DESCRIPTION

Title of Invention: A METHOD OF RELEASING THE SUPPRESSION OF EGG MATURATION IN ECONOMICALLY-IMPORTANT PRAWN/SHRIMP SPECIES

Technical Field

[0001]

The present invention relates to a method for regulating inherent biological functions *in vivo* in shrimps using a molecular biology technique. Specifically, the present invention relates to a method for releasing oocyte maturation inhibition by suppressing expression of a gene capable of regulating oocyte maturation in shrimps.

Background Art

[0002]

Currently, about 200 billion young shrimps are produced worldwide to support 3.5 million tons of annual whiteleg shrimp (*Litopenaeus vannamei*) farming production, and 800,000 shrimps are produced as parent shrimps and exported even only by Hawaii in the United States. It is difficult to artificially mature *Penaeidae* shrimps including whiteleg shrimps. Under the present circumstances, eyestalk ablation is performed to promote maturation. However, eyestalk ablation is problematic because the maturity success rate is low. Besides, it is also getting criticized for animal cruelty because one of eyestalks of is removed from a shrimp by ablation. It is an urgent task to develop maturation promotion technology to replace eyestalk ablation and realize planned and efficient production of young shrimps.

[0003]

RNA interference (RNAi) is a phenomenon in which mRNA is cleaved in a sequence-specific manner by double-stranded RNA (dsRNA) such that gene expression is suppressed. It has been reported that RNAi is a form of defense

common in organisms at the nucleic acid level (see Non Patent Literature 1). In RNAi, when dsRNA is processed by the action of Dicer, siRNA (short interfering RNA) is formed, and siRNA serves as guide RNA to recognize a target sequence and cleave the target mRNA, thereby suppressing gene expression.

[0004]

At present, in order to artificially allow adult shrimps to mature and spawn in a hatchery or the like, there is no alternative to eyestalk ablation. However, some research cases aimed at suppressing vitellogenesis-inhibiting hormone (VIH) expression in shrimps such as *Penaeus monodon* using the RNA interference method have been recently reported (see Non Patent Literature 1, 2, and 3). In addition, there are patent applications stating that it is possible to promote maturation in the same way (see Patent Literature 1).

Citation List

Patent Literature

[0005]

Patent Literature 1: U.S. Patent Application Publication No. US2015/0099702

Non Patent Literature

[0006]

Non Patent Literature 1: Tiu and Chan, 2007, FEBS Journal, 274: 4385-4395

Non Patent Literature 2: Treerattrakool et al., 2008, FEBS Journal, 275: 970-980

Non Patent Literature 3: Treerattrakool et al., 2013, Aquaculture, 404-405: 116-121

Summary of Invention

Technical Problem

[0007]

An objective of the present invention is to provide a method for releasing oocyte maturation inhibition, by suppressing the expression of a gene capable of regulating oocyte maturation in shrimps based on RNA interference.

Solution to Problem

[0008]

The present inventors made intensive studies to arrive at a method for regulating oocyte maturation in shrimps, comprising suppressing the expression of a gene capable of regulating oocyte maturation in shrimps using dsRNA that binds to mRNA of the gene capable of regulating oocyte maturation in shrimps based on the principle of RNA interference. It was the first step to enabling efficient maturation regulation based on biochemical research, thereby blocking the expression of a hormone gene inhibiting *in vivo* maturation. Accordingly, the present inventors found that it is possible to efficiently suppress the expression of a gene capable of regulating oocyte maturation in shrimps using dsRNA as described above, thereby releasing oocyte maturation inhibition.

[0009]

Further, the present inventors found that a plurality of genes are involved in oocyte maturation inhibition, and the use of a mixture of a plurality of dsRNAs each capable of suppressing the expression of the relevant gene makes it possible to suppress the expression of each gene capable of regulating oocyte maturation with improved efficiency. This has led to the completion of the present invention.

[0010]

Specifically, the present invention is described as follows.

[1] A method for releasing oocyte maturation inhibition in farmed shrimps to be used as spawners (hereafter, “farmed shrimp”), comprising suppressing the expression of a vitellogenesis-inhibiting hormone (VIH) gene in shrimps by RNA

interference using double-stranded RNA (dsRNA) targeting mRNA of the vitellogenesis-inhibiting hormone gene in farmed shrimps.

[2] The method according to [1], wherein the farmed shrimps are shrimps belonging to the family *Penaeidae*.

[3] The method according to [2], wherein the farmed shrimps are adult or subadult whiteleg or kuruma shrimps.

[4] The method according to any one of [1] to [3], wherein the double-stranded RNA (dsRNA) targeting mRNA of the vitellogenesis-inhibiting hormone (VIH) gene has a nucleotide sequence identical to a part of the nucleotide sequence of the VIH gene, and wherein a sense strand capable of hybridizing with the gene and an antisense strand having a nucleotide sequence complementary to the nucleotide sequence of the sense strand are bound to each other in the dsRNA.

[5] The method according to any one of [1] to [4], wherein the vitellogenesis-inhibiting hormone gene is one or more types of genes selected from the group consisting of whiteleg shrimp sinus gland peptides SGP-A, SGP-B, SGP-C, SGP-F, and SGP-G.

[6] The method according to any one of [1] to [5], comprising using one or more types of double-stranded RNAs (dsRNAs) selected from the group consisting of: double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene; a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene; and a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 39 for targeting the SGP-G gene.

