table 1). The ZP proteins of *Xenopus (Silurana) tropicalis* registered in NCBI GenBank are the proteins of 375-690 amino acids, suggesting that most base sequences for ZP proteins in the RNAseq data were nearly full size.

In contrast, some base sequences for the $\alpha 1$ subunits of VDCCs were too small to encode full amino acid sequences (Table 2). In this case, one to five base sequences were identified for each subunit (Table S2). This feature in the $\alpha 1$ subunits of VDCCs may be caused by the presence of multiple isoforms expressed in distinct cell types of the testes as well as by assembly error due to the lack of reference sequences. In the case of protamine genes that had been identified in spermatids (Yoshinobu *et al.*, 1997), full base sequences obtained by expression screening of a cDNA library were found in the RNAseq data from the testis samples (comp59911 for protamine 1 and comp43001 for protamine 2) with 98% identity (Fig. S1). Although comp59911 had an additional 1012 base sequence in the 5'-region that resulted in nonsense coding, the 3'-region potentially encoded the full amino acid sequence of protamine 1. Thus, comp59911 is supposed to be the protamine 1 cDNA sequence fused with that of an unknown gene due to assembly error. The high homologies of comp59911 and comp43001 to the protamine 1 and 2 genes, respectively, indicate that reliable genetic information can be obtained at least in protein coding regions from the *de novo* assembled RNAseq data.

Zona pallucida (ZP) proteins

Base sequences homologous to all six subgroups of ZP proteins were found in the RNAseq data from ovary (Table 1). They included a base sequence annotated as a ZP4 homologue (Table 1). This is the first finding of ZP4 expression in amphibians. They also included every base sequence for ZPB or the other subgroups of ZP proteins except for ZPC/ZP3: six distinct base sequences

TABLE 1

CONTIG SEQUENCES HOMOLOGOUS TO ZP PROTEINS IN THE RNASEQ DATA FROM OVARY

contig	length (bp)	zp domain (bp)	accession	species	E-value	bit score	identical (%)
ZP1							
comp38331	1942	672-1455	ELK13491.1	*Pteropus alecto*	2.63E-116	371.7	40.7
			EHH22824.1	*Macaca mulatta*	6.68E-115	369.0	39.7
			ELV10238.1	*Tupaia chinensis*	4.84E-114	366.7	39.0
ZPA/ZP2							
comp46414	2316	792-1572	NP_001081858.1	*Xenopus laevis*	6.37E-142	445.3	41.7
			AAD12172.1	*Xenopus laevis*	1.22E-140	442.2	41.5
			AAK84067.1|AF401322_1	*Discoglossus pictus*	4.06E-137	432.9	39.4
ZPB/ZP4							
comp60329	2012	573-1383	EMP28460.1	*Chelonia mydas*	1.02E-143	440.7	51.2
			NP_001081068.1	*Xenopus laevis*	2.99E-142	437.6	47.2
			NP_988854.1	*Xenopus (Silurana) tropicalis*	4.97E-137	424.1	45.2
comp67568 (partial)	1068	2-256	EMP33668.1	*Chelonia mydas*	3.88E-91	289.7	56.0
			EMP28460.1	*Chelonia mydas*	4.28E-88	285.8	58.8
			NP_990210.1	*Gallus gallus*	6.73E-86	280.4	56.3
ZPC/ZP3							
comp68248	1528	213-987	AAV35105.1	*Meleagris gallopavo*	6.03E-115	358.2	45.1
			AAB39079.1	*Xenopus laevis*	1.40E-114	357.5	47.1
			AAV35193.1	*Gallus gallus*	2.90E-114	356.3	52.3
comp64853	1565	318-1095	AAV35105.1	*Meleagris gallopavo*	2.66E-118	367.1	64.1
			AAV35107.1	*Lyrurus tetrix*	4.28E-117	364.0	62.4
			AAV35103.1	*Centrocercus urophasianus*	1.03E-116	363.2	62.3
comp62734	1342	93-831	EMP27581.1	*Chelonia mydas*	1.07E-103	331.6	50.2
			AAI58454.1	*Xenopus (Silurana) tropicalis*	3.89E-94	300.8	40.0
			ADH93592.1	*Acipenser sinensis*	5.51E-62	216.9	36.4
comp60094 (partial)	2438	15-705	AAV35105.1	*Meleagris gallopavo*	5.64E-76	264.2	47.0
			AAH72326.1	*Xenopus laevis*	2.96E-75	260.4	51.2
			AAV35103.1	*Centrocercus urophasianus*	4.39E-75	261.9	46.7
comp46343 (partial)	1098	186-552	EMP27581.1	*Chelonia mydas*	4.45E-28	107.8	42.4
			ADH93592.1	*Acipenser sinensis*	4.21E-19	95.10	37.7
			AAG28398.1|AF192407_1	*Pimephales promelas*	9.14E-16	85.90	37.4
comp38201	1485	219-993	AAB39079.1	*Xenopus laevis*	2.48E-120	371.7	50.3
			AAH61428.1	*Xenopus (Silurana) tropicalis*	1.01E-117	365.5	52.7
			AAV35105.1	*Meleagris gallopavo*	1.80E-115	359.0	51.9
ZPX							
comp64828	3002	1506-2268	AAI69766.1	*Xenopus laevis*	0	843.2	49.1
			NP_988851.1	*Xenopus (Silurana) tropicalis*	0	814.7	47.1
			ABQ9459.1	*Numida meleagris*	0	703.4	42.8
ZPD							
comp68322	1528	339-1092	NP_998741.2	*Gallus gallus*	2.11E-69	238.8	37.4
			BAF56912.1	*Coturnix japonica*	5.01E-68	235.3	37.1
			AAI57425.1	*Xenopus laevis*	1.09E-55	201.1	38.4

