CHAPTER III

RESULTS

3.1 Tissues and total RNA preparation

Antennal gland, epipodite and gill of shrimp acclimated in 3, 25 and 40 ppt salinities at various time points were collected for total RNA isolation. The A_{260}/A_{280} ratio of total RNA samples was 1.5 to 1.8 which were within the expected ratio for the acceptable quality of total RNA. The average quantity of total RNA obtained from gill, epipodite, and antennal gland was approximately 20, 10 and 8 μg per individual shrimp, respectively. The quality of total RNA was also monitored by running on a denaturing formaldehyde/agarose/EtBr gel. The total RNA from antennal gland, epipodite and gill revealed a predominant band of 18S rRNA (1.9 kb) as shown in Figure 3.1.

3.2 Identification of functionally relevant genes controlling osmorality in *P. monodon* by using Differential Display PCR (DD-PCR)

3.2.1 Differential Display PCR (DD-PCR) profiles

Black tiger shrimp genes that are regulated by salinity stress were identified by differential mRNA display. Shrimp acclimatized in 25 ppt salinity (control group) were transferred to 3 and 40 ppt salinities (stressed group). Time course experiments were carried out to determine how rapidly the changes in the mRNA population occurred following the onset of a salt treatment. RNA isolated from gill, epipodite and antennal gland at 6, 24 h and 2 weeks following the transfer, was pooled from 4 individuals for each experiment and used for first-strand cDNA synthesis. The cDNAs were used as DNA templates for DD-PCR reactions. Amplified products were separated on 4% denaturing polyacrylamide gel by size and clearly visualized by silver staining.

The differential display products were selected according to their changes in the level of expression in response to salinity stress. The level of expression was evaluated from the intensity of bands.

Before parallel comparisons of stressed and control DD-PCR profiles of shrimp were performed, 54 combinations of primers (10 arbitary primers in random combination with 9 oligo dT primers) were screened using cDNAs generated from shrimp gill as template cDNAs for DD-PCR reactions. Twenty-six primer combinations were selected for further use to identify the salinity regulated genes because they showed high number of DD-PCR products.

cDNAs generated from antennal gland, epipodite and gill of control and stressed shrimp at various time points (6 h, 24 h and 2 weeks) were amplified with the 26 primer combinations. For each primer combination, DD-PCR profiles derived from the three tissues were rather similar. The profile from gill had the highest number of DD-PCR products followed by those from epipodite and antennal gland, respectively. The majority of differentially expressed fragments showed strong intensity in the profiles from stressed samples (3 or 40 ppt salinities) but was absent or showed low intensity in the profile from control samples. The approximate size of differentially expressed bands ranged from 200 to 1,500 bp.

Seventeen from twenty six primer combinations demonstrated 97 differentially expressed bands. These bands consisted of 25, 24 and 48 bands from antennal gland, epipodite and gill, respectively. No differentially expressed band could be readily identified by comparisons of displaying patterns generated from the remaining primers. Changes in the mRNA level of differential display bands are shown in Table 3.1. Figure 3.2 demonstrated representative gel of DD-PCR products generated from RNA isolated from antennal gland, epipodite and gill of the control shrimp.

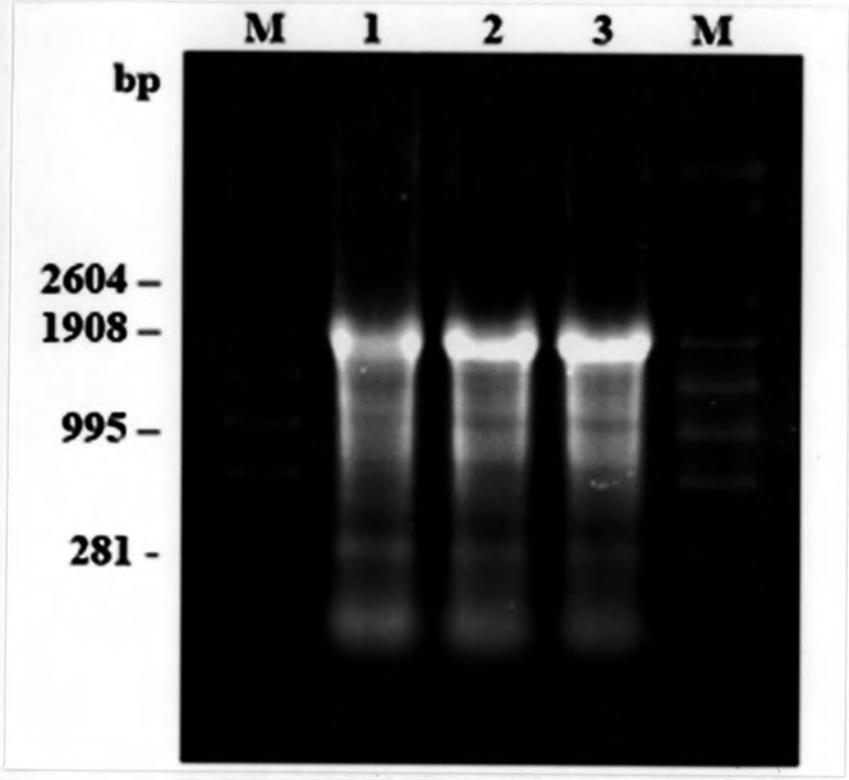

Figure 3.1 Total RNA isolated from antennal gland, epipodite and gill of control shrimp.

The total RNA were electrophoresed on a 1% formaldehyde agarose gel.

Lane M : RNA marker

Lane 1 : Total RNA from antennal gland

Lane 2 : Total RNA from epipodite

Lane 3 : Total RNA from gill

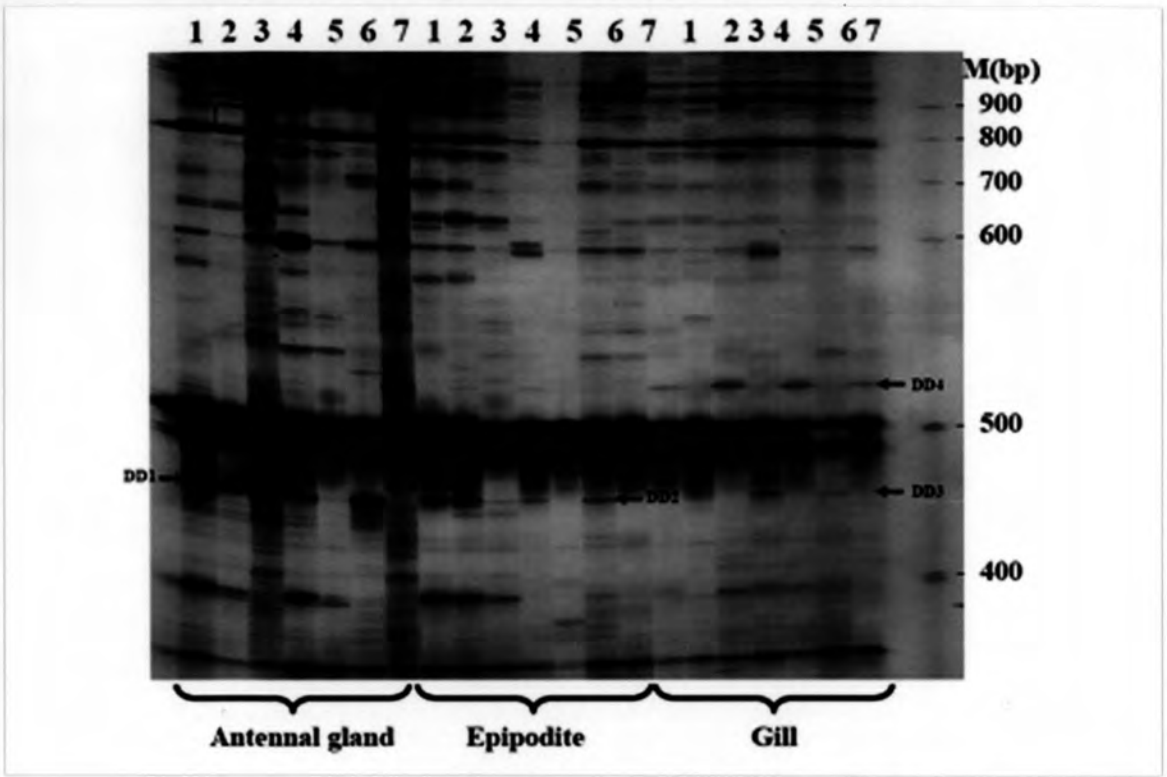

Figure 3.2 A differential display PCR profile of salinity stressed shrimp.