[7] A composition for releasing oocyte maturation inhibition in farmed shrimps by suppressing the expression of a vitellogenesis-inhibiting hormone (VIH) gene in shrimps, comprising double-stranded RNA (dsRNA) targeting mRNA of a vitellogenesis-inhibiting hormone (VIH) gene, wherein the dsRNA has a nucleotide sequence identical to a part of the nucleotide sequence of the VIH gene, and wherein a sense strand capable of hybridizing with the gene and an antisense strand having a nucleotide sequence complementary to the nucleotide sequence of the sense strand are bound to each other in the dsRNA.

[8] The composition according to [7], wherein the farmed shrimps are shrimps belonging to the family *Penaeidae*.

[9] The composition according to [8], wherein the farmed shrimps are adult or subadult whiteleg or kuruma shrimps.

[10] The composition according to any one of [7] to [9], wherein the vitellogenesis-inhibiting hormone gene is one or more types of genes selected from the group consisting of whiteleg shrimp sinus gland peptides SGP-A, SGP-B, SGP-C, SGP-F, and SGP-G.

[11] The composition according to any one of [7] to [10], comprising one or more types of double-stranded RNAs (dsRNAs) selected from the group consisting of: double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene; a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene; and a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 39 for targeting the SGP-G gene.

[0011]

The present description includes part or all of the contents as disclosed in the description and/or drawings of Japanese Patent Application No. 2016-214411, which is a priority document of the present application.

Advantageous Effects of Invention

[0012]

The use of double stranded RNAs (dsRNAs) according to the present invention makes it possible to suppress the expression of genes responsible for inhibiting oocyte maturation in shrimps based on the principle of RNA interference, thereby releasing oocyte maturation inhibition. As described above, by promoting oocyte maturation in shrimps in which oocyte maturation is successfully inhibited, it is possible to produce young shrimps in a planned and efficient manner.

[0013]

The use of the double-stranded RNAs (dsRNAs) according to the present invention makes it possible to completely knockdown gene expression in all individuals in 10 days. This state can be maintained for at least 30 days. In addition, in the case of using dsRNAs according to the present invention, the gene expression knockdown rate is from 76.9% to 99.9%. Meanwhile, according to the results obtained by the other groups based on RNA interference disclosed in, for example, Feijo et al. (2016), Mar Biotechnol. 18:117-123, the gene expression knockdown rate is merely from 64% to 73% after administration.

Brief Description of Drawings

[0014]

[Figure 1] Figure 1 depicts the concept of the method of the present invention.

[Figure 2] Figure 2 depicts the nucleotide sequence and deduced amino acid sequence of VIH (SGP-G) cDNA in whiteleg shrimps. Each arrow (▼) represents a

putative cleavage site, and each dsRNA primer site is double-underlined. The number of nucleotides is indicated on the right.

[Figure 3] Figure 3 depicts the measurement results of VIH gene expression in eyestalks by semiquantitative PCR. "Initial" represents a plot without injection, "TE" represents a plot with TE (Tris-EDTA) buffer injection, "GFP" represents a plot with GFP-dsRNA injection, and "VIH" represents a plot with VIH-dsRNA injection.

[Figure 4] Figure 4 depicts the measurement results of VIH gene expression in adult shrimp eyestalks by quantitative PCR. (N) represents the number of individuals analyzed, and "*" indicates a significant difference with respect to "Initial" ($P < 0.05$).

[Figure 5] Figure 5 depicts the nucleotide sequence and deduced amino acid sequence of SGP-C cDNA in whiteleg shrimps. Each arrow (↓) represents a putative cleavage site, and each dsRNA primer site is double-underlined. The number of nucleotides is indicated on the right, the number of amino acids is indicated on the left.

[Figure 6] Figure 6 depicts the nucleotide sequence and deduced amino acid sequence of SGP-A cDNA in whiteleg shrimps. Each arrow (↓) represents a putative cleavage site, each dsRNA primer site is double-underlined, and each dsRNA site to be synthesized is written in bold. The number of nucleotides is indicated on the right.

[Figure 7] Figure 7 depicts the nucleotide sequence and deduced amino acid sequence of SGP-B cDNA in whiteleg shrimps. Each arrow (↓) represents a deduced cleavage site, each dsRNA primer site is double-underlined, and each dsRNA site to be synthesized is written in bold. The number of nucleotide sequences is shown on the right side. The underlined portion indicates that a deduced amino acid sequence from cDNA differs from the reported peptide sequence.

[Figure 8] Figure 8 depicts the nucleotide sequence and deduced amino acid sequence of SGP-F cDNA in whiteleg shrimps. Each arrow (↓) represents a putative cleavage site, each dsRNA primer site is double-underlined, and each dsRNA site to be synthesized is written in bold. The number of nucleotides is indicated on the right. The underlined portion indicates that a deduced amino acid sequence from cDNA differs from the reported peptide sequence.
[Figure 9] Figure 9 depicts the measurement results of VIH gene expression in subadult shrimp eyestalks by quantitative PCR. (N) represents the number of individuals analyzed, and "*" indicates a significant difference with respect to "Initial" ($P < 0.05$).
Description of Embodiments
[0015]

The present invention will be described in detail below.

[0016]

The present invention provides a method for releasing oocyte maturation inhibition, comprising suppressing the expression of a gene capable of regulating oocyte maturation in shrimps based on RNA interference.

