Chapter 41 Screening for Genes Participating in the Formation of Prismatic and Nacreous Layers of the Japanese Pearl Oyster *Pinctada fucata* by RNA Interference Knockdown



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Abstract Many genes have been identified to participate in the shell formation so far. Nevertheless, the whole picture of the molecular mechanisms underlying the shell formation has remained unknown. In our previous study, we analyzed comprehensively genes expressed in the shell-producing tissues and identified 14 genes to be involved in the shell formation by the RNA interference (RNAi) method. In the present study, we performed further screening to find additional novel genes involved in the formation of the nacreous and prismatic layers. We here selected 80 genes from the EST data as candidates to function in the shell formation, conducted knockdown experiments by the RNAi method, and observed surface appearances on the nacreous and prismatic layers. We newly identified 64 genes that could participate in the shell formation. Taken together with our previous study, 78 genes were

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© The Author(s) 2018 K. Endo et al. (eds.), *Biomineralization*, https://doi.org/10.1007/978-981-13-1002-7_41 supposed to function in the shell formation. These findings indicate that the combination of transcriptome and knockdown analyses is a powerful tool to screen novel genes involved in the shell formation.

Keywords EST · Knockdown · Nacreous layer · Pearl oyster · Prismatic layer · RNAi · Shell

41.1 Introduction

Many genes have been identified to participate in the shell formation so far. In classical ways, proteins were purified from shells after decalcification and their properties were analyzed. Nacrein, for instance, was purified from shells of the Japanese pearl oyster *Pinctada fucata* and characterized in detail (Miyamoto et al. 1996). Suzuki et al. (2009) employed the RNA interference (RNAi) method to elucidate possible functions of Pif discovered as an aragonite-binding protein in the shell of P. fucata. Knockdown of the Pif gene by the RNAi method induced an abnormal crystal structure of aragonite. This finding confirmed that Pif is really involved in the nacreous layer formation and proved that the RNAi method is useful to study genes involved in shell formation. We obtained the EST data of nacreous and prismatic layer-producing tissues of *P. fucata*, which contained 29,682 genes, and found novel 29,550 genes (Kinoshita et al. 2011). Genes involved in the shell formation must be contained in these genes. Thus, we compared gene expression patterns among mantle pallium, edge, and pearl sac tissues using the EST data to find genes expressed in a tissue-specific manner. We selected five genes specifically expressed in the mantle pallium, three highly expressed in the mantle pallium and pearl sac, and six specifically expressed in the mantle edge as candidates to function in shell formation. Knockdown experiments for these candidate genes induced abnormal appearances on the inner surface of the shells in the oysters (Funabara et al. 2014). These findings demonstrated that a combination of transcriptome analyses and RNAi knockdown is a powerful tool to screen genes involved in the shell formation. In the present study, we conducted further screening for genes involved in the shell formation of *P. fucata* using the above method.

41.2 Materials and Methods

We selected 195 genes having more than 200 reads from the EST data (Kinoshita et al. 2011) of the shell-forming tissues, along with 9 genes expressed similarly to those known to be involved in the shell formation from genes having less than 200 reads in the EST data. We conducted cDNA cloning of the selected genes with primers designed using the nucleotide sequences of respective genes. dsRNAs of the selected genes were synthesized using the cDNA clones as templates with a ScriptMAXTM Thermo T7 Transcription Kit (Toyobo, Osaka, Japan). About 40 μ g of dsRNA/100 μ l H₂O were injected into adductor muscles of 2-year-old pearl

oysters (n = 3), followed by rearing them in artificial seawater at 23 °C for 8 days with feeding plankton once a day. The green fluorescence protein (GFP) and *Pif* genes were used as negative and positive references, respectively, to verify the RNAi experiments. Surface appearances of the prismatic and nacreous layers on the shells of the knockdown oysters were observed with a scanning electron microscope (SEM), S-4000 (Hitachi, Tokyo, Japan).