The cDNAs from antennal gland, epipodite and gill were amplified with P2 and T5 primers and separated on a 4% high-resolution polyacrylamide gel under denaturing (urea) conditions. The arrows indicate the differentially expressed bands.

Lane M : 100 bp DNA marker

Lane 1 : shrimp acclimated in 25 ppt saliity (control)

Lanes 2, 4, 6: shrimp acclimated in 3 ppt salinity for 6, 24 h and 2 weeks

Lanes 3, 5, 7: shrimp acclimated in 40 ppt salinity for 6, 24 h and 2 weeks

Table 3.1 Changes in mRNA level of differential display bands identified in the osmoregulation organs of salinity stressed *P. monodon*

Differential display bands	Primer pair	Size (bp)	Tissue*	Change in expression**					
				3 ppt salinity			40 ppt salinity		
				6 h	24 h	2 weeks	6 h	24 h	2 weeks
DD1	P1-T3	200	E	+	+	+	=	+	+
DD2	P1-T3	200	E	+	+	+	=	+	+
DD3	P1-T3	200	G	=	+	+	=	+	+
DD4	P1-T3	250	G	=	+	+	=	+	+
DD5	P2-T1	270	A	+	+	+	+	+	+
DD6	P2-T1	250	A	-	+	+	-	-	-
DD7	P2-T1	250	A	=	+	+	-	+	-
DD8	P2-T1	220	A	=	+	+	-	+	-
DD9	P2-T1	550	G	+	+	+	-	-	-
DD10	P2-T3	500	G	+	+	+	-	-	-
DD11	P2-T4	450	G	+	+	+	-	-	-
DD12	P2-T1	300	G	-	+	+	+	-	-
DD13	P2-T1	330	G	-	+	+	+	-	-
DD14	P2-T1	260	G	-	+	+	+	-	-
DD15	P2-T2	240	G	-	-	-	+	+	+
DD16	P2-T2	260	G	-	-	-	+	+	+
DD17	P9-T1	230	G	-	-	+	-	-	+
DD18	P9-T1	210	G	-	-	+	-	-	+
DD19	P2-T1	260	E	-	-	-	+	+	+
DD20	P2-T1	260	E	-	-	-	+	+	+

*A = Antennal gland, E = Epipodite, G = Gill

** +, - and = indicate up, down and no change compared with control (25 ppt salinity), respectively.

Table 3.1 (continued)

Differential display bands	Primer pair	Size (bp)	Tissue*	Change in expression**					
				3 ppt salinity			40 ppt salinity		
				6 h	24 h	2 weeks	6 h	24 h	2 weeks
DD21	P9-T1	260	E	-	+	+	+	-	+
DD22	P9-T1	250	E	-	+	+	+	-	+
DD23	P9-T1	250	E	-	+	+	+	-	+
DD24	P2-T4	250	E	-	-	-	+	+	+
DD25	P2-T4	230	E	-	-	-	+	+	+
DD26	P2-T4	230	E	-	-	-	+	+	+
DD27	P1-T1	240	A	-	-	-	+	+	+
DD28	P1-T1	250	E	=	+	+	+	+	+
DD29	P9-T4	340	A	+	+	+	-	+	--
DD30	P9-T4	340	A	+	+	+	-	+	--
DD31	P2-T6	450	G	+	+	+	-	-	-
DD32	P2-T6	440	G	+	+	+	=	+	+
DD33	P2-T6	430	G	+	+	+	=	+	+
DD34	P2-T6	450	A	+	+	+	-	-	-
DD35	P2-T6	420	A	+	+	+	-	-	-
DD36	P2-T5	450	G	+	+	+	-	-	-
DD37	P2-T5	330	G	=	-	+	+	+	+
DD38	P2-T5	450	E	+	+	+	-	-	-
DD39	P2-T5	330	E	=	-	-	+	+	+
DD40	P2-T5	450	A	+	+	+	-	-	-
DD41	P7-T3	890	A	=	-	-	+	=	+

*A = Antennal gland, E = Epipodite, G = Gill

** +, - and = indicate up, down and no change compared with control (25 ppt salinity), respectively.

Table 3.1 (continued)

Differential display bands	Primer pair	Size (bp)	Tissue*	Change in expression**					
				3 ppt salinity			40 ppt salinity		
				6 h	24 h	2 weeks	6 h	24 h	2 weeks
DD42	P7-T3	800	A	+	+	+	=	+	+
DD43	P7-T3	650	A	+	+	+	-	-	-
DD44	P7-T3	450	A	+	+	-	-	-	=
DD45	P7-T3	440	A	+	+	=	-	=	-
DD46	P7-T4	420	A	+	+	+	-	-	-
DD47	P7-T4	410	A	-	=	-	-	-	=
DD48	P7-T4	420	E	+	+	+	--	-	-
DD49	P7-T4	410	E	--	-	-	-	=	=
DD50	P7-T4	420	G	+	+	+	-	-	-
DD51	P7-T4	690	G	+	+	-	-	-	-
DD52	P1-T3	650	A	+	+	+	-	-	-
DD53	P1-T3	750	E	-	-	-	=	+	+
DD54	P1-T3	740	E	+	+	+	-	-	=
DD55	P1-T3	750	G	+	+	+	+	+	=
DD56	P1-T3	740	G	+	=	=	=	-	-
DD57	P1-T3	730	G	+	=	=	=	-	-
DD58	P1-T3	700	G	+	+	-	+	-	-
DD59	P1-T3	650	G	+	+	-	=	=	-
DD60	P2-T3	>1,000	G	+	-	-	-	+	+
DD61	P2-T3	>1,000	G	+	-	-	-	+	+
DD62	P2-T3	950	G	+	-	-	-	+	+

*A = Antennal gland, E = Epipodite, G = Gill

** +, - and = indicate up, down and no change compared with control (25 ppt salinity), respectively.

Table 3.1 (continued)