[0017]

Oocyte maturation inhibition occurs in farmed shrimps. Conventionally, eyestalk ablation is performed to promote oocyte maturation. According to the present invention, oocyte maturation inhibition is released by suppressing the expression of a gene capable of regulating oocyte maturation in farmed shrimps.

[0018]

Shrimps in which oocyte maturation inhibition is released by the method of the present invention are shrimps belonging to the family *Penaeidae*, including kuruma shrimps (*Penaeus japonicus*), whiteleg shrimps (*Litopenaeus vannamei*), Chinese white shrimps (*Penaeus chinensis*), green tiger shrimps (*Penaeus semisulcatus*), giant tiger shrimps (*Penaeus monodon*), and western king shrimps

(*Melicertus latisulcatus*). Of these, whiteleg shrimps or kuruma shrimps, which are mass-produced as edible shrimps, are preferable. In addition, either adult shrimps or subadult shrimps may be used. Here, subadult shrimps are defined as having body weights of 10 g to less than 25 g, and adult shrimps are defined as having body weights of 35 g or more.

[0019]

Genes inhibiting oocyte maturation in shrimps, which are treated by the method of the present invention so as to suppress the expression of the genes, are genes belonging to the crustacean hyperglycemic hormone (CHH) family. Examples of genes belonging to the CHH family include crustacean hyperglycemic hormone (CHH) genes, vitellogenesis-inhibiting hormone (VIH) genes, and molt-inhibiting hormone (MIH) genes. The term "vitellogenesis-inhibiting hormone" (VIH) is also referred to as "gonad-inhibiting hormone."

[0020]

According to the present invention, the expression of vitellogenesis-inhibiting hormone (VIH) genes belonging to the crustacean hyperglycemic hormone (CHH) family is suppressed. Examples of vitellogenesis-inhibiting hormone (VIH) genes include sinus gland peptide (SGP) genes. There are seven sinus gland peptides which are SGP-A to SGP-G. In the present invention, by suppressing the expression of one or more types of genes of five sinus gland peptides referred to as SGP-A, AGP-B, SGP-C, SGP-F, and SGP-G, it is possible to suppress the expression of vitellogenesis-inhibiting hormones so as to facilitate yolk protein production for oocyte maturation, thereby regulating oocyte maturation.

[0021]

As described below, the nucleotide sequences of the genes and the amino acid sequences of peptides encoded by the genes are set forth in SEQ ID NOS: 1 to 10.

SEQ ID NO: 1 SGP-A Nucleotide sequence

SEQ ID NO: 2 SGP-A Amino acid sequence

SEQ ID NO: 3 SGP-B Nucleotide sequence

SEQ ID NO: 4 SGP-B Amino acid sequence

SEQ ID NO: 5 SGP-C Nucleotide sequence

SEQ ID NO: 6 SGP-C Amino acid sequence

SEQ ID NO: 7 SGP-F Nucleotide sequence

SEQ ID NO: 8 SGP-F Amino acid sequence

SEQ ID NO: 9 SGP-G Nucleotide sequence

SEQ ID NO: 10 SGP-G Amino acid sequence

In addition, the nucleotide sequence and amino acid sequence of SGP-A are depicted in Figure 6, the nucleotide sequence and amino acid sequence of SGP-B are depicted in Figure 7, the nucleotide sequence and amino acid sequence of SGP-C are depicted in Figure 5, the nucleotide sequence and amino acid sequence of SGP-F are depicted in Figure 8, and the nucleotide sequence and amino acid sequence of SGP-G are depicted in Figure 2.

[0022]

In order to suppress the expression of any of the above genes, double stranded RNA (dsRNA) consisting of double strands targeting mRNA for the gene can be used. dsRNA causes suppression (silencing) of the expression of a target gene by RNA interference. One of strands of such dsRNA has a nucleotide sequence identical to a part of the nucleotide sequence of the relevant gene, which is a sense strand that can hybridize with the gene. The other strand is an antisense strand having a nucleotide sequence complementary to the nucleotide sequence of the sense strand. The sense strand and the antisense strand binds to each other complementarily, thereby forming dsRNA. Note that the sense strand and the antisense strand are not necessarily exactly complementary to each other. As long as they bind to each other complementarily, there may be one or more mismatches, for example, 1 to 10 mismatches, preferably 1 to 5 mismatches, more preferably 1

to 3 mismatches, and particularly preferably 2 or 1 mismatch. Target sequences in the above sinus gland peptide genes may be either coding regions or non-coding regions. Each target sequence may comprise both a coding region and a non-coding region.

[0023]

According to the present invention, dsRNA comprises small hairpin RNA (shRNA) and small interfering RNA (siRNA). shRNA has a stem loop structure containing a double-stranded portion in which a sense strand and an antisense strand are bound to each other via a loop sequence. In shRNA, the 3' end of the sense strand and the 5' end of the antisense strand are bound to each other via a loop (hairpin loop sequence). Examples of such hairpin loop sequence include, but are not limited, a sequence comprising 5 to 12 nucleotides starting with UU, for example, UUCAAGAGA. dsRNA and shRNA are treated by *in vivo* processing carried out by Dicer, thereby forming siRNA. In addition, dsRNA may form miRNA. According to the present invention, in a case in which dsRNA having a length of 30 nucleotides or more is provided, the dsRNA is treated by processing due to the action of an RNaseIII-like enzyme called "Dicer" such that an siRNA molecule comprising 21 to 27 nucleotides and having an overhang comprising two nucleotides at the 3' end can be formed.