41.3 Results

41.3.1 Selection of Candidate Genes Functioning in Shell Formation

We selected candidate genes having more than 200 reads in the EST data (Kinoshita et al. 2011) to be possibly involved in the shell formation, except for 14 genes which we analyzed in our previous study (Funabara et al. 2014) (Table 41.1). cDNAs of 71 genes out of the selected 181 genes above were successfully cloned and used for synthesizing dsRNAs as templates. We selected additionally 9 genes showing expression patterns similarly to those of known shell formation-related genes such as PFMG1, KRMP1, N19, and N16 series from those having less than 200 reads (Table 41.1). cDNAs of all the nine genes were cloned and used for the synthesis of dsRNAs. A total of 80 genes were subjected to the knockdown experiments.

41.3.2 Observation of the Appearances on the Inner Surface of the Knockdown Oyster Shells

Knockdown of 64 out of 80 genes induced abnormal appearances on the inner surface of the shells (Table 41.2). Among them, 18 knockdown oysters had abnormal appearances on both the prismatic and nacreous layers, 45 only on the nacreous layers, and 1 only on the prismatic layers. The data combined with our previous study are shown in Fig. 41.1. Ninety-four genes, 80 in the present and 14 in our previous studies, contained 78 genes that are suggested to be involved in the shell formation processes. Only one gene changed the surface appearance on the prismatic layer.

41.4 Discussion

We have obtained the data of gene expression patterns and genes possibly involved in shell formation (Tables 41.1 and 41.2). It is not easy to discuss how genes play roles in shell formation based on expression patterns in the EST and knockdown data. We have only short sequences of the respective genes in the EST data. Full-length sequences or at least open reading frame (ORF) regions of the interest genes

Table 41.1 Gene expression patterns in shell- and pearl-forming tissues

TPM TPM <th></th>																				
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	26^{b}	2286	2306	4459		77b	2373	2414	65	372	134	888	930	962	243	301	1616		0	219

207	244	346	276	290	275	250	214	304	205	205	228	218	222	211	212	162	105	55	48	4	123	48	4	55
83	1221	916	1203	2618	333	296	259	2812	1896	1896	2109	2017	2054	1952	1961	0	259	0	204	0	1138	0	0	28
1147	442	1876	1040	36	1673	1517	211	0	0	0	0	0	0	0	0	968	574	585	36	550	0	574	48	0
1485	1092	1310	859	58	1441	1325	131	0	0	0	0	0	0	0	0	1267	422	87	335	0	0	0	0	0
323	336	344	384	395	399	407	411°	3840	3969	4121	4600	5656	7101	7147	11,232	390♭	493b	496b	1362 ^b	3968 _b	4254 ^b	95099	14278 ^b	16419 ^b
396	428	233	448	218	284	292	235	244	501	437	219	223	344	225	287	405	402	354	335	361	215	245	273	240
2054	1600	99	1767	0	65	0	361	0	2461	3275	1129	814	1591	0	1480	1230	1304	0	1489	2785	1175	1499	1674	
944	2031	1434	1517	1577	2031	167	1398	1601	1374	442	609	741	806	1804	645	1792	1458	2019	1243	454	442	382	663	1040 749
1383	1237	1558	1893	1252	1558	4047	1150	1601	1747	029	029	1063	1398	1077	1063	1776	2024	2693	1019	320	742	742	495	1048
136	137	138	139	141	143	145°	147	148	150	152	154	155	157	161	162	164	165	166	167	168	170	171	172	176
)3	5	0,	_	22	6,	93	2.2	0	74	9(4	15	0,	80		75	0	13	90	4,	94	09	72	
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14		26	364	20	17	13	16	14	16	11	14	10	12		21	15	21.	437	20		18			
78	79 ^b	80	81°	82	83	84	85	98	87	88	86	8	91	92 _b	93	94	95	396	97	98c	66	101	102	103
561	584	761	928	421	752	705	407	1251	673	735	488	638	427	830	683	672	372	869	643	705	390	371	770	732
3432	0	3867	5005	0	2877	3719	982	6642	2655	0	1970	2997	0	2711	2396	3867	194	3210	2600	3034	0	157	3451	3608
1744	3967	2115	2270	1267	3202	1613	2151	2784	2258	4756	1685	1995	2485	3465	2772	1458	2629	1649	1900	2342	2856	2222	2330	2103
641	3669	2417	2868	4586	2519	2446	2067	4367	2868	4906	1951	2140	3188	3596	2795	1922	1907	1645	2955	2636	2198	2446	2941	2417
27°	28 ^b	29 ^b	30b	31°	32b	33 ^b	34 ^b	35b	36^{b}	37b	38b	39b	40	41	42b	43	44 _b	45b	46 ^b	47b	48b	49	$50^{\rm b}$	51 ^b