Differential display bands	Primer pair	Size (bp)	Tissue*	Change in expression**					
				3 ppt salinity			40 ppt salinity		
				6 h	24 h	2 weeks	6 h	24 h	2 weeks
DD63	P2-T4	880	G	+	=	=	+	+	=
DD64	P2-T4	620	G	-	=	+	+	+	+
DD65	P2-T4	570	G	-	-	-	-	-	-
DD66	P2-T4	550	G	+	+	+	+	+	+
DD67	P2-T4	540	G	+	+	+	+	+	+
DD68	P1-T4	650	E	+	+	=	--	-	-
DD69	P1-T4	450	E	=	=	=	+	+	+
DD70	P1-T4	700	G	=	=	-	-	-	-
DD71	P1-T4	650	G	+	+	=	-	-	-
DD72	P2-T3	500	G	+	+	+	-	-	--
DD73	P2-T3	450	G	+	+		-	-	-
DD74	P2-T4	550	G	--	--	-	+	+	=
DD75	P2-T4	450	G	+	+	+	-	-	-
DD76	P2-T4	440	G	+	+	+	=	=	-
DD77	P7-T1	900	A	-	+	+	=	=	=
DD78	P7-T1	550	A	-	-	-	+	+	+
DD79	P7-T1	450	A	+	+	+	=	+	+
DD80	P7-T1	550	E	=	=	=	+	+	=
DD81	P7-T1	460	E	-	-	-	+	+	+
DD82	P7-T2	>1,000	A	+	+	-	-	-	=
DD83	P7-T2	730	A	=	=	=	-	-	-

*A = Antennal gland, E = Epipodite, G = Gill

** +, - and = indicate up, down and no change compared with control (25 ppt salinity), respectively.

Table 3.1 (continued)

Differential display bands	Primer pair	Size (bp)	Tissue*	Change in expression**					
				3 ppt salinity			40 ppt salinity		
				6 h	24 h	2 weeks	6 h	24 h	2 weeks
DD84	P7-T2	650	A	=	=	=	-	-	-
DD85	P7-T2	430	A	+	+	+	-	-	-
DD86	P7-T2	850	E	-	-	=	+	+	+
DD87	P7-T2	550	E	=	=	=	+	+	=
DD88	P7-T2	580	G	+	+	+	-	--	+
DD89	P10-T1	790	G	+	+	+	-	-	-
DD90	P10-T1	580	G	+	+	+	+	+	+
DD91	P10-T1	450	G	+	+	=	-	+	=
DD92	P10-T2	900	E	+	=	-	+	+	+
DD93	P10-T2	900	G	+	+	+	-	-	-
DD94	P10-T2	890	G	+	+	+	=	+	+
DD95	P10-T2	880	G	-	=	=	-	-	-
DD96	P10-T2	700	G	+	+	+	=	=	=
DD97	P10-T2	680	G	+	+	+	=	-	-

*A = Antennal gland, E = Epipodite, G = Gill

** +, - and = indicate up, down and no change compared with control (25 ppt salinity), respectively.

3.2.2 Reamplification and sequence analysis of differentially expressed bands

The differentially expressed bands of interest were reamplified, cloned and sequenced. The bands were carefully scratched from the gel. The DNAs were eluted and reamplified with the corresponding combination of primers. The reamplified products were checked for their size by EtBr-stained agarose gel and eluted from the gel. An example of PCR products derived from reamplification of differentially expressed bands are shown in Figure 3.3. The purified PCR products were subsequently cloned and recombinant plasmids were isolated.

The mixture of sequences obtained from one cDNA band may arise due to amplification of similar sized products from differential genes co-migrating in the gel or due to a technical error while excising bands from the gel. For elimination these problems, three to six representative clones from each band of 97 differentially expressed bands were randomly selected. A total of 506 clones were sequenced by Macrogen Inc. sequencing service (Korea). All sequences from each band were aligned and clustered to determine their sequence discrepancy. After sequence analysis, a total of 129 unique different sequences were found. Each of the 129 unique different sequences was submitted to the GenBank database to conduct homology searches with BLASTX and BLASTN programs available on-line at http://www.ncbi.nlm.nih.gov/BLAST.

Homology search showed 37 different sequences significantly matched the GenBank database entries whereas 92 different sequences did not significantly matched. Among the matched sequences, 22, 6 and 9 gene homologues were known gene, ribosomal protein and hypothetical protein, respectively. Gene homologues of unique differentially expressed fragments identified from salt stress of *P. monodon* are demonstrated in appendix A.

Twenty three of the 129 unique sequences originally identified from DD-PCR as potentially regulated by salinity stress were chosen to confirm their differential expression by RT-PCR. These cDNA fragments were divided into two groups. The first group was composed of cDNA fragments that were homologous to known genes whose functions were potentially regulated by salinity stress. The cDNA fragments included in the first group showed similarity to carbonic anhydrase, corin isoform 2, sarcolemmal associated protein 2, NIMA-family kinase Nek 7, karyopherin alpha 4, vacuolar protein sorting 18, CHK1 checkpoint, Rps 16 protein and integrin alpha. The second group was composed of hypothetical proteins and unknown genes which demonstrated high differential expression.

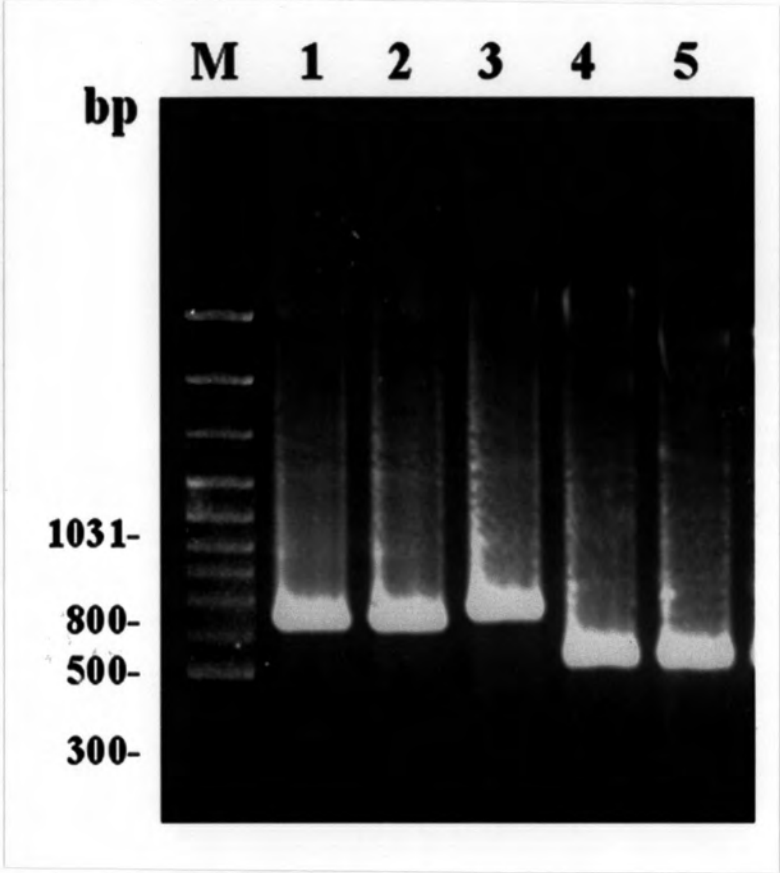

Figure 3.3 Reamplification products of eluted DD-PCR bands with corresponding primer pairs.

PCR products were separated on 1.5% agarose gel.

Lane M : DNA ladder (100 bp marker)

Lane 1 : reamplified product of DD65

Lane 2 : reamplified product of DD66

Lane 3 : reamplified product of DD71

Lane 4 : reamplified product of DD80

Lane 5 : reamplified product of DD87

3.3 Confirmation of cDNA fragments of interest by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Since it was likely that more than one fragment of cDNAs were recovered by eluting a band from the gel, the confirmation of the differential expression by reverse transcription-PCR (RT-PCR) analysis was performed to exclude the likelihood of selecting the false positives. Twenty three of the 129 unique sequences originally identified from DD-PCR were chosen to confirm their differential expression through RT-PCR. Twenty-three specific primers were named according to the names of the unique sequences that were used to design their primers. For example, P-S8 primers were specific primers designed from the S8 unique sequence. Details of gene specific primers and β-actin for semi-quantitative RT-PCR are listed in Table 3.2.