[0024]

The number of nucleotides of dsRNA is, but is not limited to, 50 to 800 nucleotides, preferably 100 to 500 nucleotides, and more preferably 100 to 300 nucleotides.

[0025]

Examples of a sense strand capable of hybridizing with a target sequence of a sinus gland peptide gene of dsRNA to suppress the expression of the sinus gland peptide gene include a sense strand having an RNA sequence comprising a nucleotide sequence which is a partial sequence of any of the nucleotide sequence

of the SGP-A gene set forth in SEQ ID NO: 1, the nucleotide sequence of the SGP-B gene set forth in SEQ ID NO: 3, the nucleotide sequence of the SGP-C gene set forth in SEQ ID NO: 5, the nucleotide sequence of the SGP-F gene set forth in SEQ ID NO: 7, and the nucleotide sequence of the SGP-G gene set forth in SEQ ID NO: 9, in which thymine has been substituted with uracil in the gene sequence.

[0026]

Specifically, for example, it is possible to use one or more types of double-stranded RNAs (dsRNAs) selected from the group consisting of: double-stranded RNA (dsRNA) having a sense strand comprising the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene; double-stranded RNA (dsRNA) having a sense strand comprising the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene; double-stranded RNA (dsRNA) having a sense strand comprising the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene; a double-stranded RNA (dsRNA) having a sense strand comprising the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene; and a double-stranded RNA (dsRNA) having a sense strand comprising the nucleotide sequence set forth in SEQ ID NO: 39 for targeting the SGP-G gene. Antisense strands comprising sequences complementary to these sense strands hybridize to the sense strands, thereby forming dsRNAs. The above sense strands may be produced using dsRNA primers. A primer for double-stranded RNA (dsRNA) comprising the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene may be a primer comprising the nucleotide sequence set forth in SEQ ID NO: 27. A primer for double-stranded RNA (dsRNA) comprising the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene may be a primer comprising the nucleotide sequence set forth in SEQ ID NO: 29. Primers for double-stranded RNA (dsRNA) comprising the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene may be a primer comprising the nucleotide sequence set forth in SEQ ID NO: 31 and a primer

comprising the nucleotide sequence set forth in SEQ ID NO: 32. Primers for double-stranded RNA (dsRNA) comprising the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene may be a primer comprising the nucleotide sequence set forth in SEQ ID NO: 34 and a primer comprising the nucleotide sequence set forth in SEQ ID NO: 35. Primers for double-stranded RNA (dsRNA) comprising the nucleotide sequence set forth in SEQ ID NO: 39 for targeting SGP-G gene may be a primer comprising the nucleotide sequence set forth in SEQ ID NO: 37 and a primer comprising the nucleotide sequence set forth in SEQ ID NO: 38.

[0027]

A sense strand that constitutes dsRNA according to the present invention is desirably identical to the nucleotide sequence of a sinus gland peptide gene, which, however, may be a substantially identical sequence. In other words, as long as the sense strand of dsRNA hybridizes with the mRNA sequence of a sinus gland peptide as an actual target, there may be mismatches due to deletion, substitution, or addition of one or more nucleotides, for example, 1 to 10 nucleotides, preferably 1 to 5 nucleotides, and more preferably 1 to 3 nucleotides or 2 or 1 nucleotide.

[0028]

In order to release oocyte maturation inhibition in shrimps, the expression of at least one type of the above-described sinus gland peptide genes, preferably a plurality of the genes, for example, two, three, four, or five types of the genes, is suppressed. In a case in which the expression of two or more types of the genes is suppressed, it is possible to release oocyte maturation inhibition with improved efficiency. In order to suppress the expression of two or more types of the genes, a plurality of dsRNAs each capable of suppressing the expression of the relevant gene can be used.

[0029]

A sense strand or an antisense strand that constitutes a dsRNA molecule may have an overhang at the 3' end thereof. The type and number of nucleotides of such overhang are not limited. For example, a sequence comprising 1 to 5 nucleotides, preferably 1 to 3 nucleotides, and more preferably 1 or 2 nucleotides may be used. Specific examples include TTT, UU, and TT. The term "overhang" used herein refers to nucleotides which are added to the end of one strand of a dsRNA molecule and do not include nucleotides that can complementarily bind to nucleotides at the corresponding positions of the other strand.

[0030]

According to the present invention, dsRNA can be synthesized via chemical synthesis or *in vitro* synthesis in a transcription system using promoters and RNA polymerases. For example, in the case of chemical synthesis, self-complementary single-stranded RNA having a sequence and another sequence that is a reverse sequence complementary thereto is synthesized, thereby allowing the sequences to bind to each other to form a self-complementary portion. Annealing of a sense strand and an antisense strand of the synthesized dsRNA can be carried out by an ordinary method known to those skilled in the art.

[0031]

In addition, in a case in which dsRNA is synthesized using a promoter and an RNA polymerase, template DNA having a structure, in which a sense strand and an antisense strand are bound to each other via a loop downstream of the promoter, is synthesized so as to transcribe RNA using the RNA polymerase. In the case of *in vitro* production, a T7 promoter, a T3 promoter, or the like can be used. In addition, in a case in which template DNA of dsRNA is introduced into a vector and the vector is administered *in vivo* to a shrimp so as to synthesize dsRNA *in vivo* according to the present invention, a PolIII promoters such as a H1 promoter or a U6 promoter can be used. In a case in which a vector is used, a plasmid vector, a viral vector, or the like can be used. Examples of a plasmid vector that can be used

include pSUPER vectors and pBAsi vectors. Examples of a viral vector that can be used include adenovirus vectors, lentiviral vectors, and retroviral vectors.