were found as ZPC/ZP3 homologues, including a homologue of *Xenopus laevis* ZPC (Makabe-Kobayashi *et al.*, 2003; accession: KM085177).
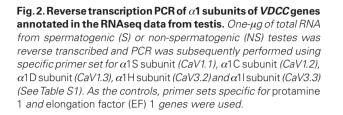
ZPC/ZP3 is a major component of the egg envelope participating in sperm binding in *X. laevis* (Vo and Hedrick, 2000; Kubo *et al.*, 2002; Hedrick, 2008), although a homologue of *X. laevis* ZPC did not participate in the sperm binding to the egg envelope in *C. pyrrhogaster* (Makabe-Kobayashi *et al.*, 2003). Thus, the molecular mechanism for sperm binding to the egg envelope differs between the species. In the fertilization process, binding to the egg envelope occurs with acrosome-intact spermatozoa in *X. laevis* (Ueda *et al.*, 2002; Kubo *et al.*, 2008) or with acrosome-reacted spermatozoa in *C. pyrrhogaster* (Nakai *et al.*, 1999; Sasaki *et al.*, 2002), as in mouse (Jin *et al.*, 2011). The sperm binding in *C. pyrrhogaster* and mouse shows an additional similarity: sperm binding is modulated by oviduct-secreted substances and heparin (Nakai *et al.*, 1999; Hiyoshi *et al.*, 2007; Coy *et al.*, 2008). From these facts, it was supposed that ZPA/ZP2 was a critical sperm receptor of the egg envelope in *C. pyrrhogaster* as in mammals (Bleil and Wassarman, 1988; Avella *et al.*, 2014).

However, in the present study we found the unexpected expression of ZP proteins including six distinct molecules of ZP3/ZPC, a ZP4 and a ZP1 in the egg envelope of *C. pyrrhogaster*. In the NCBI GenBank, *X. laevis* and *X. (S.) tropicalis* possess one and

two ZPC homologues, respectively. This indicated that the *ZPC/ZP3* gene might be highly multiplicated, specifically in the *C. pyrrhogaster* genome. Furthermore, the egg envelope of *C. pyrhogaster* was suggested to possess a ZP4 and a ZP1 homologue, which are not identified in that of *X. laevis*. Those newly identified ZP proteins should be considered in order to understand the molecular mechanism underlying sperm binding to the egg envelope in *C. pyrrhogaster*.
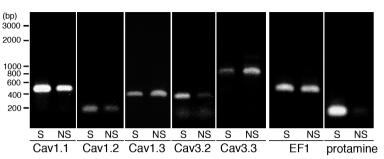
Voltage-dependent Ca²⁺ channel (VDCCs)