3.3.1 Determination of condition parameters

In most cases, when RNA analysis is required, a qualitative study is not sufficient to deliver a satisfactory answer. A common question is the quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions. To select the suitable conditions for semi-quantitative RT-PCR, many condition parameters must be taken into consideration.

For optimization of the cDNA quantity used in semi-quantitative RT-PCR reaction, several dilutions of cDNA samples were tested to eliminate the effect of the cDNA concentration. Limiting cDNA template amounts often result in sample to sample variations in both the sensitivity and reproducibility of the RT-PCR. Each specific primer was tested with cDNA dilution of 1:5, 1:10 and 1:25. The lowest dilution that yielded the highest band intensity was chosen for the RT-PCR reaction. Optimal dilutions of the cDNA templates for P-S14, P-S21, P-S25 and P-S71 were found at 1:5, whereas for P-S22 was found at 1:10. The amplification reaction for the remaining sequences used 1:25 dilution of template.

Table 3.2 Gene specific primers for semi-quantitative RT-PCR

Primer name	Differential display band	Sequence homology	Primer sequence*
P-S5	DD32 DD33	unknown gene	F = 5' CATCCACAGCCAGAGGAGTT 3' R = 5' AGCCAGTTCCGAGTTGGTAG 3'
P-S8	DD53 DD55	carbonic anhydrase	F = 5' CTCTGTCGGACGAATACCTA 3' R = 5' CCTCGAAGACAATCCAAGTG 3'
P-S9	DD63	ced-6 CG11804-PC, isoform C	F = 5' AGCCGTGATCCTCAAGTGTA 3' R = 5' GCCTTGTCATCAGCACAGTA 3'
P-S11	DD52 DD59	CG14476-PB	F = 5' ACCAGAGCATGCGGACTCTT 3' R = 5' TGCCAGACTCGGAGAACCAA 3'
P-S12	DD54 DD56 DD57	CG14982-PB	F = 5' CCTTAGGCCGAGGTGTGATT 3' R = 5' GAGCGCGGTTCAGTTGTTAC 3'
P-S13	DD71	CHK1 checkpoint homolog	F = 5' CAATGGCTATCACGGGTAAC 3' R = 5' GGAGCAGAAGTCAAACTACG 3'
P-S14	DD95	corin isoform 2	F = 5' GAATCTCGGACTGTGTTGAC 3' R = 5' GAACCTGCTCATAGGCTTTG 3'
P-S17	DD59	ENSANGP00000017400	F = 5' GTCAGACCTGGAAGTGGATA 3' R = 5' CATCCTTACTGCGTGGAATG 3'
P-S21	DD55	integrin alpha	F = 5' TTACTGTCACGGAGGGTTTG 3' R = 5' ACTGAAGGCGGATAGGATTG 3'

Table 3.2 (continued)

Primer name	Differential display band	Sequence homology	Primer sequence*
P-S22	DD42 DD49 DD79	karyopherin (importin) alpha 4	F = 5' TATTGACGCAGGTCTCATCC 3' R = 5' TTGGCTACCACTTCCACATC 3'
P-S25	DD55	NIMA-family kinase Nek7	F = 5' CTGGGAGTGGTGGATAATCA 3' R = 5' CATCACAGCACAAGGCATAG 3'
P-S26	DD58	ORF2	F = 5' CGTTTCCGTAGCTCTGTGTA 3' R = 5' GACAGGCAACTGGATGTCTT 3'
P-S30	DD35	Rps16 protein	F = 5' TCGGCGTGTTGAGCACAATG 3' R = 5' TTGGATATGGCCTGGCGGAT 3'
P-S34	DD70	sarcolemmal associated protein-2	F = 5' TGCGTATGGTCTCTGCCATT 3' R = 5' AAGCCAGTCGCATTAGTGTC 3'
P-S38	DD69	vacuolar protein sorting 18	F = 5' TTCCATGCTTTACACCTTTGG 3' R = 5' GGGAGTGCCTCTACTGTGGT 3'
P-S56	DD51	unknown gene	F = 5' ATGGCACTGTGGTGATCGTT 3' R = 5' ACTCTGAATGGCGTGACGTT 3'
P-S71	DD64	unknown gene	F = 5' ATCGGTCATAGGACTTAGGG 3' R = 5' TCATGGCCTACTGTAACCTG 3'

Table 3.2 (continued)

Primer name	Differential display band	Sequence homology	Primer sequence*
P-S111	DD83 DD84	unknown gene	F = 5' GTTCACCTATGCCTTACG 3' R = 5' CAACCACCTTCTCTTCAC 3'
P-S112	DD93 DD95	unknown gene	F = 5' TCCACAAGCCAGTCGTGTTC 3' R = 5' CTTCTCTATCCGCTGTGACC 3'
P-S114	DD92 DD94	unknown gene	F = 5' ACTTTGCCCCATCACTCATC 3' R = 5' CAGGGAAATGAAAATGGTGTG 3'
P-S118	DD47 DD48 DD49	unknown gene	F = 5' CAGCACATTTCGACCGAAAG 3' R = 5' TACCGCAGACTAACCGAATC 3'
P-S126	DD60 DD61 DD62 DD63	unknown gene	F = 5' GTAAGCTGGTTGTGCGTTCA 3' R = 5' AGCGATCTACTGCATCTTGG 3'

Table 3.2 (continued)

Primer name	Differential display band	Sequence homology	Primer sequence*
P-S129	DD9	unknown gene	F = 5' GGAATGCGTGTCTACAGGTT 3'
	DD10		R = 5' TTGAAGATATGCGCGTCCGA 3'
	DD11		
	DD31		
	DD32		
	DD34		
	DD35		
	DD36		
	DD38		
	DD40		
	DD46		
	DD48		
	DD50		
	DD73		
	DD75		
	DD89		
β-actin			5'GCTTGCTGATCCACATCTGCT3'
			5'ACTACCATCGGCAACGAGA3'

* F = Forward primer, R = Reverse primer

It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified. However, amplification product should be in the exponential range and has not reached a plateau yet. The cycles that indicated the highest band intensity before the product reached a plateau phase was chosen for quantization. Among primers used, the suitable cycle numbers of each specific RT-PCR primer and internal control, β-actin primer range from 18 to 35 consisting of 18, 20, 23, 25, 26, 27, 30 and 35 cycles.

The optimal $MgCl_2$ and primer concentrations are also essential factor for the semi-quantitative RT-PCR assay. Mg^{2+} is a co-factor of the *Taq* DNA polymerase but efficiency of amplification with specific primers is strictly sequence-dependent. The approximate concentration for each primer was determined. Lower primer concentration may be not quantitative, whereas higher concentration may leave a large amount of unused primers which could give rise to none specific amplification product. In this experiment, $MgCl_2$ and primer concentrations were varied between 2.0 to 2.5 mM and 0.15 to 0.25 μM, respectively. DD-PCR experiments were replicate at least thrice with the same condition to reduce the effect of parameter errors.

The optimum PCR conditions of interested cDNA sequences and β-actin gene are shown in Table 3.3.