[0032]

The present invention also encompasses vectors capable of expressing the above-described dsRNA molecules.

[0033]

According to the present invention, dsRNA can cleave mRNA of a sinus gland peptide gene in a sequence-specific manner, thereby inducing RNA interference (RNAi) for suppressing the expression of the sinus gland peptide gene so as to suppress the expression of the sinus gland peptide gene.

[0034]

In order to suppress the expression of a gene capable of regulating oocyte maturation in shrimps using dsRNA according to the present invention so as to release oocyte maturation inhibition, dsRNA of the present invention can be administered *in vivo* to shrimps. Administration can be carried out by the oral route as well as injectable or parenteral routes such as intravenous, intramuscular, subcutaneous and intraperitoneal routes. For example, dsRNA can be intraperitoneally injected. In such case, it is preferable to use a thin insulin needle of about 30G to 33G. It is also possible to administer dsRNA by mixing the dsRNA with feed and allow shrimps to ingest the feed according to the present invention.

[0035]

According to the present invention, dsRNA can be prepared with a pharmacologically acceptable carrier, diluent, or excipient for use. Examples of such carrier include physiological saline, phosphate buffered saline, a phosphate buffered saline glucose solution, and buffered saline. In a case in which dsRNA is prepared with a carrier, the content of dsRNA according to the present invention may be appropriately determined depending on a preparation, and it is preferably 0.001% to 1% by weight, which may differ variously depending on a preparation.

According to the present invention, 0.0001 to 1 mg of dsRNA can be administered to shrimps once or in divided amounts for several times a day. In addition, the dsRNA molecular weight per administration unit is 1 nM to 100 μM, preferably 10 nM to 50μM, and more preferably 100 nM to 20 μM.

[0036]

According to the present invention, suppression (silencing) of the expression of a target gene by RNA interference also includes a case in which when the expression of a gene is determined based on the expression level of mRNA or protein of the gene, the expression is suppressed not only at a rate of 100% but also at a rate of 75% or more, 50% or more, or 20% or more as compared with a case in which siRNA according to the present invention is not introduced. The degree of suppression of gene expression may be determined by comparing the production of mRNA or protein of the gene before and after introduction of siRNA. In the case of mRNA, measurement can be carried out by northern hybridization, RT-PCR, *in situ* hybridization, or the like. In the case of a protein, measurement can be carried out by a conventional method such as western blotting or ELISA.

[0037]

Oocyte maturation inhibition can be released by suppressing the expression of a gene capable of regulating oocyte maturation in shrimps using dsRNA according to the present invention. In other words, the present invention encompasses a method for releasing oocyte maturation inhibition in shrimps by administering dsRNA according to the present invention into shrimps, thereby suppressing the expression of a gene capable of regulating oocyte maturation in shrimps. The method is also referred to as a method for producing shrimps in which oocyte maturation inhibition is released by administering dsRNA according to the present invention into shrimps, thereby suppressing the expression of a gene capable of regulating oocyte maturation in shrimps.

[0038]

In a case in which dsRNA according to the present invention is administered to shrimps, it is possible to completely suppress the expression of a gene capable of regulating oocyte maturation within several days to more than ten days. The state of suppressing gene expression can be maintained for 10 to 50 days and preferably 30 days. The gene expression suppression rate in the case of using dsRNA according to the present invention is from 76.6% to 99.9%.

[0039]

As described above, it is possible to produce artificially matured shrimps by promoting oocyte maturation in shrimps which are free from oocyte maturation inhibition. Young shrimps can be produced in a planned and efficient manner from such shrimps.

Examples

[0040]

The present invention will be specifically described by the Examples described below, but the present invention is not limited by these Examples.

Example 1: Suppression of the expression of the vitellogenesis-inhibiting hormone (VIH; SGP-G) gene in whiteleg female adult shrimps

1. Production of the double-stranded RNA (ds-RNA) of vitellogenesis-inhibiting hormone (VIH) gene

Cloning of the VIH gene was carried out based on the sequence (SGP-G gene sequence) reported in Tsutsui et al. (2013), Fish. Sci., 79: 357-365, thereby preparing a plasmid. The prepared plasmid was used as a template, and the mature VIH site was amplified using gene specific primers (T7-VIH-L, T7-VIH-R, see Table 1) bound to a T7 promoter. The resulting product was used as a template to produce VIH-dsRNA using MEGAscript RNAi Kits (Figure 2). Among the sequences listed in Table 1, the sequence represented by TAATACGACTCACTATAGGG (SEQ ID NO: 20) is the sequence of a T7 promoter.

