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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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 ScriptMAX™ Thermo T7 Transcription Kit (Toyobo, Osaka, Japan). About 40 μg of dsRNA/100 μl H_2O 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			Total		TPM			Total		TPM			Total		TPM			Total
Gene[a]	ME	MP	PS	reads	Gene	ME	MP	PS	reads	Gene	ME	MP	PS	reads	Gene	ME	MP	PS	reads
1[b]	6042	6381	7207	1728	52[b]	1456	1386	93	226	104	1922	908	148	224	179	1456	1028	1129	308
2[b]	12,142	17,827	1240	24,620	53[b]	1922	1529	3099	595	105	1267	1063	805	263	187	1834	2282	0	317
3[b]	4833	4672	1351	869	54[b]	1485	1159	3238	549	106	1150	920	1933	365	188	772	1123	2396	406
4	2737	1410	5301	879	55[b]	1470	1326	2923	528	107	2329	2342	2156	589	190	903	836	805	219
5[b]	4062	4146	28	629	56[b]	1776	1529	0	250	108[b]	815	729	2535	391	191	466	430	1480	228
6[b]	3479	2449	5458	1034	57[b]	2538	2951	0	409	109	961	478	1628	282	193	1267	1195	1018	297
7[b]	3130	3644	4200	974	58[c]	2635	275	0	204	110	1616	1482	1286	374	194[c]	2751	1028	0	275
8[b]	1994	2892	185	399	59[b]	2140	2426	629	418	111[b]	1601	1864	1008	375	196	597	347	3349	432
9[b]	3261	2366	19	424	60[b]	2519	1937	1415	463	112[b]	975	1135	601	227	197	1092	1267	722	259
10[b]	2737	2892	111	442	61[b]	1529	1338	3654	612	113[c]	1019	2653	56	298	200[c]	2227	789	0	219
11[b]	3494	2844	65	485	62[b]	2373	1852	2563	595	114[b]	2504	1470	2082	520	209	189	72	1878	222
12[b]	2227	3597	315	488	63[b]	3712	2438	0	459	115[b]	1529	1302	1758	404	215	1631	1338	0	224
13[b]	3217	1972	130	400	64[b]	3217	2461	1878	630	116	2009	1517	0	265	216	932	1111	490	210
14[b]	2125	1816	3173	641	65[b]	3072	1972	0	376	118[c]	641	1924	102	216	218	670	789	860	205
15[b]	5008	3023	1739	785	66[c]	2853	48	0	200	121	830	657	1813	308	228	1325	1350	0	204
16[b]	2009	2593	5107	907	67[b]	2053	1625	3312	635	122	1077	1171	786	257	237	1194	1446	28	206
17[b]	2504	2665	4431	874	68[b]	2737	1470	2285	558	123	1077	753	1147	261	243	1529	1075	361	234
18	1732	1995	3007	611	69[b]	3523	3190	4496	995	124	728	693	1452	265	248	1441	1099	333	227
19	2533	1613	5097	860	70[b]	757	1051	833	230	125	1689	1147	2720	506	250	1296	1338	1332	345
20[b]	2038	1804	3626	683	71[b]	1878	1972	1480	454	127	1791	2246	2017	529	252	1252	1506	0	212
21	1470	1995	5190	829	72[b]	3028	143	0	220	128	1820	1852	1092	398	268	903	1016	1425	301
22[b]	2475	1697	3423	682	73	1354	2031	481	315	129	2038	1482	2044	485	272	1936	1386	37	253
23[b]	2358	2210	3830	761	74[b]	2795	2043	111	375	130	1034	1816	648	293	274	684	633	1471	259
24[b]	2198	1888	4330	777	75[b]	2562	1936	2868	648	132	1194	1565	259	241	292	903	789	1018	238
25[b]	2737	3405	0	473	76[b]	2082	1912	2646	589	133[c]	903	1804	46	218	300	611	203	1369	207
26[b]	2286	2306	4459	832	77[b]	2373	2414	65	372	134	888	930	962	243	301	1616	1290	0	219