Table 3.3 Optimal conditions of gene specific primers for semi-quantitative RT-PCR

Primer name	Annealing Temperature (°C)	Cycle	Dilution of template	Product size (bp)	$MgCl_2$ concentration (mM)	Primer concentration (μM)
P-S5	57	30	1:25	293	2.5	0.25
P-S8	55	25	1:25	396	2.5	0.25
P-S9	55	27	1:25	448	2.5	0.25
P-S11	57	30	1:25	305	2.5	0.25
P-S12	55	30	1:25	344	2.5	0.25
P-S13	55	18	1:25	306	2.5	0.15
P-S14	55	25	1:5	291	2.5	0.25
P-S17	55	30	1:25	413	2.5	0.25
P-S21	55	30	1:5	269	2.5	0.25
P-S22	55	25	1:10	261	2.5	0.25
P-S25	55	35	1:5	275	2.5	0.25
P-S26	55	30	1:25	224	2.5	0.25
P-S30	55	20	1:25	286	2.0	0.25
P-S34	55	26	1:25	339	2.5	0.25
P-S38	55	25	1:25	229	2.5	0.25
P-S56	55	26	1:25	327	2.5	0.25
P-S71	55	30	1:5	359	2.5	0.25
P-S111	53	30	1:25	260	2.5	0.25
P-S112	55	20	1:25	396	2.5	0.25
P-S114	55	26	1:25	211	2.5	0.25
P-S118	55	23	1:25	226	2.5	0.15
P-S126	55	26	1:25	270	2.5	0.25
P-S129	55	23	1:25	369	2.0	0.15
β-actin	55	25	1:25	317	2.5	0.25

3.3.2 Semi-quantitative RT-PCR

A total of 23 primer pairs corresponding to the interested cDNA fragments was quantified the expression level in control and stressed samples using β-actin as an internal control gene. The cDNA templates of the RT-PCR analysis were prepared from the total RNA of different group of shrimp, used in the DD-PCR experiment. The amplified products of target fragments and the internal control (β-actin) were electrophoresed on the same agarose gel and stained with ethidium bromide. Level of the expression of RT-PCR products were evaluated by measuring the intensity of bands. The relative expression of interested gene was determined as the signal ratio of the interest fragment : β-actin with the expression of β-actin normalized to 100.

The confirmation of the 23 interested cDNA fragments were tested in the particular tissues which were observed the differential expression from the DD-PCR analysis. Expression patterns of the cDNA fragments in response to salinity stress determined by semi-quantitative RT-PCR are shown in appendix B. Example of up-, down-, and no change of gene expression in response to salinity stress are in Figure 3.4. The RT-PCR results showed that the relative expression of 21 cDNA fragments including carbonic anhydrase, corin isoform 2, NIMA-family kinase Nek 7, karyopherin alpha 4, vacuolar protein sorting 18, CHK1 checkpoint, Rps 16 protein, integrin alpha, 5 hypothetical proteins and 8 unknown gene products, were regulated by salinity stress. These cDNA fragments exhibited differential expression either early or late in the time course. Expression patterns of each cDNA fragment in response to salinity stress of 3 and 40 ppt were divided into six groups. List of cDNA fragments in each group are shown in Tables 3.4 to 3.9.

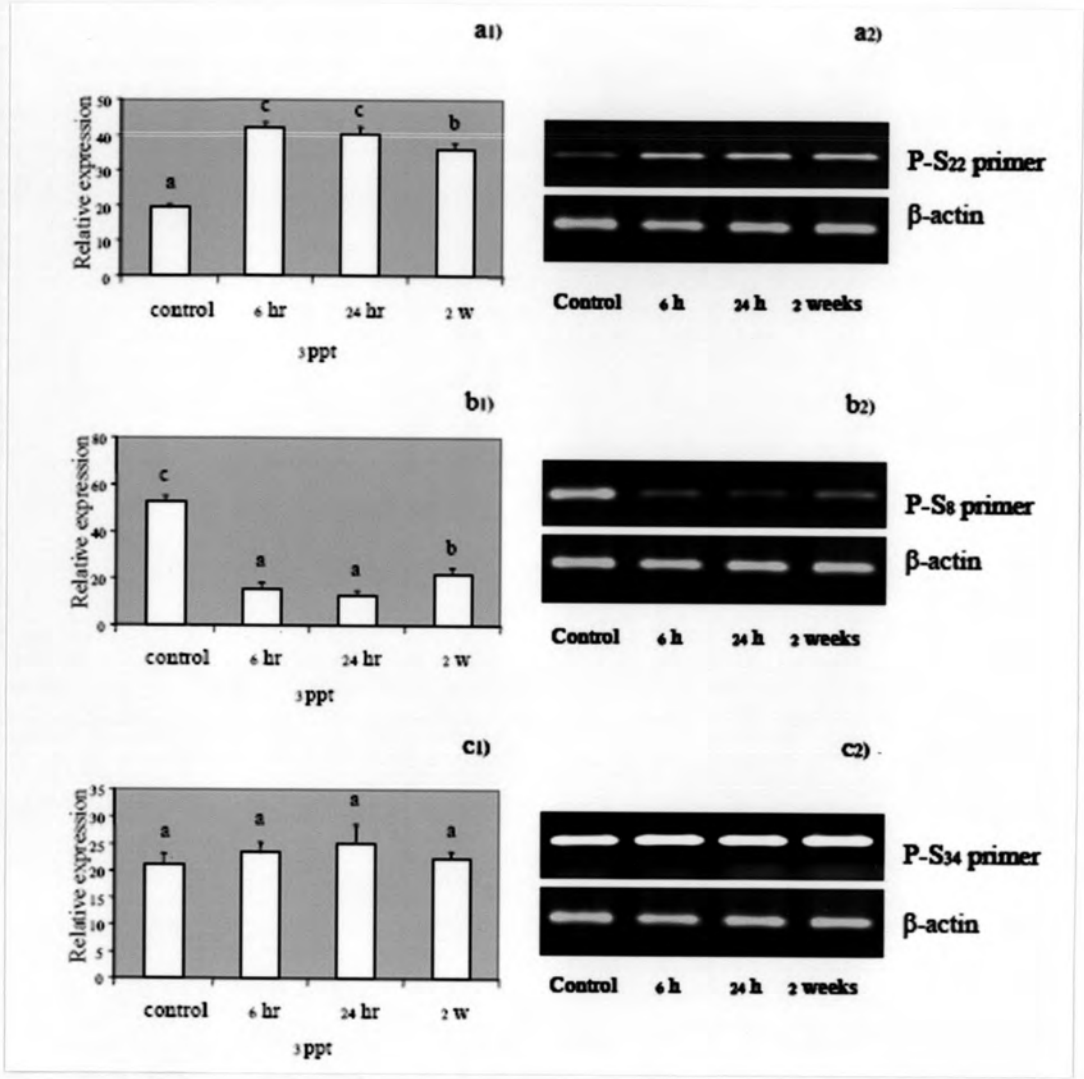

Figure 3.4 Semi-quantitative RT-PCR of salinity stressed *P. monodon* showing examples of up- (a1, a2), down- (b1, b2), and no change (c1, c2) of gene expression.

Table. 3.4 Relative expression patterns of group I determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S9	ced-6 CG11804-PC, isoform C	G	3	++	+	+
P-S11	CG14476-PB	A	3	+	+	+
P-S11	CG14476-PB	A	40	+	+	++
P-S13	CHK1 checkpoint homolog (S. pombe)	G	3	++	+	++
P-S13	CHK1 checkpoint homolog (S. pombe)	G	40	++	++	+
P-S22	karyopherin (importin) alpha 4	A	3	++	++	+
P-S30	Rps16 protein	A	40	++	+	+++
P-S71	unknown gene	G	40	+	+	+
P-S112	unknown gene	G	3	+	+	+
P-S114	unknown gene	G	3	++	+	+
P-S129	unknown gene	A	3	+	+++	++
P-S129	unknown gene	E	3	+	++	++
P-S129	unknown gene	G	3	++	+++	+

Group I are putative genes whose expression was up-regulated at both early (6 or 24 h) and late (2 weeks) time points when compared with the control group (25 ppt salinity).