2. Administration of VIH-dsRNA

Female adult whiteleg shrimp individuals (each having a body weight of 39 g to 70 g) in the intermolt stage (Stage C0-C1) were selected and used in experiments. The produced VIH-dsRNA was dissolved in TE buffer (10 mM Tris-HCl pH 7, 1 mM EDTA) (1 μg/μL) and injected in an amount of 3 μg per body weight (g) (VIH-dsRNA plot). In addition, in order to examine the influence of buffer, TE buffer was injected at the same dose (3μL) per body weight (g) (TE buffer plot). dsRNA (1μg/μL) for the green fluorescent protein (GFP) gene, which does not exist *in vivo* in shrimps, was produced as a negative control, and it was injected in an amount of 3 μg per body weight (g) (GFP-dsRNA plot). Sampling from each experimental plot was conducted on days 10 and 20 after injection so as to examine VIH expression in eyestalks. In addition, in order to set the original *in vivo* VIH expression level as a standard, non-injected female adult shrimp individuals (Initial plot) were also sampled so as to examine the expression of the VIH gene in eyestalks. Note that individuals sampled in each experimental plot were labeled in the order of the experimental plot, date of sampling, and reference number. Specifically, individuals sampled 10 days after injection of TE buffer (TE buffer plot) are simply referred to as TE10-1 to TE10-5, and individuals sampled 20 days after injection are simply referred to as TE20-1 to TE20-5.

3. Gene expression level analysis (total RNA extraction, semiquantitative PCR, quantitative PCR)

Total RNA was purified from each sampled eyestalk using RNeasy mini kits (Qiagen) and Clean-up kits (Qiagen). In some purified total RNA samples (TE10-3, TE10-4, VIH10-2), a remaining hue was observed. Purified total RNA was reverse-transcribed by High capacity RNA to cDNA kits (Applied Biosystems) to synthesize cDNA. The synthesized cDNA was diluted 4-fold, and the dilution was used as a template so as to examine the expression level of the VIH gene by semiquantitative PCR and quantitative PCR.

[0041]

Under semiquantitative PCR, in order to examine the expression level of the gene of interest, PCR (denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds) was performed using specific primers (Liv-SGP-G-F:CGGAGTGCAGGAGCAACTG (SEQ ID NO: 21), Liv-R3:CCTCTCTGTGTCTTCTGGCCGTTGG (SEQ ID NO: 22)) for the VIH gene and TaqMan Fast Universal PCR Master Mix (2x NoAmpErase UNG, Applied Biosystems), thereby analyzing the expression level. In addition, PCR was performed in the same manner using primers (Liv-act-Fw1:CGACCTCACAGACTACCTGATGAAGAT (SEQ ID NO: 23), Liv-act-Rv2:GTGGTCATCTCCTGCTCGAAG (SEQ ID NO: 24)) for amplifying the beta-actin gene (ACTB) serving as an internal standard gene, followed by relative comparison.

[0042]

Under quantitative PCR, in addition to the gene specific primers and kits described above, probes (Liv-SGP-G-Prb:TCTACAACCCCGTGTTCGTCCAGTGC (SEQ ID NO: 25) for VIH and Liv-act-Prb:CGACCACCGCCGAGCGAGAAATCGTTCGT (SEQ ID NO: 26) for ACTB) were used so as to perform real-time PCR (7500 Fast Real-Time PCR system, Applied Biosystems) under the following conditions: denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 62°C for 30 seconds. The expression level of the VIH gene was obtained as a relative quantitative value calculated based on the expression level of the gene of interest (VIH; SGP-G) with respect to the expression level of the internal standard gene (ACTB).

4. Results

Among total RNA samples purified from eyestalks, a remaining hue was observed in RNA samples of TE10-3, TE10-4, VIH10-2. After semiquantitative PCR, DNA electrophoresis was performed for confirmation. As a result, the

internal standard gene was not amplified in TE10-3 and TE10-4 (Figure 3), and therefore, the results were excluded from gene expression level analysis. The VIH10-2 sample showed an increase in the internal standard gene. However, as the remaining hue could interfere amplification of VIH, the results were also excluded from gene expression level analysis. After 30 cycles of semiquantitative PCR, the VIH expression was not confirmed in individuals of Initial-2, GFP10-2, 5, TE20-1, and GFP20-5. However, the VIH expression was observed entirely in the initial plot, the TE buffer plot, and the GFP-dsRNA plot. Meanwhile, in the experimental plot of VIH-dsRNA injection, the VIH expression was not observed in all individuals on days 10 and 20.

[0043]

Further, as depicted in Figure 4, the results of quantitative PCR showed that the VIH expression level did not significantly vary on both days 10 and 20 in the TE buffer plot and the GFP-dsRNA plot as compared with the initial plot while the VIH expression level was significantly reduced in the VIH-dsRNA plot on both days 10 and 20. As described above, it was demonstrated that VIH gene expression can be suppressed in adult shrimps by injection of VIH-dsRNA.

[0044]

[Table 1]

List of gene specific primers used for RNA interfering

target gene	Oligo Name	Sequence (5' to 3')
GFP	T7-EGFP-2L	TAATACGACTCACTATAGGGAGAGCATCGACTTCAAGGAGGAC (SEQ ID NO: 11)
GFP	T7-EGFP-2R	TAATACGACTCACTATAGGGAGATGGGTGCTCAGGTAGTGGTT (SEQ ID NO: 12)
SGP-G	T7-VIH-L	TAATACGACTCACTATAGGGAGAAAGCGAGCAAACTTCGAC (SEQ ID NO: 13)
SGP-G	T7-VIH-R	TAATACGACTCACTATAGGGAGACTACTTGCCCACCGTCTG (SEQ ID NO: 14)
SGP-C/SGP-A	T7_sgpC-L	TAATACGACTCACTATAGGGAGACTCGCTCTTCGACCCTTCC (SEQ ID NO: 15)
SGP-C	T7_sgpC-R	TAATACGACTCACTATAGGGAGACTATTTCCCGACCATCTGG (SEQ ID NO: 16)
SGP-B	T7-sgpB-L2	TAATACGACTCACTATAGGGAGACGCAGCATATCCTTCGACTCGT (SEQ ID NO: 17)
SGP-F	T7-sgpF-L	TAATACGACTCACTATAGGGAGAAAGCGCTCCCTCTTCGACC (SEQ ID NO: 18)
SGP-F	T7-sgpF-R	TAATACGACTCACTATAGGGAGACTTTATTTGCCGACGGTCTGCAGG (SEQ ID NO: 19)
	T7 promoter	TAATACGACTCACTATAGGG (SEQ ID NO: 20)