27[c]	641	1744	3432	561	78	1441	1410	1721	403	136	1383	944	2054	396	323	1485	1147	83	207
28[b]	3669	3967	0	584	79[b]	2795	2497	2812	705	137	1237	2031	1600	428	336	1092	442	1221	244
29[b]	2417	2115	3867	761	80	2679	2139	1915	570	138	1558	1434	56	233	344	1310	1876	916	346
30[b]	2868	2270	5005	928	81[c]	364	1625	463	211	139	1893	1517	1767	448	384	859	1040	1203	276
31[c]	4586	1267	0	421	82	2053	2605	583	422	141	1252	1577	0	218	395	58	36	2618	290
32[b]	2519	3202	2877	752	83	1776	1972	851	379	143	1558	2031	65	284	399	1441	1673	333	275
33[b]	2446	1613	3719	705	84	1339	2210	453	326	145[c]	4047	167	0	292	407	1325	1517	296	250
34[b]	2067	2151	786	407	85	1689	2402	1295	457	147	1150	1398	361	235	411[c]	131	211	259	214
35[b]	4367	2784	6642	1251	86	1412	1840	546	310	148	1601	1601	0	244	3840	0	0	2812	304
36[b]	2868	2258	2655	673	87	1645	693	1878	374	150	1747	1374	2461	501	3969	0	0	1896	205
37[b]	4906	4756	0	735	88	1150	36	1147	206	152	670	442	3275	437	4121	0	0	1896	205
38[b]	1951	1685	1970	488	89	1456	2031	1610	444	154	670	609	1129	219	4600	0	0	2109	228
39[b]	2140	1995	2997	638	90	1005	621	1018	231	155	1063	741	814	223	5656	0	0	2017	218
40	3188	2485	0	427	91	1208	801	1110	270	157	1398	908	1591	344	7101	0	0	2054	222
41	3596	3465	2711	830	92[b]	1631	2342	0	308	161	1077	1804	0	225	7147	0	0	1952	211
42[b]	2795	2772	2396	683	93	2198	2449	1795	550	162	1063	645	1480	287	11,232	0	0	1961	212
43	1922	1458	3867	672	94	1514	1398	1425	375	164	1776	1792	1230	405	390[b]	1267	896	0	162
44[b]	1907	2629	194	372	95	2140	1756	2738	590	165	2024	1458	1304	402	493[b]	422	574	259	105
45[b]	1645	1649	3210	598	96[c]	437	1446	2701	443	166	2693	2019	0	354	496[b]	87	585	0	55
46[b]	2955	1900	2600	643	97	2096	1792	851	396	167	1019	1243	1489	335	1362[b]	335	36	204	48
47[b]	2636	2342	3034	705	98[c]	1194	2760	194	334	168	320	454	2785	361	3968[b]	0	550	0	4
48[b]	2198	2856	0	390	99	1893	2175	315	346	170	742	442	1175	215	4254[b]	0	0	1138	123
49	2446	2222	157	371	101	1718	2306	2211	550	171	742	382	1499	245	6605[b]	0	574	0	48
50[b]	2941	2330	3451	770	102	1310	1350	1563	372	172	495	693	1674	273	14278[b]	0	48	0	4
51[b]	2417	2103	3608	732	103	2315	2067	1832	530	176	1048	1040	749	240	16419[b]	0	0	28	55

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

[a]Data and gene numbers from Kinoshita et al. (2011)

[b]Genes subjected to RNAi experiments in the present study

[c]Genes 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

Gene[a]	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	27[b]	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
[a]Gene numbers from Kinoshita et al. (2011)
[b]Data from Funabara et al. (2014)

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

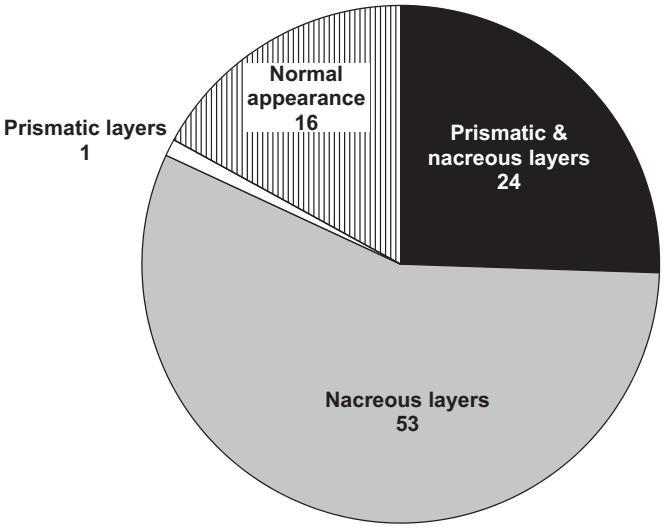

Fig. 41.1 The numbers of individuals having normal and abnormal appearances on the inner surface of the shells of the Japanese pearl oysters *Pinctada fucata* subjected to the RNAi experiments as observed by SEM. "Prismatic and nacreous layers," "nacreous layers," "prismatic layers," and "normal appearances"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