Homologous base sequences to α1B, C, D, H, L, and S subunits were found in the testis RNAseq data (Table 2). α1C, D, H, L, and S subunits were detected in both spermatogenic and nonspermatogenic testes by reverse transcription-polymerase chain reaction (Fig. 2) with stronger expression of α1C, H, and S in spermatogenic testes. Elongation factor (*EF*) *1* gene expression was detected at similar levels in both testes, and a spermatid-specific gene, *protamine 1* (Yoshinobu *et al.*, 1997), was detected only in spermatogenic testes. These results suggest that the L-type VDCCs, CaV1.1 and 1.2, and a T-type VDCC, CaV3.2. were expressed in spermatozoa.

Calcium ion is a significant intracellular mediator for multiple sperm functions, such as sperm acrosome reaction (Darszon *et al.*, 2006; Florman *et al.*, 2008), initiation/activation of sperm

Fig. 2. Reverse transcription PCR of α**1 subunits of *VDCC* genes annotated in the RNAseq data from testis.** *One-*μ*g of total RNA from spermatogenic (S) or non-spermatogenic (NS) testes was reverse transcribed and PCR was subsequently performed using specific primer set for* α*1 S subunit (CaV1.1),* α*1 C subunit (CaV1.2),* α*1 D subunit (CaV1.3),* α*1 H subunit (CaV3.2) and* α*1 I subunit (CaV3.3) (See Table S1). As the controls, primer sets specific for* protamine 1 *and* elongation factor (EF) 1 *genes were used.*

motility (Morisawa *et al.*, 1999; Darszon *et al.*, 2006; Kirichok and Lishko, 2011), and sperm binding to the egg envelope (Saito *et al.*, 2012). Spermatozoa possess multiple types of cation channels in the plasma membrane and Ca^{2+} stores, which are critical for increasing cytoplasmic Ca^{2+} in every step of sperm-egg interaction (Darszon *et al.*, 2006; Marquez *et al.*, 2007). In the internal fertilization of *C. pyrrhogaster*, a unique mechanism for acrosome reaction-associated sperm motility initiation acts on the surface of the jelly layer (Fig. 1). When spermatozoa in the sperm reservoir of the female are inseminated onto the surface of the jelly layer, they undergo acrosome reaction in response to acrosome reaction-inducing substances (ARIS) in the sheet structure covering the outer surface of the jelly layer (Watanabe *et al.*, 2010). The sheet structure is digested possibly by acrosomal enzymes released by the acrosome reaction, which results in the exposure of granules containing sperm motility-initiating substance (SMIS) (Watanabe *et al.*, 2010; Yokoe *et al.*, 2014). A pharmacological study using T-type VDCC blockers, including mibefradil, and L-type VDCC blockers, including nitrendipine, suggests that CaV3.1 or 3.3 of the spermatozoa is involved in the induction of the acrosome reaction by ARIS and CaV3.2 in the initiation of SMIS-induced sperm motility (Takayama-Watanabe *et al.*, 2015). In addition, a nitrendipine-sensitive Ca^{2+} channel and a mibefradil-sensitive Ca^{2+}

channel predominantly mediate the increase of intracellular Ca^{2+} to enhance sperm motility in the anterior and posterior midpiece, respectively (Takahashi *et al.*, 2013). The local enhancements are suggested to cause a specific state of motility crucial for spermatozoa to penetrate the jelly matrix.

The results of the present study suggest that CaV3.2 is actually present in spermatozoa and may involve in the initiation of sperm motility, and CaV1.1 and/or 1.2 can mediate the enhancement of sperm motility to penetrate the jelly layer. On the other hand, the expression in spermatozoa of CaV 3.1 or 3.3, each of which are thought to be involved in the induction of the acrosome reaction, was unclear (Fig. 2). This may indicate the involvement of the CatSper channel in the sperm function. Mibefradil is a major blocker of the CatSper channel in mammalian spermatozoa (Strünker *et al.*, 2011; Lishko *et al.*, 2011). Although we could not find base sequences annotated as CatSper in the RNAseq data, it may be difficult to identify homologues of the rapidly evolving *CatSper* by a BLAST search (Cai and Clapham, 2008). It is worth trying to identify the *CatSper* gene of *C. pyrrhogaster* using another method.