TPM templates per million, ME mantle edge, MP mantle pallium, PS pearl sac

^aData and gene numbers from Kinoshita et al. (2011) ^bGenes subjected to RNAi experiments in the present study ^cGenes analyzed in our previous study Funabara et al. (2014)

Table 41.2 Appearances of the inner surface of shells injected with dsRNAs of the subject genes

Genea	Prismatic	Nacreous	Gene	Prismatic	Nacreous	Gene	Prismatic	Nacreous
1	n	a	39	a	a	79	a	a
2	a	a	42	n	a	92	a	a
3	a	a	44	n	a	108	n	a
5	n	a	45	n	a	111	n	a
6	a	a	46	a	a	112	n	a
7	a	a	47	n	a	114	a	n
8	a	a	48	n	a	115	n	n
9	n	a	50	n	a	390	n	n
10	a	a	51	n	a	493	n	n
11	a	a	52	n	a	496	n	n
12	n	a	53	n	a	1362	n	n
13	a	a	54	n	n	3968	n	n
14	a	a	55	n	a	4254	n	n
15	a	a	56	n	a	6605	n	a
16	n	a	57	n	a	14,278	n	n
17	n	a	59	a	a	16,419	n	n
20	n	a	60	n	a	27ь	a	a
22	n	a	61	n	n	31 ^b	a	a
23	a	a	62	n	a	58 ^b	n	a
24	n	a	63	n	a	66 ^b	n	a
25	n	a	64	n	n	81 ^b	a	a
26	n	a	65	n	a	96 ^b	a	a
28	n	a	67	n	n	98 ^b	a	a
29	a	a	68	n	a	113 ^b	n	a
30	n	a	69	n	a	118 ^b	n	a
32	n	a	70	n	a	133 ^b	n	a
33	n	a	71	n	n	145 ^b	n	a
34	n	a	72	n	n	194 ^b	a	a
35	n	a	74	n	a	200 ^b	n	a
36	a	a	75	n	a	411 ^b	n	a
37	n	a	76	n	n			
38	n	a	77	n	a			

n normal appearance, a abnormal appearance

are required to discuss their function. To determine the full-length sequences, it is reasonable that we choose genes in descending order of the numbers of their reads in the EST data. We can also search the genome database for their gene models by BLAST searching using the EST sequence data (Takeuchi et al. 2012).

Many studies on shell formation-related proteins have focused on those secreted from mantle tissues into shells. This way is incapable of analyzing regulatory

^aGene numbers from Kinoshita et al. (2011)

^bData from Funabara et al. (2014)

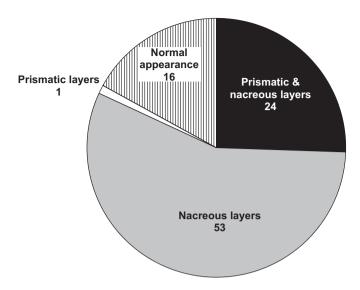


Fig. 41.1 The numbers of individuals having normal and abnormal appearances on the inner surface of the shells of the Japanese pearl oysters *Pinctada fucata* subjected to the RNAi experiments as observed by SEM. "Prismatic and nacreous layers," "nacreous layers," "prismatic layers," and "normal appearances" indicate individuals having abnormal appearances on "both the prismatic and nacreous layers," "only on the nacreous layers," "only on the prismatic layers," and "normal appearances" on the shell inner surface, respectively. Numerals indicate the numbers of the genes

pathways to form shells. We found in our previous study that some shell formation-related genes encoded proteins lacking a signal peptide, suggesting that such cyto-plasmic proteins function in shell formation together with secretory ones (Funabara et al. 2014). We have not determined the full-length sequences for the newly identified 64 genes to be involved in shell formation yet. They may contain cytoplasmic proteins which function in shell formation. The combination of transcriptome and knockdown analyses would give us some useful information on the shell formation processes from genes to shells.

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D. Funabara et al.

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