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

Table. 3.5 Relative expression patterns of group II determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S8	carbonic anhydrase	E	3	--	--	-
P-S22	karyopherin (importin) alpha 4	E	3	-	-	-
P-S25	NIMA-family kinase Nek7	G	3	-	-	--
P-S26	ORF2	G	3	-	-	---
P-S26	ORF2	G	40	-	-	-
P-S38	vacuolar protein sorting 18	E	3	--	-	--
P-S56	unknown gene	G	40	-	-	-
P-S129	unknown gene	E	40	-	-	--

Group II are putative genes whose expression was down-regulated at both early (6 and 24 h) and late (2 weeks) time points when compared with the control group (25 ppt salinity).

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

Table. 3.6 Relative expression patterns of group III determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S9	ced-6 CG11804-PC, isoform C	G	40	+	=	=
P-S12	CG14982-PB	E	3	-	-	=
P-S12	CG14982-PB	E	40	-	=	=
P-S12	CG14982-PB	G	3	+	=	=
P-S14	corin isoform 2	G	40	-	+	=
P-S17	ENSANGP00000017400	G	3	+	=	=
P-S17	ENSANGP00000017400	G	40	-	-	=
P-S21	integrin alpha	G	3	-	=	=
P-S22	karyopherin (importin) alpha 4	E	40	--	-	=
P-S56	unknown gene	G	3	+	+	=
P-S71	unknown gene	G	3	-	+	=
P-S114	unknown gene	G	40	+	+	=
P-S118	unknown gene	A	3	+	++	=
P-S118	unknown gene	E	3	-	=	=
P-S118	unknown gene	E	40	--	-	=

Group III are putative genes whose response was observed at early time points (6 or 24 h) and decrease to the basal level as the control group (25 ppt salinity) at a late time point (2 weeks).

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

Table. 3.7 Relative expression patterns of group IV determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S5	unknown gene	G	40	=	=	+
P-S112	unknown gene	G	40	=	=	-
P-S114	unknown gene	E	3	=	=	-

Group IV are putative genes whose response was observed only at late time points (2 weeks) when compared with the control group (25 ppt salinity).

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

Table. 3.8 Relative expression patterns of group V determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S5	unknown gene	G	3	++	-	+
P-S8	carbonic anhydrase	E	40	-	=	+
P-S8	carbonic anhydrase	G	3	-	=	+
P-S8	carbonic anhydrase	G	40	=	+	-
P-S11	CG14476-PB	G	3	++	+	-
P-S21	integrin alpha	G	40	=	+	+
P-S22	karyopherin (importin) alpha 4	A	40	+	=	+
P-S25	NIMA-family kinase Nek7	G	40	=	++	+
P-S30	Rps16 protein	A	3	+	=	++
P-S38	vacuolar protein sorting 18	E	40	=	-	+
P-S114	unknown gene	E	40	+	-	+
P-S118	unknown gene	A	40	-	-	+
P-S126	unknown gene	G	3	+	-	-
P-S126	unknown gene	G	40	-	=	-
P-S129	unknown gene	A	40	+	-	-

Group V are putative genes whose expression modified differentially at various time points (6 h, 24 h and 2 weeks) when compared with the control group (25 ppt salinity).

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

Table. 3.9 Relative expression patterns of group VI determined by semi-quantitative RT-PCR

Primer	Sequences homology	Tissue*	Salinity (ppt)	Expression patterns**		
				6 h	24 h	2 weeks
P-S11	CG14476-PB	G	40	=	=	=
P-S12	CG14982-PB	G	40	=	=	=
P-S14	corin isoform 2	G	3	=	=	=
P-S34	sarcolemmal associated protein-2	G	3	=	=	=
P-S34	sarcolemmal associated protein-2	G	40	=	=	=
P-S111	unknown gene	A	3	=	=	=
P-S111	unknown gene	A	40	=	=	=
P-S129	unknown gene	G	40	=	=	=

Group VI are putative genes which showed no change in expression at both early (6 and 24 h) and late (2 weeks) time points when compared with the control group (25 ppt salinity)

*A = Antennal gland

E = Epipodite

G = Gill

** +, - and = indicate up, down and no change when compared with control (25 ppt salinity), respectively. The double and triple symbols indicate change in expression much more than a symbol, respectively.

3.3.3 Identification of protein domain from unknown gene sequences

Differentially expressed cDNA fragments of unknown genes regulated by salinity from RT-PCR analysis were performed for domain prediction using SMART (http://smart.embl-heidelberg.de) programs. In this experiment, The SMART results suggested that the predicted amino acid sequences of several cDNA fragments had potential to be a transmembrane region. There was no other type of domain were predicted by SMART. For SMART program, TMHMM2 analytical method is used to predict the location and topology of transmembrane helicase, since this method demonstrate 90% accuracy for transmembrane prediction (Krogh et al., 2001).

Kyte-Doolittle hydropathy plots (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm) were also used for detecting hydrophobic region. By using Kyte-Doolittle hydropathy plots, transmembrane regions were identified by peaks with scores greater than 1.8 using a window size of 19. In this experiment, hydrophobicity analysis suggested that the predicted amino acid sequences of S5, S114 and S126 cDNA fragments had potential to be a transmembrane region. Transmembrane prediction of S5 cDNA fragment showed transmembrane domain from residues 20 to 30. Transmembrane domain of S114 and S126 cDNA fragments were shown as residues 40 to 50 and 50 to 60, respectively. Hydrophobicity profiles of the amino acid sequence from unknown genes are shown in Figures 3.5 to 3.9.