[0045]

Example 2: Regulation of VIH gene expression in whiteleg female subadult shrimps

1. Cloning of sinus gland peptides

For whiteleg shrimps, seven sinus gland peptides (sinus gland peptides: SGP- A to G) have been identified. It has been reported that among them, six sinus gland peptides (A, B, C, E, F, G) can suppress yolk protein expression (Tsutsui et al., 2007, Mar. Biotechnol., 9:360-369). Since the abundance of SGP-G is largest, SGP-G has been considered to be the major VIH to target in further studies. However, since it has been revealed that the nucleotide sequence of SGP-C is structurally similar to that of the crustacean hyperglycemic hormone (CHH) and

SGP-C has an action similar to that of CHH, SGP-C has been determined to have properties of both hormones (Lago-Leston et al., 2007, Aquaculture, 270: 343-357; Liu et al., 2014, Peptides, 53: 115-124). According to the present invention, the VIH expression level can be suppressed via *in vivo* injection of dsRNAs of not only SGP-G (VIH) but also SGP-C and other similar SGP genes into shrimps. For such reasons, the sequence of SGP-C was obtained from the eyestalk cDNA library, and cloning of cDNAs of SGP-A, SGP-B, and SGP-F was further conducted.

2. Production of dsRNA of sinus gland peptides

As in the case of adult shrimps, a gene fragment of the mature VIH site was amplified by PCR based on the plasmid of the VIH gene, and GFP-dsRNA was produced by MEGAscript RNAi Kits using the amplified product as a template and VIH-dsRNA as a negative control. In addition, as in the case of VIH-dsRNA, in order to synthesize dsRNAs targeting the mature sites of SGP-A, SGP-B, SGP-C, and SGP-F genes, gene fragments were amplified using gene specific primers bound to a T7 promoter and a T7 promoter primer (see Table 1) by PCR using each cloned plasmid as a template by the same method used for VIH-dsRNA, thereby synthesizing dsRNA of each gene. The thus obtained GFP-dsRNA, VIH-dsRNA, and SGP-C-dsRNA were dissolved in TE buffer at a concentration of 3 μg/4 μL for use. Three dsRNAs, namely, SGP-A-dsRNA, SGP-B-dsRNA, and SGP-F-dsRNA, were mixed such that the concentration of each dsRNA was adjusted to 1 μg/4 μL. The resulting product was dissolved in TE buffer to yield a total dsRNA concentration of 3 μg/4 μL for use.

3. Administration of dsRNA

VIH-dsRNA was injected at a concentration of 3μg per body weight (g) into randomly selected female subadult shrimp individuals (each having a body weight of 15 g to 25 g) only once (VIH-1 plot) or three times at 7-day intervals (VIH-2 plot). SGP-C-dsRNA was injected at a concentration of 3 μg per body weight (g) only once (SGP-C plot). SGP-A-dsRNA, SGP-B-dsRNA, and SGP-F-dsRNA were

mixed at equivalent concentrations, and the mixture was injected at a concentration of 3 μg per body weight (g) only once (Mix plot). GFP-dsRNA was injected as a negative control at a concentration of 3 μg per body weight (g) only once (GFP plot). Sampling was conducted on days 10, 20, and 30 after the initial injection, and the VIH expression level in eyestalks was examined.

4. Gene expression level analysis (total RNA extraction, semiquantitative PCR, quantitative PCR)

Gene expression levels were analyzed in the same manner as in 3 in Example 1.

5. Results

The SGP-C gene cloned from the eyestalk cDNA library was found to encode an amino acid sequence of 115 residues, and its primary structure was composed of a signal peptide (24 residues), CPRP (a CHH precursor-related peptide; 16 residues), and a mature SGP-C (75 residues) (Figure 5). In addition, cDNAs which were considered to correspond to the SGP-A, SGP-B, and SGP-F genes were also successfully cloned. Each cDNA was compared with the peptide sequence previously extracted from eyestalks. In the case of SGP-A, there was consistency in the putative amino acid sequence of the mature site (Figure 6), while two amino acid residues were different in SGP-B (Figure 7) and SGP-F (Figure 8). These cDNAs were each used as a template for synthesis of the corresponding dsRNA. In Figures 2 and 5 to 8, the sequence of each dsRNA primer used for dsRNA production is double-underlined. Each primer was used for producing dsRNA for suppressing the expression of the relevant sinus gland peptide gene. The dsRNA sequence for SGP-G in Figure 2 is set forth in SEQ ID NO: 39, and the sequences of dsRNA primers for producing dsRNA of SGP-G are set forth in SEQ ID NOS: 37 and 38. The dsRNA sequence for SGP-C in Figure 5 is set forth in SEQ ID NO: 33, and the sequences of dsRNA primers for producing dsRNA of SGP-C are set forth in SEQ ID NOS: 31 and 32. The dsRNA sequence for SGP-A

in Figure 6 is set forth in SEQ ID NO: 28, and the sequence of a dsRNA primer for producing dsRNA of SGP-A is set forth in SEQ ID NO: 27. The dsRNA sequence for SGP-B in Figure 7 is set forth in SEQ ID NO: 30, and the sequence of a dsRNA primer for producing dsRNA of SGP-B is set forth in SEQ ID NO: 29. The dsRNA sequence for SGP-F in Figure 8 is set forth in SEQ ID NO: 36, and the sequences of dsRNA primers for producing dsRNA of SGP-F are set forth in SEQ ID NOS: 34 and 35.