The presence of multiple VDCCs is a common feature in the spermatozoa of the animal species examined. In mammalian spermatozoa, seven types of VDCCs, including L-type, N/P/Q-type, and T-type, are expressed (Darszon *et al.*, 2006). The role

TABLE 2

CONTIG SEQUENCES HOMOLOGOUS TO α**1 SUBUNITS OF VDCCs IN THE RNASEQ DATA FROM TESTIS**

contigs	length (bp)	accession	species	E-value	bit score	identical (%)
testis						
VDCC						
α1 S (CaV1.1)						
comp387802	443	XP_002809678.1	*Pongo abelii*	2.62E-82	266.9	95.4
		EMP32289.1	*Chelonia mydas*	8.58E-81	268.1	97.0
		XP_002941452.1	*Xenopus (Silurana) tropicalis*	1.90E-80	266.9	95.4
α1 C (CaV1.2)						
comp648010	320	BAD92253.1	*Homo sapiens*	5.08E-24	105.1	71.7
		XP_003313479.1	*Pan troglodytes*	1.51E-23	104.8	71.7
α1 D (CaV1.3)						
comp158103	761	NP_990365.1	*Gallus gallus*	4.95E-126	404.8	84.5
		EMC88796.1	*Columba livia*	1.43E-125	403.7	84.5
		NP_001072929.1	*Xenopus (Silurana) tropicalis*	2.75E-122	374.4	81.8
α1 B (CaV2.2)						
comp161304	981	DAA24110.1	*Bos taurus*	0	543.5	89.6
		EAW88415.1	*Homo sapiens*	3.77E-179	533.9	88.0
		AAD51821.1|AF173018_1	*Gallus gallus*	1.74E-178	556.2	92.3
α1 H (CaV3.2)						
comp55396	5503	XP_002932520.1	*Xenopus (Silurana) tropicalis*	0	1508	73.3
		XP_414830.3	*Gallus gallus*	0	1359	66.9
		XP_002187949.2	*Taeniopygia guttata*	0	1321	68.1
α1 I (CaV3.3)						
comp42249	1288	XP_002933803.1	*Xenopus (Silurana) tropicalis*	8.72E-70	251.5	60.2
		XP_425474.2	*Gallus gallus*	2.08E-64	235.7	49.7
		XP_003221064.1	*Anolis carolinensis*	2.32E-54	206.5	52.1

TABLE 3

CONTIG SEQUENCES HOMOLOGOUS TO CRISPS IN THE RNASEQ DATA FROM UTERUS

contig	length (bp)	homologous protein	accession	E-value	bit score	identical (%)
comp48729	1396	cysteine-rich secretory protein 2 precursor [Mus musculus]	NM_009420.2/NP_033446.1	8.00E-57	191	48.0
		Serotriflin [Caprimulgus carolinensis]	KFZ46955.1	9.00E-57	188	54.0
		cysteine-rich secretory protein 2 [Bos mutus]	ELR49508.1	1.00E-55	188	49.0
comp47835	1182	MGC108118 protein precursor [Xenopus (Silurana) tropicalis]	NM_001030355	1.27E-69	232.6	50.0
		natrin-2 [Gallus gallus]	XP_420051.3	2.15E-66	221.9	50.5
		cysteine-rich seceretory protein Az-CRP [Azemiops feae]	ACE73558.1	3.19E-66	221.5	47.0

of each VDCC appears to be redundant, because mice in which one VDCC gene is disrupted are sometimes fertile (Sakata *et al.*, 2002; Chen *et al.*, 2003; Stamboulian *et al.*, 2004). Compared with mammalian spermatozoa, only 2 L-type and 1 T-type VDCC appeared to be expressed in those of *C. pyrrhogaster* (Fig. 2) and thus act with less redundancy. In internal fertilization, mammalian spermatozoa, unlike those of *C. pyrrhogaster*, make long trips through the oviduct. In that process, spermatozoa are deposited in the isthmus and capacitated with increasing intracellular Ca^{2+} (Kopf *et al.*, 1999). The expression of redundant VDCCs in mammalian spermatozoa may correlate with the appropriate maintenance of intracellular Ca^{2+} in spermatozoa passing through the oviduct.