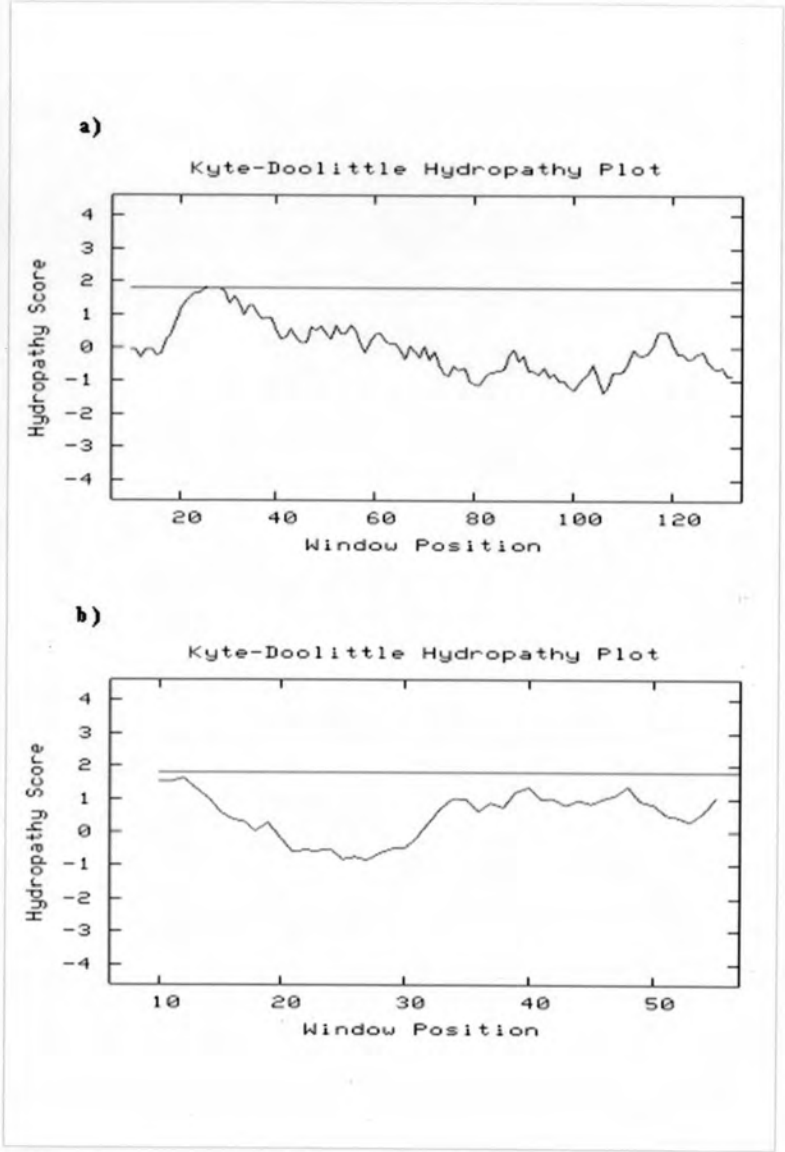

Figure 3.5 Kyte and Doolittle hydrophobicity/hydrophilicity plot analysis of predicted amino acid sequences from S5 cDNA sequence (a) and S56 cDNA sequence (b).

When the window size is 19, peaks with scores greater than 1.8 (straight line) indicate possible transmembrane regions. Window position refers to the number of amino acids examined at a time to determine a point of hydrophobic character.

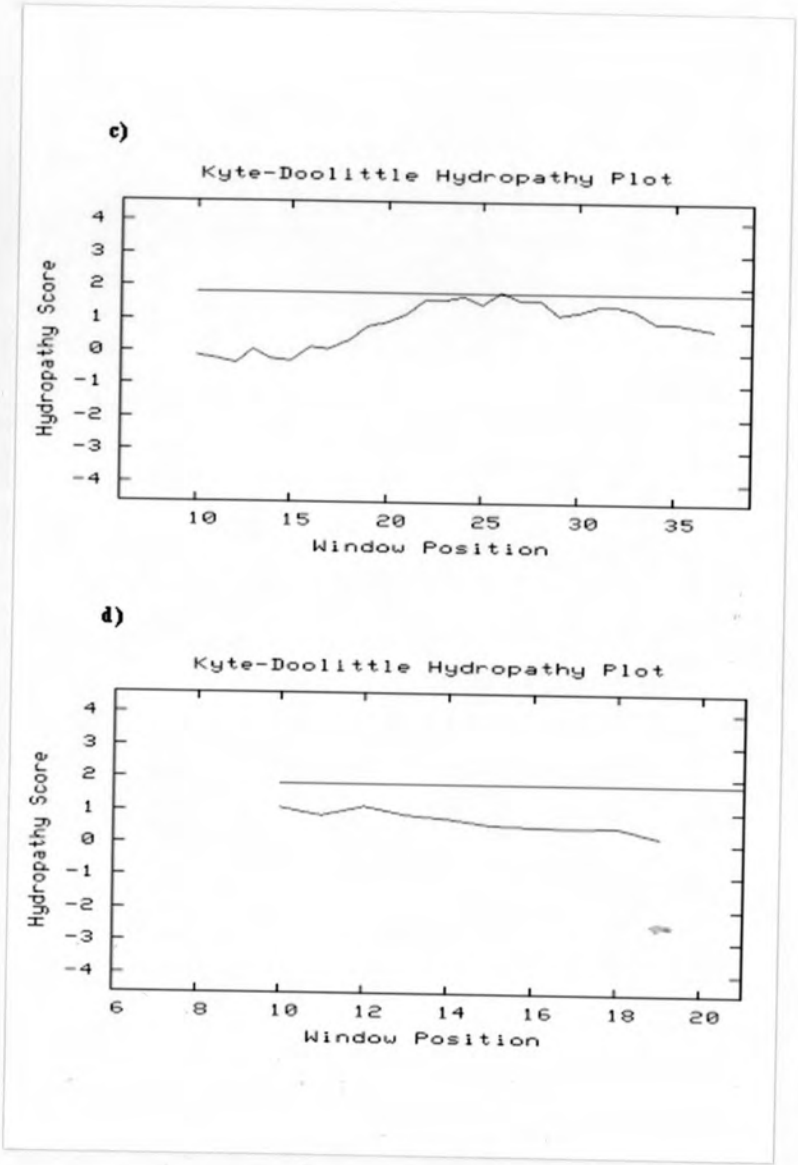

Figure 3.6 Kyte and Doolittle hydrophobicity/hydrophilicity plot analysis of predicted amino acid sequences from S71 cDNA sequence (c) and S111 cDNA sequence (d).

When the window size is 19, peaks with scores greater than 1.8 (straight line) indicate possible transmembrane regions. Window position refers to the number of amino acids examined at a time to determine a point of hydrophobic character.

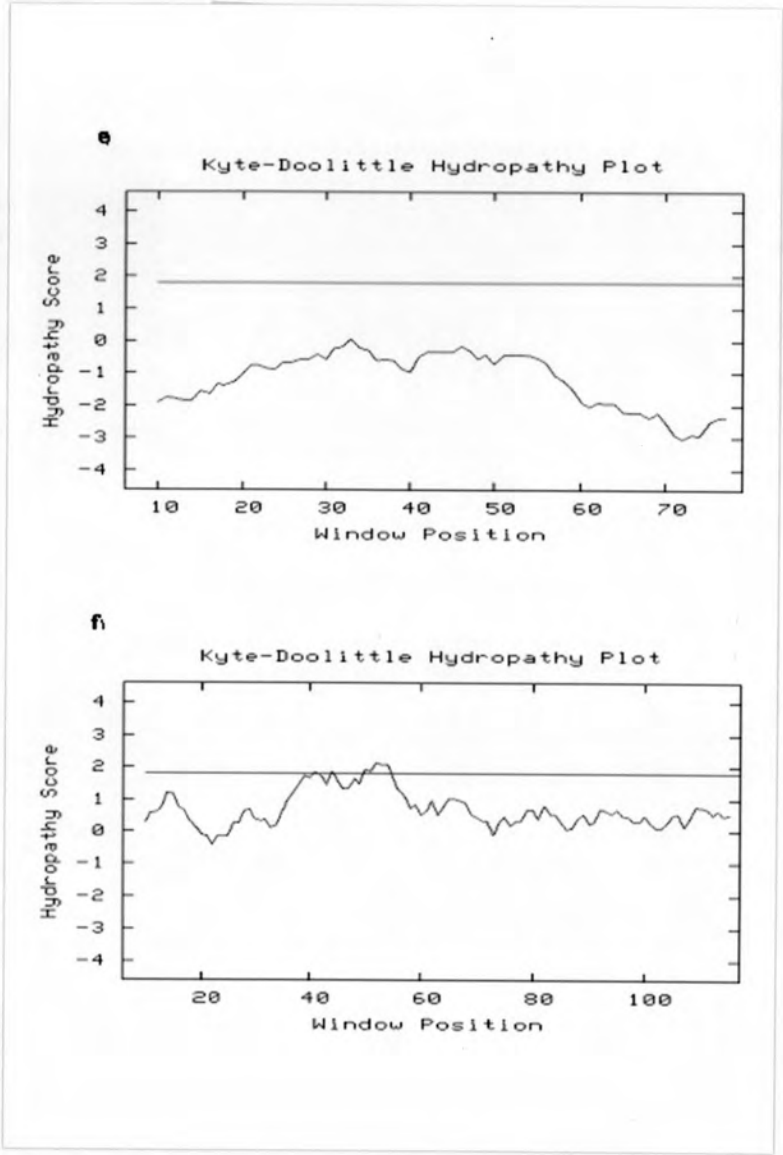

Figure 3.7 Kyte and Doolittle hydrophobicity/hydrophilicity plot analysis of predicted amino acid sequences from S112 cDNA sequence (e) and S114 cDNA sequence (f).