[0046]

VIH gene expression in eyestalks followed by dsRNA injection was analyzed by quantitative PCR. As a result, with respect to the initial plot (without injection), the GFP plot in which GFP-dsRNA was injected, there was no significant decrease in the VIH expression level on days 10, 20, and 30. However, in the VIH-1 plot (1 injection) and VIH-2 plot (3 injections) in which VIH-dsRNA was injected, there was a significant decrease in the VIH expression level on days 10, 20, and 30 after injection (Figure 9). In the SGP-C plot in which dsRNA of a similar SGP-C gene was injected, although the VIH expression level significantly declined on day 10 after injection, there was no significant difference as compared with the VIH expression level of the initial plot on days 20 and 30. In addition, in the Mix plot in which the mixture of dsRNAs of the SGP-A, SGP-B, and SGP-F genes was injected, although the VIH expression level significantly declined on days 10 and 30 after injection, there was no significant difference as compared with the VIH expression level of the initial plot on day 20.

[0047]

As described above, it is possible to remarkably reduce the expression level of the VIH gene using dsRNA of VIH (SGP-G) in subadult shrimps as well. In addition, it is also possible to obtain similar effects using other similar VIH genes.

Industrial Applicability

[0048]

Shrimps can be produced in a planned and efficient manner using the dsRNAs of the present invention.

Sequence Listing Free Text

[0049]

SEQ ID NOS: 11-19, 21-27, 29, 31, 32, 34, 35, 37, and 38 for primers

All publications, patents and patent applications cited in the present description are incorporated herein by reference in their entirety.

CLAIMS

[Claim 1]

A method for releasing oocyte maturation inhibition in farmed shrimps to be used as spawners (hereafter, “farmed shrimp”), comprising suppressing the expression of a vitellogenesis-inhibiting hormone (VIH) gene in shrimps by RNA interference using double-stranded RNA (dsRNA) targeting mRNA of the vitellogenesis-inhibiting hormone gene in farmed shrimps.

[Claim 2]

The method according to claim 1, wherein the farmed shrimps are shrimps belonging to the family *Penaeidae*.

[Claim 3]

The method according to claim 2, wherein the farmed shrimps are adult or subadult whiteleg or kuruma shrimps.

[Claim 4]

The method according to any one of claims 1 to 3, wherein the double-stranded RNA (dsRNA) targeting mRNA of the vitellogenesis-inhibiting hormone (VIH) gene has a nucleotide sequence identical to a part of the nucleotide sequence of the VIH gene, and wherein a sense strand capable of hybridizing with the gene and an antisense strand having a nucleotide sequence complementary to the nucleotide sequence of the sense strand are bound to each other in the dsRNA.

[Claim 5]

The method according to any one of claims 1 to 4, wherein the vitellogenesis-inhibiting hormone gene is one or more types of genes selected from the group consisting of whiteleg shrimp sinus gland peptides SGP-A, SGP-B, SGP-C, SGP-F, and SGP-G.

[Claim 6]

The method according to any one of claims 1 to 5, comprising using one or more types of double-stranded RNAs (dsRNAs) selected from the group consisting of: double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene; a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene; and a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 39 for targeting the SGP-G gene.

[Claim 7]

A composition for releasing oocyte maturation inhibition in farmed shrimps by suppressing the expression of a vitellogenesis-inhibiting hormone (VIH) gene in shrimps, comprising double-stranded RNA (dsRNA) targeting mRNA of a vitellogenesis-inhibiting hormone (VIH) gene, wherein the dsRNA has a nucleotide sequence identical to a part of the nucleotide sequence of the VIH gene, and wherein a sense strand capable of hybridizing with the gene and an antisense strand having a nucleotide sequence complementary to the nucleotide sequence of the sense strand are bound to each other in the dsRNA.

[Claim 8]

The composition according to claim 7, wherein the farmed shrimps are shrimps belonging to the family *Penaeidae*.

[Claim 9]

The composition according to claim 8, wherein the farmed shrimps are adult or subadult whiteleg or kuruma shrimps.

[Claim 10]

The composition according to any one of claims 7 to 9, wherein the vitellogenesis-inhibiting hormone gene is one or more types of genes selected from the group consisting of whiteleg shrimp sinus gland peptides SGP-A, SGP-B, SGP-C, SGP-F, and SGP-G.

[Claim 11]

The composition according to any one of claims 7 to 10, comprising one or more types of double-stranded RNAs (dsRNAs) selected from the group consisting of: double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 28 for targeting the SGP-A gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 30 for targeting the SGP-B gene; double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 33 for targeting the SGP-C gene; a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 36 for targeting the SGP-F gene; and a double-stranded RNA (dsRNA) having a sense strand consisting of the nucleotide sequence set forth in SEQ ID NO: 39 for targeting the SGP-G gene.