Cysteine rich secretory proteins (CRISPs)

CRISP proteins are reported to be expressed in the male re-productive tract of mammals and to modulate sperm maturation in the epididymis, as well as capacitation and fusion with the egg plasma membrane in the oviduct, at fertilization (Cohen *et al.*, 2000; Roberts *et al.*, 2003; Gibbs *et al.*, 2006; Busso *et al.*, 2007a; 2007b; Burnett *et al.*, 2008a; Cohen *et al.*, 2008). In amphibians, allurin, a member of the CRISP family proteins, is expressed in the oviduct (Al-Anzi and Chandler, 1998; Olson *et al.*, 2001; Xiang *et al.*, 2004). This protein is accumulated as a jelly component and diffuses into the medium to attract spermatozoa (Xiang *et al.*, 2004). Allurin possesses a unique structure lacking an ion channel regulatory domain, which is conserved in the 3'-region of CRISP family proteins (Burnett *et al.*, 2008a). The *allurin* gene is not functionally conserved in higher vertebrates, suggesting that allurin mediates a fertilization mechanism specific to *Xenopus* species. Allurin-mediated chemotaxis is not involved in the internal fertilization of *C. pyrrhogaster*, which lacks sperm attraction to eggs in the fertilization process, although CRISP family proteins including allurin may contribute to some other events in the egg jelly of urodele species. Thus, we surveyed the RNAseq data from the uterus of the oviduct, where components of the outermost region of the jelly layer are secreted at ovulation (Okimura *et al.*, 2001), and found two base sequences homologous to CRISP family proteins (Table 3). Comp48729 was annotated as CRISP 2, whose expression in the oviduct had not been reported until now. The other contig, comp47835, here called CRISP-like protein, displays high homology to several proteins belonging to the CRISP family. It contains both a pathogenesis-related domain and an ion channel regulatory domain. Reverse-transcription PCR revealed that mRNA having both contig sequences was expressed in the uterus and pars convoluta (Fig. 3). Allurin is reported to be the only CRISP family protein expressed in the *X. (S.) tropicalis* oviducts (Burnett *et al.*, 2008b), and no CRISP family protein apparently expresses in mammalian oviducts (Burnett *et al.*, 2008a). Thus, it is remarkable that the putative CRISP 2 and CRISP-like protein were expressed in the *C. pyrrhogaster* oviducts.

The expression of comp48729 was increased in the uterus but reduced in the pars convolute, whereas the expression of comp47835 was decreased in both the uterus and the pars convoluta of HCG-injected females (Fig. 3). In the controls, the expression of EF1 was ubiquitous, whereas that of α-type estrogen receptor was reduced in the oviduct following HCG injection. The reduced expression of the estradiol receptor by HCG treatment has been reported in mammalian tissue (Chiang *et al.*, 2000), suggesting that the RNA obtained from the oviduct of HCG-treated females actually contained mRNA whose transcription was affected by the HCG signal. Because HCG induced the secretion of jelly components as well as ovulation, the upregulation of CRISP 2 gene expression by HCG indicates that CRISP 2 protein may be abundantly present in the outer surface of the egg jelly. This protein may modulate the acrosome reaction-associated motility initiation on the surface of the egg jelly. Alternatively, it may be responsible for antibacterial effects after females spawned eggs into fresh water, as the CRISP family proteins function as reptile venom (Burnett *et al.*, 2008a). Further study is needed to evaluate the significance of the newly found CRISP family protein in the internal fertilization and embryonic development of *C. pyrrhogaster*.