When the window size is 19, peaks with scores greater than 1.8 (straight line) indicate possible transmembrane regions. Window position refers to the number of amino acids examined at a time to determine a point of hydrophobic character.

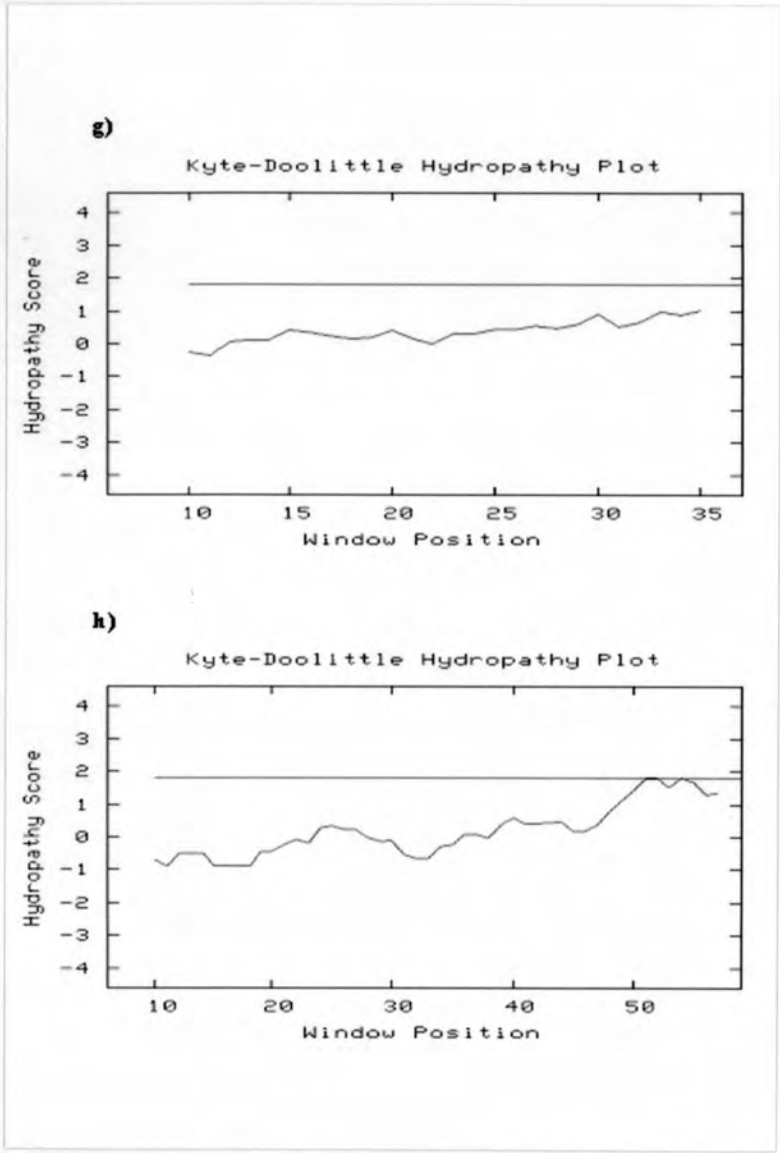

Figure 3.8 Kyte and Doolittle hydrophobicity/hydrophilicity plot analysis of predicted amino acid sequences from S118 cDNA sequence (g) and S126 cDNA sequence (h).

When the window size is 19, peaks with scores greater than 1.8 (straight line) indicate possible transmembrane regions. Window position refers to the number of amino acids examined at a time to determine a point of hydrophobic character.

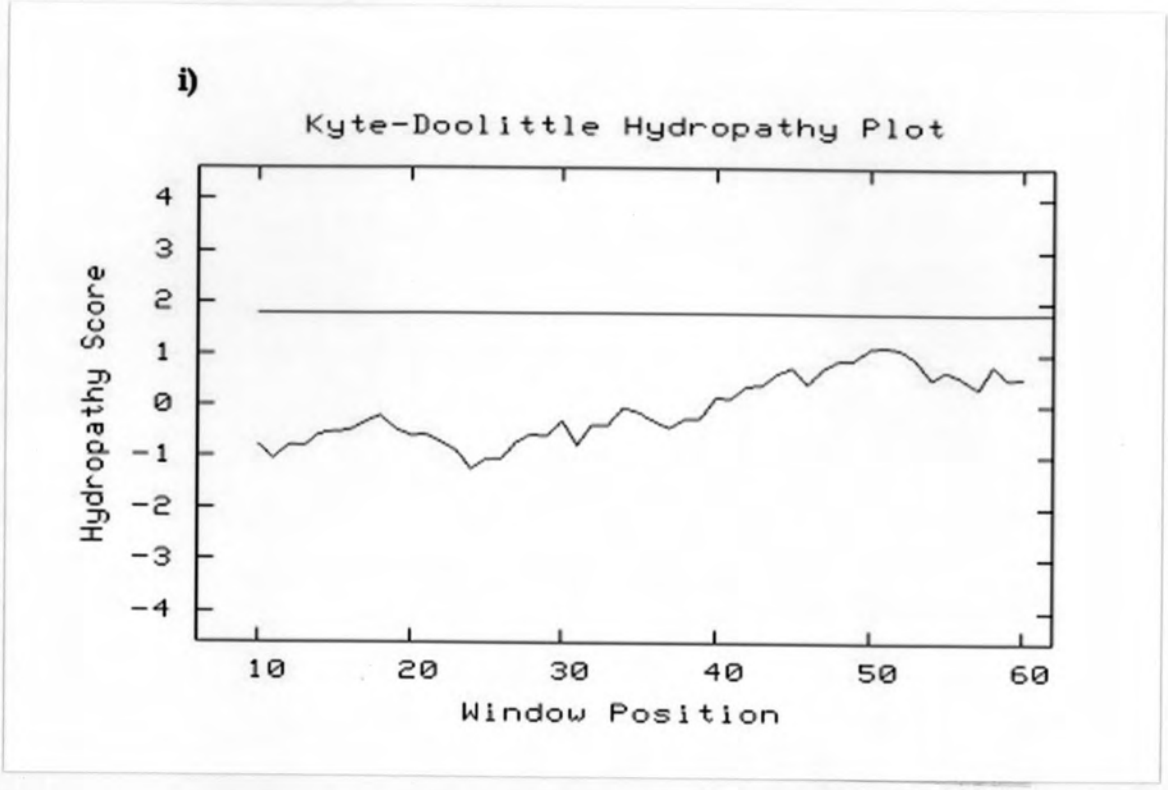

Figure 3.9 Kyte and Doolittle hydrophobicity/hydrophilicity plot analysis of predicted amino acid sequences from S129 cDNA sequence (i).

When the window size is 19, peaks with scores greater than 1.8 (straight line) indicate possible transmembrane regions. Window position refers to the number of amino acids examined at a time to determine a point of hydrophobic character.