RNAseq data from ovary, testis, and oviduct samples demon-strated its usefulness in providing gene lists for fertilization research in *C. pyrrhogaster*. In the present study, we first found that 1) egg envelopes were composed of a unique combination of ZP proteins, including ZP1, ZP4, and six isoforms of ZPC, which are thought to be responsible for the *C. pyrrhogaster*-specific mechanism underlying sperm binding to the egg envelope; 2) two L-type VDCCs, CaV1.1 and 1.2, and a T-type VDCC, CaV3.2, which are proposed to act in the initiation and enhancement of sperm motility (Takahashi *et al.*,
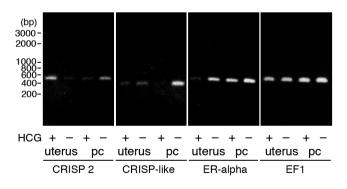

Fig. 3. Reverse transcription PCR of *CRISP* genes annotated in the RNAseq data from uterus. *One-μg of total RNA from uterus or pars convolute (pc) of oviduct dissected from ovulation-induced (+) or not-induced (-) females was reverse transcribed and PCR was subsequently performed using specific primer sets for CRISP 2 and CRISP-like protein. Primer sets of* estrogen receptor (ER)- *α and* elongation factor (EF) 1 *genes were used as the controls.*

2013; Takayama-Watanabe *et al.*, 2015), were actually present in spermatozoa; and 3) CRISP 2 might participate in the acrosome reaction-associated initiation of sperm motility critical for the success of internal fertilization.

Construction of omics data has already been reported in the newt *Nothophtalmus viridescens*, and the presence of many newt-specific transcripts as well as genes conserved in vertebrates has been revealed (Looso *et al.*, 2013). Furthermore, advances in gene technology allowed DNA targeting in newts using TAL effector-like nuclease (Hayashi *et al.*, 2014; Flowers *et al.*, 2014). These technologies will contribute to the further elucidation of the molecular mechanisms underlying fertilization events in nonmodel amphibian species.

Materials and Methods

RNAseq data

De novo assembled RNAseq data that were constructed using Trinity (http://trinityrnaseq.sourceforge.net) and annotated using NCBI GenBank were provided by Dr. Chiba, C. of Tsukuba University and Dr. Toyama, F. of Utsunomiya University (http://antler.is.utsunomiya-u.ac.jp/imori/). Messenger RNA for the data construction was from the spermatogenic testes, ovaries containing all stages of oocytes, and the most posterior portion of the oviduct, the uterus. The uterus is the portion where most components of the outer surface of jelly layer are secreted (Okimura *et al.*, 2001). For the RNA preparation from the uterus, females in which secretion of jelly components was stimulated by human chorionic gonadotoropin (HCG) was used.

Animals

Sexually mature newts were captured in Yamagata Prefecture in late autumn. Spermatogenic and non-spermatogenic testes were dissected from males immediately and after a 1-year hibernation at 10°C in labo. It has been reported that in *C. pyrrhogaster*, incubation of males at low temperature results in cessation of meiosis and spermiogenesis (Yazawa *et al.*, 2003). Oviducts were obtained from females in which ovulation was induced by two injections of 200 IU HCG (ASKA Pharmaceutical Co., Tokyo Japan) at a 24-h interval. The uterus and second posterior portion, the pars convolute, where components of the inner jelly layer are secreted (Okimura *et al.*, 2001) were dissected. Total RNA was prepared from the testis, uterus, and pars convolute using Trizol® reagent (Invitrogen).

Reverse-transcription PCR

One-μg of total RNA was reverse transcribed using SMARTScript™ reverse transcriptase (Clontec), and subsequent PCR was performed using 2 μl and 4 μl of the reacted solution from testis and oviduct (uterus or pars convolute) samples, respectively. The reaction was performed with 30 or 35 cycles of denaturing at 95°C for 30 sec, annealing at 58 or 60°C for 30 sec, and extension at 72°C for 1 min. Gene-specific primers were designed from the limited region of the base sequence of RNAseq data that have high homology to the examined gene of the other animals (Table S1). The amplified DNA was electrophoresed in 1% agarose gels and visualized using 10 μg/ml ethidium bromide.

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